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PLATELET KINETICS AND THROMBOGENESIS

By

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Synopsis

This synopsis focuses primarily on platelet kinetics, the role of platelets in thrombosis and the effect of inhibition of platelet function on thrombosis. It highlights our contribution to these fields of study. Key references are given as footnotes. Our own publications are cited in parentheses. For purposes of clarity, the publications are grouped under three headings. Those in the first group deal with the methods that we developed to isolate a representative and viable population of platelets from blood, to effectively label them with In-111 and to accurately quantify their in vivo distribution. They also address normal platelet kinetics and explain what information can be deduced from kinetic studies. Those in the second group describe how we used In-111-platelets as an investigative tool to study various aspects of platelet function utilising several approaches in patients and experimental animals. Those in the third group describe our contribution to assess the effective use of agents developed to inhibit specific components of the thrombotic process.

1. Introduction

Blood platelets originate from megakaryocytes in the bone marrow and circulate as disc-shaped fragments at a concentration of $150 - 400 \times 10^9/l$. Although by far the smallest, they are biologically amongst the most active of the blood cells. They are pivotal to the maintenance of haemostasis and vascular integrity and plays a key role in various thrombotic processes.

2. Platelet kinetics

The term platelet kinetics include all the processes of platelet production, distribution, utilisation, and destruction as well as the haemostatic mechanisms which control them. A clear knowledge of these processes is useful to understand the mechanisms of thrombocytopenia and the part platelets play in vascular diseases such as thrombosis, hypercoagulability and interaction with foreign surfaces such as biomaterials. In order to study these processes, platelets are preferably labelled with a radioisotope.¹ Since its introduction in 1976,² In-111 has become the platelet label of choice. It is a highly suitable label for platelets and its high gamma production enables the imaging of the in vivo distribution of labelled platelets by means of a scintillation camera.³

¹ I.C.S.H. Panel on diagnostic application of radioisotopes in Haematology: recommended methods for radioisotope platelet survival studies. *Blood*, 50: 1137, 1977.

² Thakur et al: Indium-111-labelled platelets: studies on preparation and evaluation of in vitro and in vivo functions. *Thrombosis Research*, 9: 345, 1976.

³ Heyns et al: Kinetics, distribution and sites of destruction of In-111-labelled human platelets. *British Journal of Haematology*: 44: 89, 1980.

Platelet labelling: Since there is no suitable cohort label for platelets, one has to resort to isolating them from a blood sample by differential centrifugation, thus obtaining platelets of all ages. We have shown that a single step wash procedure isolates a platelet population that does not represent the circulating platelet population. The mean platelet life span (MPLS) of a population obtained by this method is significantly shorter than that of a representative population, i.e. platelets of all ages isolated by a multiwash procedure [S-1]. Oxine and tropolone are chelates used to internalise the In-111. Labelling with In-111-oxine is done in saline. In the case of In-111-tropolone, labelling is done in plasma. We have shown that neither the medium of labelling nor the in vitro handling and manipulation of the platelets during the labelling process have any adverse effects on their function and kinetics [S-1 - S-3]. Moreover, since the kinetics of labelled platelets are not affected by the chelate used, results are comparable [S-2]. We also developed a method to isolate sufficient platelets from the blood of patients with severe thrombocytopenia to enable us to study the kinetics of autologous labelled platelets in these patients [S-4].

Determination of the MPLS: Since platelets of all ages are isolated by differential centrifugation, the MPLS can be calculated from the disappearance of labelled platelets from the circulation. There are numerous mathematical equations to calculate the MPLS. We have evaluated these models, compared them with one another and also developed a computer program (in Basic) to calculate the MPLS [S-5 - S-8].

Quantification of the in vivo distribution of labelled platelets: Initially the in vivo distribution of labelled platelets was determined using a cumbersome method.³ This necessitated the development of a simpler, but accurate method of quantification. The geometric mean method of quantification was developed using a baboon model [S-9 - S-12]. This requires the acquisition of an anterior and a posterior image of the whole body by means of a scintillation camera, interfaced with a data processing system. The proportion of injected labelled platelets in a particular organ is then calculated by dividing the square root of the product of the anterior and posterior count rate in that organ by the square root of the product of the anterior and posterior whole body count rate. By using this approach, we are able to estimate the organ distribution of radiolabelled platelets to within 3% of the true content [S-9]. We have also shown that, although some elution of In-111 from the liver and spleen takes place after senescent platelets are sequestered in these organs, it has no significant effect on the measurement of in vivo platelet kinetics [S-

13]. In addition, the radiation dose from In-111, and contaminating In-114M, is such that relatively large amounts of radioisotopes can be used without exceeding the specified maximum permissible dose.⁴

In summary, we developed methods to isolate a representative population of platelets from whole blood and to label them with In-111 without adversely affecting their function. We were also able to calculate the MPLS and to accurately quantify the in vivo distribution of labelled platelets. This enabled us to study the in vivo kinetics of labelled platelets in normal subjects, experimental animals and in patients who have increased platelet consumption (see Part 2 of this Synopsis, page 5).

Normal Mean Platelet Life Span, Distribution and Destruction: In this section, which is based on various studies that we performed [S-1 - S-22], I want to summarise our contribution to the knowledge of platelet kinetics. When labelled platelets are injected, they circulate for 240 - 288 hours in humans and approximately 146 hours in baboons [S-1 - S-9, S-14 - S-18]. Much information can be derived from the shape of the survival curve and the MPLS. Removal (sequestration) of platelets from the circulation either takes place as an age-dependent process or as a result of increased destruction or random utilisation [S-16 - S-18]. Age-dependent removal takes place at the end of the life span of a platelet and the resulting survival curve best fits a linear function. If platelet removal is age-independent, platelets of all ages are randomly removed and the survival curve best fits an exponential function. Platelet turnover, as an estimate of platelet production, can be calculated from the MPLS and platelet count and by correcting for the platelet pool in the spleen.⁵ This gives an indication of increased, normal or decreased platelet production (see later).

Circulating platelets are distributed in two compartments. Approximately 33% of platelets in humans and 15% in baboons are pooled in the spleen while the remainder is in the circulation [S-1, S-9, S-13 - S-18, S-22, S-23]. The mechanism of pooling is unknown, and may be related to the circulation in and perhaps the size of the spleen. Many platelets apparently enter the splenic cords and flow through the sinuses. This delays their transit through the spleen by 8 - 10 minutes so causing platelet accumulation out of proportion to splenic plasma and red cell volume [S-14]. Those platelets present in the spleen are in

⁴ Task group of the Committee of the International commission on Radiological Protection. Publication 26, Pergamon Press, Elmsford, New York, 1977.

⁵ Harker and Finch: Thrombokinetis in man. Journal of Clinical Investigation: 48: 963, 1969.

equilibrium with circulating platelets, and are freely exchangeable.⁶ It was believed that platelets also pool in the liver since the recovery of injected labelled platelets in asplenic patients is approximately 90%.⁷ By comparing the distribution of labelled platelets and labelled red cells during the initial 90 minutes following injection, we could clearly show that platelets only pool in the spleen [S-14]. Our results in healthy humans and baboons further support the view that the size of the spleen has little effect on the size of splenic platelet pool and that blood flow through the spleen is the important determinant [S-14].

Isolation and labelling of platelets cause a "collection" injury [S-15]. With results obtained by compartmental analysis, we estimate that the collection injury involve approximately 10% of the labelled platelets. Of these, approximately 91% accumulate transiently in the liver with a transit time of approximately 27 minutes. The remaining 9% of the "collection injured" platelets accumulate transiently in the spleen with a transit time of approximately 208 minutes [S-21, S-22].

When platelets reach the end of their life span in the circulation, the macrophages of the spleen, liver and bone marrow recognise them as "old" and remove them from the circulation [S-9, S-16, S-19, S-20]. The spleen and liver are the main sites of sequestration of senescent platelets, with 38 - 40% and approximately 28% respectively. It was generally accepted that the remainder are sequestered by the macrophages of the bone marrow.³ We have shown in baboons that approximately 15% of the non-liver/non-spleen platelets are present in the muscle, skin, intestines and kidneys [S-9], thereby indicating that the bone marrow sequesters approximately 15% of senescent platelets. The platelets not present in the spleen, liver and bone marrow are most likely used to maintain vascular integrity.⁸ By using compartmental analysis, we estimated that this component is approximately 11% [S-21, S-23], similar to the estimate in patients with bone marrow hypoplasia.⁸

The mechanism whereby macrophages recognise senescent platelets is not known. However, there are strong indications that loss of sialic acid from the platelet membrane plays a role and that recognition of senescence may well involve immunologic mechanisms [S-19, S-20]. A possible mechanism is that platelets lose sialic acid from their membranes during their circulation in the blood and that this could expose senescent cell antigens.

⁶ Heyns et al: Kinetics and mobilisation from the spleen of In-111-labeled platelets during platelet apheresis. *Transfusion*: 25: 215, 1985.

⁷ Heyns et al: Kinetics and fate of In-111-oxine labelled platelets in asplenic subjects. *Thrombosis and Haemostasis*: 44: 100, 1980.

⁸ Hanson and Slichter: Platelet kinetics in patients with bone marrow hypoplasia: Evidence for a fixed platelet requirement. *Blood*: 66: 1105, 1985.

Antibodies recognise and bind to these antigens to form an antibody-antigen complex. Once sufficient antibody is bound to the platelet membrane, it signals that the platelet is old. This signal is recognised by the macrophages and the senescent platelets are phagocytosed. This hypothesis suggests that macrophages recognise senescent platelets as "foreign" as part of performing their normal task of surveillance against foreign cells in the circulation [S-19].

3. Application of platelet kinetic studies in patients and baboon models of thrombogenesis.

Radiolabelled platelets also be used to study, at least in part, platelet involvement in conditions characterised by increased platelet consumption, and to monitor treatment. What follows are our attempts to study increased platelet consumption.

In order to determine the sensitivity of platelet function tests to diagnose *in vivo* platelet activation and consumption, we inflicted limited and substantial injury to the vascular endothelium of baboons [S-24]. The endothelium of a segment of the right carotid artery was removed with a balloon catheter to inflict the limited injury while segments of the left carotid artery, the abdominal aorta and left femoral artery were removed to achieve the substantial injury. Of the nine tests evaluated, the most sensitive tests are measurement of the circulating platelet aggregate ratio (sensitivity 0.63), plasma levels of beta-thromboglobulin (sensitivity 0.63), and the estimate of the MPLS (sensitivity 0.50). The positive tests clearly show that exposure of the subendothelium activates circulating platelets; promotes the formation of platelet aggregates the circulation, and increases platelet consumption.

Platelets deposit onto aortic aneurysms⁹ and Dacron prostheses that are commonly used in aortofemoral reconstruction [S-25, S-26]. We have shown that the thrombogenicity of arterial grafts is not the only cause of the increase in platelet consumption since the shortening in MPLS did not correlate with factors known to be associated with graft platelet deposition, i.e. graft size, peak graft radioactivity, and the time to attain peak radioactivity. It is likely that concomitant atherosclerosis plays a substantial role in the increase in consumption [S-26], a conclusion that we verified using compartmental analyses [S-27].

⁹ Heyns et al: Kinetics and fate of In-111-oxine labelled platelets in patients with aortic aneurysms. *Archives of Surgery*: 117: 1170, 1982.

A study where we used compartmental analyses to determine the MPLS and dynamics of platelet deposition onto aortic aneurysms and grafts is of particular interest [S-27]. In both groups of patients the MPLS is comparably shortened, but the patterns of deposition of platelets are markedly different. The aneurysms are more thrombogenic and accumulate platelets more rapidly and to a greater extent than the grafts, thereby suggesting that the MPLS should be shorter than in patients with grafts. However, the fraction of platelets utilised by mechanisms other than the aneurysm or grafts, i.e. unrecognised sites of vascular disease, differs markedly. Thus, in patients with aneurysms, this component is approximately 17% of random platelet destruction compared to approximately 55% in patients with grafts. This explains the comparable shortening in MPLS. The results clearly show that useful information can be gathered from the sites of increased platelet consumption by combining MPLS and imaging data, and that it is not always wise to use only the MPLS to assess platelet participation in patients with vascular disease.

In non-human primates, the feeding of diets rich in saturated fat to non-human primates promotes atherosclerosis, while decreasing dietary saturated fat or supplementing the diet with n-3 fatty acid from fish oil may halt the progress of lesions or even cause them to regress due to inhibition of platelet function.¹⁰ In vervet monkeys with confirmed atherosclerosis, we have shown that platelet function is not inhibited after 20 months of supplementation with fish oil [S-28]. MPLS is not longer, and the circulating platelet aggregate ratio and in vitro aggregation in response to ADP and collagen is not affected. In view of studies which showed that fish oil supplementation over the short term (<1 month) inhibits platelet function, our results strongly suggest that the platelet inhibitory effects of fish oil may have been transient, and that platelets probably adapt to the lower levels of arachidonic acid in the platelet membrane. Interestingly, we found that supplementation of both an atherogenic and therapeutic diet with n-6 fatty acids from sunflower oil significantly lengthens the MPLS. This suggests that n-6 enrichment regresses atherosclerosis, or, that platelet function is markedly inhibited by the n-6 fatty acids.

We used a baboon model, where a shunt consisting of silicone rubber tubing that contains a thrombogenic surface is implanted in the right femoral artery, to evaluate the

¹⁰ Fincham et al: Atherosclerosis: Chronic effects of fish oil and a therapeutic diet in nonhuman primates. *Arteriosclerosis and Thrombosis*: 11: 205, 1991.

thrombogenicity and subsequent embolisation from the thrombogenic surface [S-29]. In this model, the microvasculature of the lower limb trapped emboli originating from the thrombogenic surface. By using In-111-platelets, we could show that knitted dacron (uncrimped external velour) material, but not reinforced expanded polytetrafluoroethylene vascular graft material, is highly thrombogenic. In addition, micro embolisation from the thrombus that formed on the dacron grafts caused occlusion of the micro circulation in the lower extremity, as was evidenced by the accumulation of labelled platelets. It takes approximately 24 hours for endogenous fibrinolysis to dissolve these microthrombi. This model is therefore also ideally suited to assess the effectiveness of different fibrinolytic treatment strategies.

A baboon model of arterial-type, platelet-dependent thrombosis was established where thrombus formation and platelet deposition after exposure to a thrombogenic surface can be studied under well-controlled conditions of blood flow and geometry [S-30]. This model forms the basis of the studies in Part 3 of this synopsis (see page 11). In brief, silastic arteriovenous (AV) shunts are implanted in the femoral vessels of the baboons. These shunts neither shorten platelet survival nor cause measurable activation of platelets or coagulation. Platelet-dependent arterial-type thrombus formation, measured as the deposition of In-111-labelled platelets, is induced using uncrimped, knitted Dacron vascular graft material that is built into the wall of silicone rubber tubing (4 mm inside diameter). Autologous blood platelets are labelled with In-111. Imaging and quantification of the deposition of In-111-platelets are done as follows. Briefly, image acquisition of the grafts, including proximal and distal silastic segments, is done with a Searle Pho scintillation camera fitted with a high resolution collimator. The images are stored on and analysed with a Medical Data Systems A³ computer, interfaced with the scintillation camera. Dynamic image acquisition is started simultaneously with the start of blood flow through the devices. An image of a 3 ml autologous blood sample is also acquired each time that the grafts are imaged to determine circulating blood radioactivity (blood standard). A region of interest of the graft segment is selected to determine the deposited and circulating radioactivity in each of the dynamic images. Radioactivity in a region of similar size in the proximal segment of the extension tubing is determined, and subtracted from the radioactivity in the graft region to calculate deposited radioactivity. Platelet deposition is expressed as the total number of platelets deposited.

Typically, when these grafts are exposed to native flowing blood, platelets rapidly deposit and deposition reaches a plateau after 60 minutes. Intermittent graft placement, which resulted in the destruction of approximately 15% of the circulating platelets each time it was placed, did not alter the survival, alpha-granule contents, or dense granule contents of those platelets that continue to circulate. This indicates that those platelets that did not interact with the surface are not adversely affected. However, since about 75% of platelets removed from the circulation are present in the thrombogenic surface when it is removed after one hour, the possibility of reversible platelet interactions in addition to removal of platelets by the thrombogenic surface by embolisation and lysis cannot be excluded. However, since the viability and the function of the circulating platelet pool is not adversely affected, such interactions must be almost totally reversible, or, affect only a small portion of circulating platelets. It is important to note that aspirin and heparin do not inhibit platelet-dependent thrombogenesis in this model.¹¹

Using this model, we showed that the blood platelet count, and not the blood flow rate, is an important determinant of the number of platelets that will deposit, i.e. that will determine the size of the thrombus [S-31]. The results suggest that a doubling in the platelet count will increase deposition by greater than twofold, whereas a doubling in the blood flow rate will result in an increase of approximately 50% in platelet deposition. Taking this onto account, one can speculate that a feasible way to treat patients at risk of developing a thrombotic episode would be to reduce their blood platelet count.

Transient thrombocytopenia develops following intravenous injection of protamine sulphate and we used In-111-labelled platelets to show that the thrombocytopenia is caused by a transient accumulation of platelets in the liver [S-32].

Extracorporeal occlusive thrombogenesis is a major problem associated with haemodialysis, and standard unfractionated heparin is currently the anticoagulant of choice to manage this. However, heparin does not completely prevent thrombogenesis while complications such as thrombocytopenia, an increased bleeding tendency, osteoporosis, increased lipolytic activity and changes in lipid patterns are associated with its long-term use. In a study where we compared the use of heparin with that of recombinant (r-) hirudin during haemodialysis, we showed that hirudin is a superior anticoagulant [S-33, S-34]. Effective dialysis with r-hirudin is achieved at a shorter activated partial thromboplastin

¹¹ Hanson and Harker. Baboon models of acute arterial thrombosis. *Thrombosis and Haemostasis*, 58:801, 1987

time, i.e. the risk of bleeding is lower. Markedly less platelets also accumulate at the inlet of the artificial kidney when r-hirudin is used, suggesting a smaller loss of hollow fibre volume and perhaps more effective dialysis [S-33]. The use of r-hirudin also has a slight, but favourable effect on complement activation, O_2 saturation and lung-diffusion capacity [S-34]. The latter two observations may be a direct result of less platelet deposition in the artificial kidney which can lead to the shedding of less micro-emboli. A matter for concern with the use of r-hirudin is that it is excreted mainly by the kidneys and therefore has a long half life in patients with renal failure [S-33]. It can therefore accumulate in the plasma with repeated use, which can increase haemorrhagic complications between haemodialysis sessions. In baboons we showed that between 50% and 60% of injected r-hirudin is excreted by the kidney. The sites of plasma elimination of the rest remains unclear, although excretion into the bile may contribute significantly [S-35].

Thrombogenesis and occlusion of the extracorporeal circuit during cardiopulmonary bypass surgery is currently prevented by the use of heparin. As in the case with haemodialysis, heparin does not completely inhibit activation of platelets and coagulation. This is evidenced by increases in the plasma levels of thrombin-antithrombin complexes, the number of circulating platelet aggregates and deposition of platelets in the oxygenator. When r-hirudin is used, none of these indices increase [S-36]. The effective inhibition of platelet-mediated thrombotic processes has important implications. There is a decreased risk of end organ damage by embolising micro-aggregates and of oxygenator occlusion by micro-emboli which, in turn, assure more effective oxygenation because a larger surface is available for gas exchange.

Determination of the platelet kinetics in patients with immune thrombocytopenic purpura (ITP) proved to be informative [S-37 - S-39]. Based on the ratio of platelet sequestration in the spleen and liver, patients with chronic ITP can be divided into those patients with a splenic sequestration pattern (spleen:liver ratio > 1.4) and those with a diffuse pattern (spleen:liver ratio < 1.4) [S-39]. We showed that those patients who have a diffuse pattern of platelet sequestration had severe ITP, characterised by pronounced thrombocytopenia, decreased platelet turnover (production) and prominent early sequestration in the liver. Patients with predominant splenic sequestration, on the other hand, have a mild form of ITP characterised by relatively high platelet counts and increased platelet turnover (production) [S-38, S-39]. We also showed that those patients with predominant splenic sequestration best responded to treatment with intravenous gammaglobulin [S-39]. This is

a useful finding, as it shows that platelet kinetic studies are able to predict which patients that will respond to gammaglobulin, which is a very expensive drug. Unfortunately, the sequestration pattern is not reliable to predict whether a splenectomy is indicated or not [S-37].

Seven to 12 % of patients with HIV present with thrombocytopenia, which is postulated to be due to increased peripheral platelet destruction, a defect in platelet production or a combination of these. In a study where we measured the MPLS in patients with HIV with and without thrombocytopenia, we found that patients with thrombocytopenia have increased peripheral platelet destruction, in contrast to other studies where ineffective platelet production was observed [S-40]. The platelet production is elevated, but is insufficient to maintain a normal platelet count. These patients also have platelet sequestration predominantly in the spleen. Of interest is the finding that some patients with a normal platelet count may also have increased platelet production. This may be an early subclinical phase in the development of full-blown HIV-associated thrombocytopenia.

Patients undergoing cardiopulmonary bypass surgery lose approximately 13% of their platelets. Of this, approximately 11% are lost through interaction with the innermost active layers of the defoaming mesh of the oxygenator. "Spent" platelets are mainly sequestered by the liver [S-41].

The MPLS is moderately shortened in patients with hypercholesterolaemia [S-42]. Platelet production is slightly elevated to maintain a normal platelet count, and the sites of sequestration of platelets are not different from normal. The important finding in this study is that platelet activation cannot solely be ascribed to hypercholesterolaemia, and that concomitant atherosclerosis may contribute significantly to activation, similar to patients with aortic grafts [S-25,S-26].

In patients with beta-thalassaemia with or without prior splenectomy, the MPLS is markedly shortened [S-43]. It strongly suggests in vivo platelet activation, probably secondary to increased consumption in these patients. The results strengthens the view that these patients may have a thrombotic tendency. Platelet activation can be secondary to injury of the vascular endothelium caused by anaemia and hypoxia and/or to chronic

haemolysis with continuous release of ADP and other thrombotic materials from haemolysing red cells.

In this section, I attempt to show how In-111-labelled platelets and scintillation camera imaging can be used as a tool to study the participation of platelets in thrombogenesis, and that useful information can be gathered from this. Biomaterials can be tested for thrombogenicity, antithrombotic drugs can be tested in various clinical and experimental settings, and the causes of thrombocytopenia determined. Different models for the study of thrombosis can also be developed in order to find answers to specific questions. Determination of the MPLS has proved useful to elucidate the mechanisms of increased platelet consumption. When determining the MPLS, one must always keep in mind that a shortening in the MPLS reflects total platelet consumption, and not only consumption by the thrombogenic surface or disease process studied. We have clearly shown in patients with abdominal aortic grafts [S-25, S-26], aortic aneurysms [S-27], hypercholesterolaemia [S-42] and beta-thalassaemia [S-43] that factors other than the disease itself increase platelet consumption. This must therefore be taken into account when a shortening in the MPLS is interpreted. Determination of the sites of sequestration of senescent platelets is useful only in patients with ITP [S-37 - S-39]. In the other studies where MPLS is shortened, the sites of sequestration of platelets were normal and of little diagnostic value.

4. Evaluation of target directed antithrombotic agents

The important role of platelets and thrombin in thrombogenesis and its sequelae of myocardial infarction and stroke, together with the rapid expansion in our knowledge of the structure-function relationships of the interactions between coagulation factors as well as the interactions of the adhesive proteins with their receptors in the platelet membrane (Figure 1) provided the impetus for the identification, synthesis and development of compounds that target specific interactions in the thrombotic process. Through collaboration with international scientists and pharmaceutical companies, we are in position to obtain some of these agents and to test their effect on thrombosis in our baboon model of platelet-dependent, arterial-type thrombosis [S-30].

Blood platelet function is almost exclusively regulated by receptors in the platelet membrane [S-18]. In the context of this synopsis, two receptor families are important, i.e. the glycoprotein receptors and the seven transmembrane receptors (Figure 1). The glycoprotein receptors and their ligands both play an important part in platelet adhesion

(platelet-subendothelial interactions) and aggregation (platelet-platelet interactions).

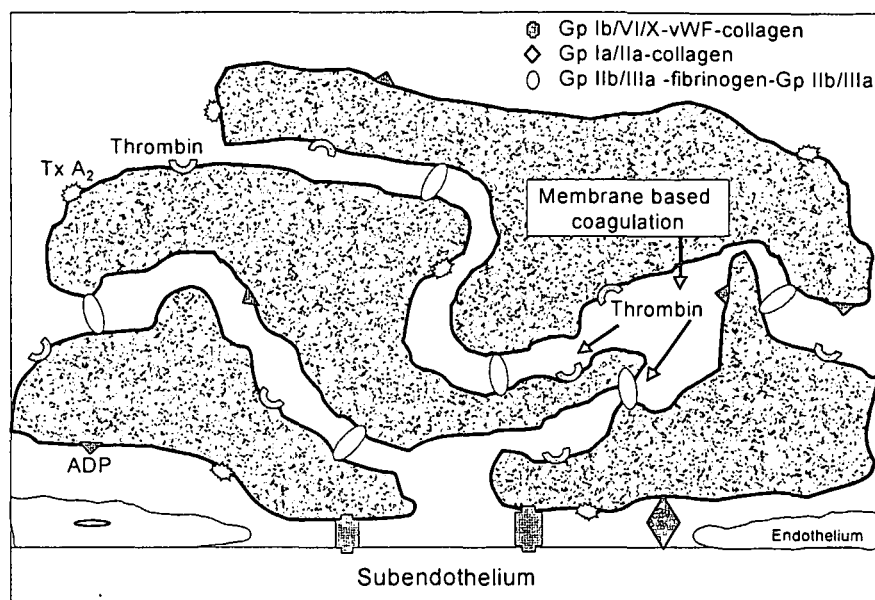


Figure 1. A schematic representation of thrombogenesis. When endothelium is damaged, circulating von Willebrand Factor (vWF) binds to exposed collagen and undergoes a conformational change to enable platelet membrane glycoprotein (Gp) Ib/V/IX to bind to it. This slows the progress of the platelet across the damaged area. Platelets are anchored (adhesion) at the site when collagen binds to Gp Ia/IIa. Binding of vWF to Gp Ib/V/IX activates platelets. This causes a conformational change in Gp IIb/IIIa to enable fibrinogen or vWF to bind to it. Fibrinogen or vWF also binds to adjacent platelets to form platelet aggregates. Platelet activation also provides a negatively charged surface, through the "flip-flop" reaction, for the coagulation complexes (tenase, prothrombinase) to bind to and so enhance the formation of thrombin approximately 10 000-fold [S18, S44]. The platelet agonists, thrombin, ADP and thromboxane A₂, each has its own seven transmembrane receptor.

Following platelet adhesion to subendothelium, the process of platelet recruitment into a growing thrombus is mediated by three independent, but interrelated platelet agonists that bind to their respective seven transmembrane receptors in the platelet membrane. Thrombin, which is generated on the phospholipid surface of activated platelets, activates the platelet thrombin receptor by a novel catalytic mechanism. ADP, secreted from storage granules of activated platelets, initiates receptor signalling of surrounding platelets. Thromboxane A₂, formed via the platelet arachidonic acid metabolic pathways, induces receptor activation.¹² It is now generally accepted that, at sites of vascular injury, thrombin is the principal mediator of platelet recruitment and plays a major part in coronary thrombosis, stroke, disseminated intravascular coagulation and thromboembolism. In addition, thrombin is the only coagulation factor that transcends all levels (plasma, blood

¹² Harker LA. Strategies for inhibiting the effects of thrombin. *Blood Coagulation and Fibrinolysis* 5: S47, 1994.

cells and fibrinolysis) and phases of haemostasis, from injury to healing. It also has several functions that are of major importance in normal and pathologic conditions.¹³ There is also strong evidence that thrombin promotes atherosclerosis because it is a mitogen that stimulates smooth muscle cell proliferation following vascular injury.

Recognition of the role of thromboxane A₂ as a physiological modulator of thrombosis, vasospasm and bronchospasm provided a strong incentive for developing thromboxane A₂-antagonising drugs.¹⁴ Two approaches have been followed: the first is to prevent thromboxane A₂ formation by inhibiting thromboxane A₂-synthetase, similar to the action of aspirin. The second is to block the thromboxane A₂ receptor. BAY U3405 is a receptor antagonist that effectively blocks the effects of thromboxane A₂ on platelets and smooth muscle cells. We have assessed the potential of BAY U3405 to inhibit acute platelet deposition in a baboon model of aspirin-resistant¹¹ platelet-dependent thrombogenesis. Platelet deposition onto native Dacron vascular graft material and onto a thrombus that was allowed to form for 60 minutes before treatment is inhibited by 33 and 58% respectively [S-44]. These results are observed despite the fact that ex vivo platelet aggregation in response to ADP and coagulation is not affected. Complete inhibition was not seen, probably because the actions of thrombin and, to a lesser extent, ADP was not affected by BAY U3405. The results strongly suggest that thromboxane A₂-receptor blockade, and not inhibition of thromboxane A₂-synthetase, is the preferred approach to inhibit the effects of thromboxane A₂ on platelets.

The initial step in haemostasis and subsequent thrombus formation is platelet adhesion through the subendothelial collagen-circulating vWF-platelet membrane Gp Ib/V/IX-axis. There are many monoclonal antibodies that were developed to bind to Gp Ib to block the binding of vWF to Gp Ib. These antibodies effectively inhibit platelet adhesion in various experimental settings in vitro. Unfortunately, they invariably cause almost immediate and profound thrombocytopenia when injected into experimental animals. As a result, no proper in vivo studies could be done to assess the effect of blockade of Gp Ib on thrombogenesis. In one of the first in vivo studies, we assessed the effect of the Fab fraction of 6B4, a monoclonal antibody that binds to Gp Ib to prevent the binding of vWF, in the baboon model of arterial thrombosis [S-45]. When injected into baboons, these fractions do not cause thrombocytopenia. Platelet dependent thrombus formation is

¹³ Fenton et al. Thrombin structure and function. Why thrombin is the primary target for antithrombotics. *Blood Coagulation and Fibrinolysis* 2: 69, 1991.

¹⁴ Ogletree. Overview of physiological and pathophysiological effects of thromboxane A₂. *Federation Proceedings*, 46:133, 1987.

dose-dependently inhibited by regulating the binding of vWF to Gp Ib with different concentrations of Fab fractions. The finding that full inhibition could not be achieved at a very high dose points to the important contributory role that Gp Ia/IIa plays in platelet adhesion. It is also important to note that inhibition of platelet adhesion leads to the formation of a much smaller thrombus than in control studies. This may have important benefits in the treatment of acute thrombosis, since these effects were observed in the absence of a bleeding tendency, i.e. the bleeding time was not prolonged by 6B4.

The binding of the adhesive proteins, fibrinogen or vWF, to Gp IIb/IIIa in the platelet membrane is the key event of thrombus growth. Binding of the adhesive proteins to Gp IIb/IIIa is partly mediated by the amino acid sequence, Arg-Gly-Asp, in the primary structure of fibrinogen or vWF [S-18]. We used a monoclonal antibody, MA-16N7C2, which contains the Arg-Gly-Asp sequence, to study the effect of Gp IIb/IIIa blockade on acute thrombosis [S-46, S-47]. MA-16N7C2, injected as a bolus, inhibits platelet deposition onto Dacron vascular graft material. The effect is dose-dependent and prolonged. Ex vivo platelet aggregation in response to ADP and collagen is also inhibited in a dose-dependent manner and the effect lasted for several days. The bleeding time is prolonged on the day of administration of 0.3 mg/kg antibody. A prolonged bleeding time is an indicator of faulty haemostasis. Of particular importance is the finding that receptor occupation with the antibody could not predict the level of inhibition that was achieved. For example, the antithrombotic effect is stronger 24 hours after a dose of 0.3 mg/kg than on the day of treatment with 0.1 mg/kg, despite the fact that the comparable numbers of Gp IIb/IIIa receptors are occupied on resting platelets. There are two possible explanations for this finding. First, with the high dose and after an extended period, occupied Gp IIb/IIIa receptors may be internalised by the platelets. Upon platelet activation, these receptors become re-exposed, but are unable to participate in thrombus formation since they are blocked with MA-16N7C2. This is in contrast to unoccupied internal Gp IIb/IIIa receptors early after the low dose. Second, the platelet α -granules may become loaded with antibody, especially after the high dose of antibody, which then would be secreted upon platelet activation in a manner similar to normal mechanism of uptake and secretion of, for example, fibrinogen. The results strongly suggest that putative "loading" of platelets with an antithrombotic can be a viable approach to safeguard patients against thrombosis.

In vivo studies with potent antithrombins made it clear that thrombin is perhaps the most important in vivo modulator of thrombosis and that inhibition of thrombin with recombinant hirudin or D-Phe-Pro-Arg Chloromethyl ketone (PPACK) or its formation with recombinant tick anticoagulant peptide (r-TAP) are effective strategies to prevent acute thrombus formation.^{15,16} We have confirmed this by using a slightly different approach [S-48-S-51]. In our studies, a thrombus was allowed to form for 15 minutes before treatment with recombinant hirudin or recombinant tick anticoagulant peptide was started. The rationale was that the 15 minutes were enough to allow for normal haemostasis following vascular surgery, and that such an approach will, at least in part, negate the danger of developing a bleeding tendency as a result of the aggressive antithrombotic treatment. Transient interruption of platelet deposition onto a fresh thrombus for four hours with r-hirudin [S-48] and two hours with r-TAP [S-50] produces a lasting and comparable effect on subsequent thrombus formation. After treatment was stopped, the rate of platelet deposition is much slower than in untreated animals, the size of the thrombus is markedly reduced and maximum deposition is reached at a later stage. These benefits are achieved despite the finding that the effect of r-hirudin and r-TAP are transient, and that after 20 - 30 hours, the thrombogenicity of the thrombus surface is similar in treated and untreated animals. The mechanism through which the long term benefit of a smaller, less thrombogenic thrombus surface is achieved is not clear. It can not be the result of residual r-hirudin or r-TAP in plasma since the half lives of both r-hirudin [S-35] and r-TAP¹⁶ are relatively short. It is possible that r-hirudin can bind to thrombin and r-TAP to Factor Xa in the thrombus and so inhibit their activity. Another possible explanation is that the thrombus undergoes autodegradation in such a manner that the thrombus surface becomes less thrombogenic over time.

r-Hirudin dose-dependently inhibits platelet-dependent thrombosis [S-49]. Although we could not define the mechanisms through which the inhibitory effect is brought about, our results suggest that two mechanisms were responsible. The one mechanism, probably the predominant one during the initial phases of thrombogenesis, is direct inhibition of thrombin through the formation of the thrombin-hirudin complex. The second mechanism is indirect. Once the complex is formed, it is reasonable to assume that there will be less thrombin to activate platelets, resulting in a decrease in platelet activation. Since thrombin

¹⁵ Hanson and Harker. Interruption of acute platelet-dependent thrombosis by the synthetic antithrombin, D-Phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone (FPRCH₂CL). *Proceedings of the National Academy of Science, USA*, 85:3184, 1988.

¹⁶ Schaffer et al. Antithrombotic efficacy of recombinant tick anticoagulant peptide; a potent inhibitor of coagulation factor Xa in a primate model of arterial thrombosis. *Circulation* 84: 1741, 1991.

mainly forms on the surface of activated platelets, it will follow that less thrombin will form.

In this study [S-49], we were able to calculate the per cent inhibition of platelet deposition and relate that to the plasma concentration of r-hirudin. The relationship is best described by an exponential association function:

$$\% \text{ Inhibition} = 95(1 - e^{-0.23 \times [\text{r-hirudin}]})$$

where $n = 21$ and $R^2 = 0.76$. The relationship implies that platelet deposition will be inhibited by 50% at a plasma concentration of 3.3 $\mu\text{g/ml}$ and by 80% at a concentration of 8.1 $\mu\text{g/ml}$ r-hirudin. In view of the reproducibility of the results, one can calculate the rate at which r-hirudin must be infused to maintain a plasma concentration that will result in a given inhibition of platelet deposition.

No satisfactory methods exist by which antithrombotic therapy can be applied to sites of vascular injury. Consequently, systemic anticoagulation remains the only option for the prevention of occlusion of, for example, vascular grafts, angioplasty sites and intravascular stents. Unfortunately, anticoagulation is associated with significant risk of haemorrhagic complications. An ideal therapy would therefore provide an effective local antithrombotic action, active only at selected sites, without altering systemic coagulation. We have produced a local antithrombotic effect by topical application of PPACK [S-52]. PPACK binds irreversibly to thrombin to inhibit its activity, and we used this characteristic to achieve a local antithrombotic effect. Pre-clotted Dacron vascular grafts were incubated for 10 minutes with PPACK (10 mg/ml) and then exposed to flowing blood in an arteriovenous shunt [S-30]. When compared to control grafts, the pretreated graft accumulated approximately 50% less platelets. Therefore, by using this approach, we could attain antithrombotic activity at the site where it is needed, without producing systemic anticoagulation. In this study, we have shown that local therapy represents a new concept in antithrombotic therapy with a wide range of potential clinical applications. Numerous questions remain to be answered: how brief an exposure period is necessary, what concentration is required, and will this influence development of neointimal hyperplasia. Based on these results, devices were developed to provide effective local antithrombotic therapy.¹⁷

¹⁷ Markou et al, A novel method for efficient drug delivery. *Annals of Biomedical Engineering*, 26:502, 1998.

Application of molecular biotechnology in the field of thrombosis and haemostasis identified the molecular basis of the reactions in the coagulation cascade, platelet adhesion and aggregation and fibrinolysis. The result is that the amino acid sequences of the epitopes in proteins that bind to their substrates are known. We have used this knowledge to design a multifunctional, antithrombotic peptide, PLATSAK [S-53] (Figure 2).

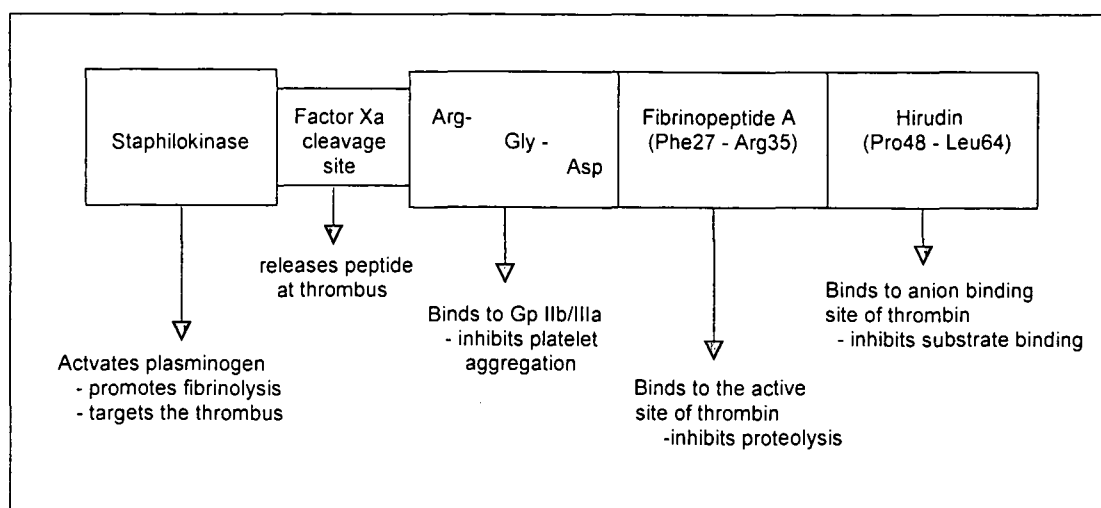


Figure 2. A schematic representation of the functional domains of PLATSAK

The peptide is designed in such a way that the staphylokinase, which is highly specific for fibrin in fibrin clots, will bind to the thrombus and enhance fibrinolysis. Since the thrombus and surrounding area are rich in coagulation factor Xa, the antiplatelet/antithrombin part will be released through Factor Xa cleavage to release it in close proximity of the thrombus. Since PLATSAK is site directed, one can postulate that much less of it will be needed to inhibit thrombogenesis, which in turn will decrease the development of haemorrhagic complications. In vitro, PLATSAK displays potent antithrombin and fibrinolytic activity. Antiplatelet activity, on the other hand, is not as expected [S-53]. We have tested the in vivo effect of PLATSAK on arterial and venous thrombosis in our baboon model [S-54]. PLATSAK inhibits both arterial and venous thrombosis markedly. The results also strongly suggest that the Arg-Gly-Asp amino acid sequence is effective to block platelet-platelet interaction in vivo, unlike the results obtained in aggregation studies in vitro [S-53]. Our results also clearly indicate that results obtained in vitro not always reflects the situation in vivo.

In conclusion, knowledge of the structure-function relationships and the use of molecular biotechnology has resulted in the identification and development of numerous peptides/proteins that target specific interactions in the thrombotic process. We and others

have clearly shown that these agents are highly effective in managing thrombosis. In addition, these studies taught us much about the mechanisms of interaction of the blood coagulation factors with each other and their interaction with platelets. They also helped to clarify the dynamics of platelet adhesion and aggregation. One of the major concerns regarding the use of these peptides/proteins is the danger of causing serious haemorrhagic complications since they virtually prevent normal haemostasis. This led to the approach of local treatment at the site of injury/thrombosis, or, the start of treatment after normal haemostasis has been attained following vascular surgery. Although these approaches have not been tested in appropriate patients, it may well be worth our while to do it. Another concern is immunogenicity of these agents since they are "foreign proteins" that are injected. This complication can be negated by humanising monoclonal antibodies, by using proteins that are too small to elicit an immune reaction, or, by using only the amino acid sequence that binds and blocks the active site of the target. In spite of these possible disadvantages, we are witnessing an era where it may become possible to effectively manage thrombosis in patients who are at risk of developing thrombotic episodes. These include patients undergoing vascular engraftment, arterial endarterectomy, stenting and balloon catheterisation.

5. General conclusions.

Our main contributions in the field of normal platelet kinetics were to develop methods to label a representative platelet population and to accurately measure its *in vivo* distribution when reinjected. Application of these techniques enabled us to establish the MPLS and size of the splenic platelet pool accurately, and to show conclusively that there is no other pool for platelets in the body. We also established the sites of sequestration of senescent platelets, and showed that approximately 11% of platelets are normally utilised to maintain the vascular integrity. We further pointed to the important role of platelet membrane sialic acid in determining platelet senescence and that the mechanism of recognition of senescent platelets by the macrophages may well be immunologic.

In patient studies and the different experimental models that we used to study increased platelet consumption, it became clear that determination of the sites of sequestration of senescent platelets was of little diagnostic value when the increased consumption was due to thrombogenesis. On the other hand, when increased consumption was immunologic in nature, determination of these sites may be helpful to determine the approach to treatment in patients with ITP. We also showed that determination of the MPLS alone may be of

little value if it is not done in conjunction with image analysis of, for example, platelet deposition onto Dacron vascular graft material or aneurysms. We have also shown that direct inhibition of thrombin with r-hirudin, rather than indirect inhibition with heparin, may be to the advantage of patients undergoing cardiac surgery and haemodialysis. Determination of platelet turnover in patients with HIV infection may be helpful to identify those patients that are at risk of developing thrombocytopenia since we could identify a group of patients with normal platelet counts but increased platelet production.

We have conclusively shown that target-directed antithrombotic agents are potent inhibitors of arterial thrombosis. Of special interest is our finding that inhibition of platelet adhesion limits the size of the thrombus that ultimately forms when native flowing blood is exposed to a collagen rich surface. Inhibition of platelet aggregation by preventing the binding of fibrinogen to its platelet membrane receptor, Gp IIb/IIIa, or, direct inhibition of thrombin and its formation are viable approaches to inhibit thrombosis. Of particular importance is our finding that transient inhibition of thrombogenesis over the short term has significant advantages with respect to the size and thrombogenicity of a thrombus that forms following the inhibition. This can have major advantages in the treatment of patients undergoing vascular engraftment, endarterectomy, stenting and balloon catheterization. We have also shown, both in vitro and in vivo, that it is viable to combine different moieties of active peptides that inhibit different components of the thrombotic process in such a way that the individual moieties retain their function.

Finally, the combination of blood cell biology, radioisotopes and the technology of the scintillation camera and computer-assisted image acquisition and analysis enabled us to study normal platelet kinetics and the kinetics and fate of platelets in patients with increased consumption extensively. We also used these methods to investigate factors that contribute to thrombogenesis as well as the effectiveness of antithrombotic agents.

5. List of publications related to this thesis

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Kinetics of In-111-Platelets in the Baboon: I. Isolation and Labelling of a Viable and Representative Platelet Population ^{S-1}

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Key words

Indium-111 – Primates – In-111-labelled platelets – Platelet labelling technique – Blood platelets

Summary

A fully representative and viable platelet population was isolated from the blood of 15 baboons by a multiwash procedure, and labelled with In-111-oxine. The recovery of the total platelet population in the circulation was $85\% \pm 9$. Mean platelet life span was $146 \text{ hr} \pm 13$. Correcting for plasma radioactivity (always less than 3.5%) did not significantly affect the estimate of platelet life span ($145 \text{ hr} \pm 16$) or recovery ($85\% \pm 12$). Platelet survival estimates, repeated at different times, were reproducible. In 5 baboons, platelets were also harvested by a single step differential centrifugation. The mean life span of a representative platelet population was significantly longer than that of platelets harvested by a single step. Recovery values of the representative and non-representative population were similar. We conclude that it may be important to harvest and label a fully representative platelet population for kinetic studies. The proposed method is simple and reproducible, and may be applied in studies in humans.

Introduction

It has been proposed that newly formed platelets are large and heavy, and that these platelets survive longer than small light platelets (1, 2). It was also suggested that the younger platelets are hemostatically more active (3), and are preferentially pooled in the spleen (4, 5). It is also known that not all platelets are harvested with the commonly employed single step differential centrifugation method (6), and those platelets not harvested are the larger, heavy population (1). Recently it has been shown that platelet buoyant density, and possibly platelet volume, are not important determinants of platelet age, and that platelet heterogeneity is a function of thrombopoiesis (7–10). Nevertheless, it would seem important to harvest a platelet population which fully represents those in the circulation for platelet kinetic studies. This would ensure optimal interaction of labelled platelets with thrombogenic surfaces and permit accurate kinetic studies of the *in vivo* redistribution of platelets during their life span.

In this study we report a simple method to isolate and label with In-111-oxine a representative and viable platelet population

from the whole blood. We compare the mean life span of this labelled population with that of platelets isolated with the usual single step differential centrifugation method. Since the results differed, we suggest that it is important to harvest a total platelet population for kinetic studies.

Materials and Methods

Experimental Animals

Fifteen baboons (*Papio ursinus*), weighing $18.6 \pm 5.7 \text{ kg}$ ($\pm 1 \text{ SD}$), apparently free of disease and in captivity for at least 6 months, were studied. Anaesthesia was with intramuscular ketamine hydrochloride (Parke-Davis), 10 mg/kg body mass.

The project was approved by the Ethical Committee of the University of the Orange Free State.

Blood Collection

Venous blood was collected with a two syringe technique from the femoral vein. Blood, 42.5 ml, was collected into a syringe containing 7.5 ml acid-citrate-dextrose, formula A (Fenwall, Baxter-Travenol Laboratories Inc., Illinois).

Harvesting a Representative Platelet Population

The platelets were isolated and labelled with In-111-oxine as described in detail elsewhere (11). All procedures were carried out at room temperature. Briefly, platelets were isolated by centrifuging the blood at 180 g for 15 min. The platelet rich plasma was removed and the red blood cells resuspended to their original volume in Plasmalyte B (Baxter, Baxter-Travenol Laboratories Inc, Deerfield, Ill). Platelets were washed from the red blood cell layer by centrifuging at 750 g for 3 min. The washing procedure was repeated a total of 4 times. Platelets were concentrated to a pellet by centrifuging at 750 g for 25 min. The platelet poor plasma (PPP) was aspirated and the platelets resuspended in 4–5 ml physiological saline. In-111-oxine (Radiochemical Centre, Amersham, United Kingdom), 25 to 29 MBq, was added and the platelets incubated at room temperature for 30 min. Two ml PPP was added, the platelets concentrated by centrifuging at 750 g for 25 min, and the supernatant decanted. The labelled platelets were finally resuspended in 5 ml PPP, and not reinjected for at least 60 min.

Single Step Platelet Harvesting

Platelets were isolated by the usual single step differential centrifugation technique recommended by the ICSH (12).

Evaluation of Platelet Populations

A fully representative platelet population was harvested in baboons 1 to 6 and platelet life span determined. The reproducibility of this method was assessed by repeating the study 2 months later.

Platelets were harvested by the single step technique in baboons 1, and 7 to 10 and platelet life span determined. This study was repeated two months after the isolation of a representative platelet population in the same baboons.

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Platelet Survival

The mean platelet survival time was estimated as recommended by the ICSH (12). Blood was collected from the saphenous vein 5 min after reinjection of the In-111-platelets, and thereafter daily for 7 days. Whole blood radioactivity was measured, and the radioactivity in an aliquot of platelet free plasma, prepared by centrifugation at $3000 \times g$ for 30 min, determined. Platelet life span was calculated by fitting the data points to a gamma function (13). Platelet life span was estimated in two ways: from whole blood radioactivity, and also after correction of whole blood radioactivity for plasma radioactivity. The recovery of In-111-platelets in the circulation at equilibrium was calculated from the blood volume, assumed to be 63 ml/kg body mass (14), and by back extrapolation of the survival curve to zero time. Platelet turnover was calculated according to the method of Harker and Finch (15).

Other Platelet Tests

Platelets were counted with an electronic particle counter (Coulter Electronics, Florida), and the percentage of platelets harvested was calculated. Mean platelet volume was estimated with an electronic particle sizer and channelizer as described by Corash et al. (1, 2). Viability of the labelled platelets was assessed by their *in vitro* response to ADP (16), and the labelled platelets were not reinjected unless 10 or more platelets per aggregate was observed.

Statistical Analysis

The significance of results were assessed with the t-test for paired data. Results are given as a mean $\pm$ 1 SD.

Results

Harvesting, Labelling and Survival of a Representative Platelet Population

The details of the results of platelet counts, mean platelet volume, harvesting and labelling are summarized in Table 1. The harvesting procedure was highly efficient and a mean of 97% of those platelets present in the whole blood were isolated. There was however, a loss of platelets, $30.0\% \pm 7$, during the steps when the platelets were concentrated and labelled with In-111-oxine. The mean volume of the harvested platelets, $5.10 \text{ fl} \pm 0.84$, and the labelled platelets, $5.01 \text{ fl} \pm 0.68$, did not differ significantly ($p > 0.70$). Platelet labelling efficiency was $83\% \pm 4$, and the final platelet product was minimally contaminated with red blood cells and plasma proteins. The platelet concentrates were contaminated with less than 1 leucocyte per 200 platelets, as

Table 1 The results on platelet isolation and labelling

Parameter	Total platelet population (n = 15)	Inefficiently harvested platelets (n = 5)
Blood platelet count ($\times 10^9/\text{l}$)	371 ± 90	295 ± 83
Labelling efficiency (%)	83 ± 4	82 ± 13
Platelet contamination (%) with labelled		
Red blood cells	1.4 ± 1.1	2.2 ± 1.6
Plasma proteins	2.1 ± 0.8	1.6 ± 1.3
In-111-platelets reinjected ($\times 10^9$)	8.5 ± 3.3	2.5 ± 1.4
² Harvesting efficiency (%)		
Final isolate	97 ± 4	28.0 ± 5.8
In-111-platelets	67 ± 6	19.4 ± 5.3
Mean platelet volume (fl)		
Final isolate	5.10 ± 0.84	4.46 ± 0.17
In-111-platelets	5.01 ± 0.68	4.51 ± 0.34

¹ Values are given as a mean $\pm$ 1 SD.

² Expressed as a percentage of platelets present in whole blood.

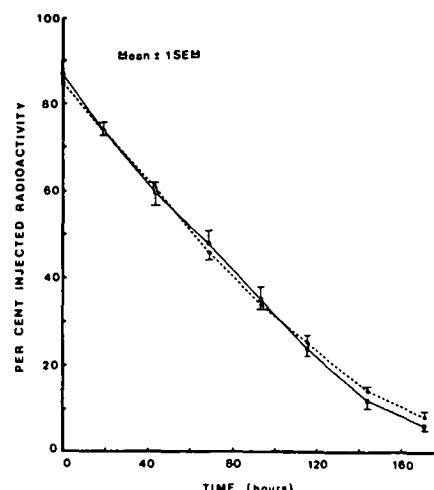


Fig. 1 The disappearance of In-111-platelets in the 6 duplicate studies where a total platelet population was harvested from the whole blood. Values are expressed as a percentage of total radioactivity injected.

assessed microscopically (results not given). In the 6 studies where the procedure was repeated after an interval of 2 months platelet harvesting and labelling results did not differ significantly ($p > 0.10$). The *in vivo* aggregation response to ADP was normal or more platelets per aggregate, in all instances.

The composite disappearance curves of the In-111-platelets from the circulation was linear (Fig. 1). Mean platelet life span was estimated to be $146 \text{ hr} \pm 13$ (Table 2). Recovery of labelled platelets in the circulation was $85\% \pm 9$. Plasma radioactivity $3.5\% \pm 1.6$ 5 min after reinjection of the In-111-platelets, remained less than 3% of that of whole blood throughout

Table 2 The recovery and survival of In-111-platelets in the circulation

Baboon	Total platelet population Recovery (%)	Life span (hr)	Repeat studies Recovery (%)	Life span (hr)
1	93	181	90	161
2	93	144	87	159
3	84	158	85	153
4	80	127	83	131
5	87	149	86	143
6	85	134	81	138
¹ Mean $\pm$ 1 SD	87 ± 5	149 ± 19	85 ± 3	148 ± 12
1	93	181	80	140
7	72	147	85	128
8	91	147	80	144
9	93	155	93	128
10	93	137	92	137
² Mean $\pm$ SD	89 ± 10	153 ± 17	86 ± 6	135 ± 7
11	81	152	-	-
12	90	139	-	-
13	76	138	-	-
14	67	148	-	-
15	92	131	-	-
³ Mean $\pm$ 1 SD	85 ± 9	146 ± 13	-	-

¹ The mean $\pm$ 1 SD of the 6 repeat studies where a total population was harvested.

² The mean $\pm$ 1 SD of the 5 studies where platelets were also harvested by a single step differential centrifugation.

³ The mean $\pm$ 1 SD of the 15 studies where a total platelet population was harvested.

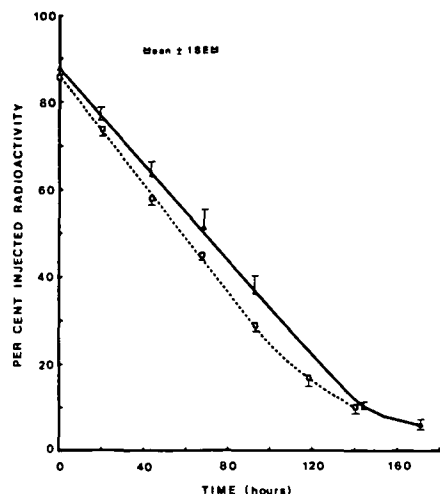


Fig. 2 The disappearance of a representative (solid line) and non-representative (broken line) population of In-111-platelets from the circulation ($n = 5$). Values are expressed as a percentage of total radioactivity injected.

platelet survival time. Correction of whole blood In-111 radioactivity for plasma radioactivity had no significant influence ($p > 0.60$) on the estimate of platelet recovery in the circulation ($85\% \pm 12$) or mean platelet survival ($145 \text{ hr} \pm 16$). Platelet turnover was $66 \pm 18 \times 10^9$ platelets/litre/day.

In the 6 studies where platelet survival was repeated 2 months later the results of the estimates of platelet recovery and mean platelet life span did not differ significantly ($p > 0.10$; Fig. 1; Table 2).

Platelets Isolated by a Single Step Differential Centrifugation

Results are summarized in Table 1. In 5 studies with this method $28.0\% \pm 5.8$ of those platelets present in whole blood were harvested. There was a further loss of platelets in the labelling procedure and only $19.4\% \pm 5.3$ of the total platelet population was labelled. The mean volume of the labelled platelets, $4.51 \text{ fl} \pm 0.34$, was significantly ($p < 0.05$) less than that of the total platelet population, $5.04 \text{ fl} \pm 0.46$.

The platelet labelling efficiency was $82\% \pm 13$. The final product was minimally contaminated with red blood cells and plasma proteins. Labelled platelets aggregated normally with ADP added *in vitro*. Disappearance of the labelled platelets from the circulation was almost linear (Fig. 2) with a mean life span of $135 \text{ hr} \pm 7$.

The mean life span of the single-step isolated platelet population was significantly shorter than that of the total platelet population in the same baboons ($p < 0.05$). Recovery in the circulation of labelled platelets, isolated by the multi- and single-step techniques, did not differ significantly ($p > 0.10$).

Discussion

A fully representative platelet population was harvested by a multiwash technique. The harvesting and labelling procedures did not affect *in vitro* platelet aggregation. The *in vivo* behaviour of the labelled platelets was apparently also unaffected since recovery of platelets in the circulation was normal and the disappearance of labelled platelets from the circulation fitted a linear function best. Also, the results obtained with this method were similar to those reported for Cr-51-labelled platelets in other baboon species (3, 14, 17). The multiwash technique is simple and

reproducible and may readily be applied for the study of platelet kinetics in other species, including man.

The multiwash isolation of the fully representative platelet population was efficient, but the final concentration steps in the labelling procedure caused a loss of about 30% of the harvested platelets. This was, however, not due to a selective loss of a specific subpopulation of platelets, since the mean volume of the platelet population did not change. There were differences between the platelets harvested by the single-step method and those procured by the multiple-wash steps. Firstly, the mean volume of the latter platelets was larger. Secondly, the mean life span of the representative platelet population was significantly longer than that harvested by a single centrifugation step. These results indicate that the efficient harvesting of platelets will include a relatively larger and young subpopulation of platelets which are not isolated by the usual recommended methods. These findings are in accordance with those reported by Corash et al. (1, 2). It has also been suggested that the large platelets are not necessarily younger platelets, but rather platelets with a longer life span (8). Our study was not designed to test this hypothesis. It should be noted that our results differ from those reported by Mezzano et al. (10) who found no difference in the mean platelet survival of representative and inefficiently harvested platelet populations. We have no ready explanation for the discrepancy, but there are major technical differences in the methodology applied in our and their studies.

Recovery of the fully representative population in the circulation did not differ from that of the platelets harvested by single-step centrifugation. This would suggest that platelet size or platelet age per se do not influence the size of the extravascular platelet pool.

We have not compared the functional effectivity of the platelet populations harvested by the two techniques. It seems reasonable, however, to consider that the study of the kinetics of a representative platelet population offers advantages. Application of this method will at least not lead to the exclusion of a subpopulation of platelets which may be functionally more effective. This view is strengthened by the recent reports that different subpopulations may have different aggregation responses (18) and that their granular content may vary (19). It is therefore important to study platelet kinetics in disease states and the interaction of platelets with foreign surfaces with a fully representative platelet population.

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Comparison of Oxine and Tropolone Methods for Labeling Human Platelets with Indium-111

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The effect of the chelates oxine and tropolone, used to label platelets, on the kinetics of indium-111- (^{111}In) labeled platelets was studied in twelve normal human subjects. Autologous platelets were labeled either in saline with ^{111}In -oxine or in plasma with ^{111}In -tropolone. Mean platelet lifespan was estimated by fitting the disappearance curve of platelets from the circulation to the multiple hit and other mathematical models. The in vivo distribution of platelets was quantitatively imaged with a scintillation camera. The in vivo recovery of ^{111}In -oxine and ^{111}In -tropolone did not differ, and the mean platelet lifespan was also similar (^{111}In -oxine: 230 ± 29 hr; ^{111}In -tropolone: 226 ± 13 hr). At equilibrium (90 min after reinjection of labeled platelets) and at the end of platelet lifespan, ^{111}In -oxine and ^{111}In -tropolone radioactivities in the spleen and liver were similar. These results demonstrate that the results of kinetics measured with ^{111}In -oxine or ^{111}In -tropolone do not differ significantly.

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Indium-111 (^{111}In) is the radionuclide recommended by the International Committee for Standardization in Hematology as the platelet label of choice (1). Although other ^{111}In -chelates have been advocated (2,3), this Committee recommends oxine and tropolone as the most suitable for platelet studies. It is, however, not clear from the results of several studies whether one of these is superior (2-12).

Theoretically, oxine is not an ideal chelate. First, some of the commercially available ^{111}In -oxine complexes contain alcohol, which may affect platelet function (5). Second, the labeling efficiency of ^{111}In -oxine is considerably higher in a medium with no plasma, than in plasma (5-8); this is unfavorable because labeling and handling platelets in an artificial medium may induce in vitro damage to platelets (9,10). These negative aspects may be overcome with ^{111}In -tropolone, a

chelate which permits labeling of platelets with relatively high efficiency in a plasma medium (4).

There is a prevailing view (2-4) that tropolone is superior to oxine because with the former, platelets are labeled in plasma and not in saline. This is considered the reason why the kinetics of ^{111}In -oxine and ^{111}In -tropolone labeled platelets have been reported to differ (2, 3,10). Thus, it has been stated that ^{111}In -tropolone labeled platelets do not accumulate transiently in the liver immediately after their reinjection (2,3). Also, the recovery fraction of ^{111}In -oxine labeled platelets in the circulation was reported to be higher than those labeled with ^{111}In -tropolone (10).

However, this view has not been substantiated by convincing scientific data. Indeed, the results of the few well-documented studies have not clarified the issue. For instance, Vallabhajosula et al. (11) conclude that platelets labeled with ^{111}In -tropolone in plasma are preserved better in the circulation and have an equal or longer lifespan than platelets labeled with ^{111}In -oxine in ACD-saline. Furthermore, there was evidence that the ^{111}In -oxine labeled platelets were temporarily sequestered in the liver to a greater extent than those labeled in tropolone. Hill-Zobel et al. (12) expressed a similar view on the basis of results in a rabbit model but were not sure that these results can be extrapolated to human conditions. In contrast, in a preliminary study, we found no evidence that the results of our method of labeling platelets with ^{111}In -oxine differs from those with tropolone (13).

It is important to establish whether there is a difference between these two chelates. Indium-111-oxine is widely used and marketed commercially as an approved platelet label. If results with oxine do indeed differ substantially from those of tropolone, it may, therefore, have important economic implications. Furthermore, the recommendation that either tropolone or oxine may be used (1) will also need to be revised if the commendable goals of standardization are to be attained.

We addressed this question by comparing, in normal human subjects, the labeling efficiency and kinetics of platelets labeled with ^{111}In -tropolone in plasma with those of platelets labeled with ^{111}In -oxine in saline. The

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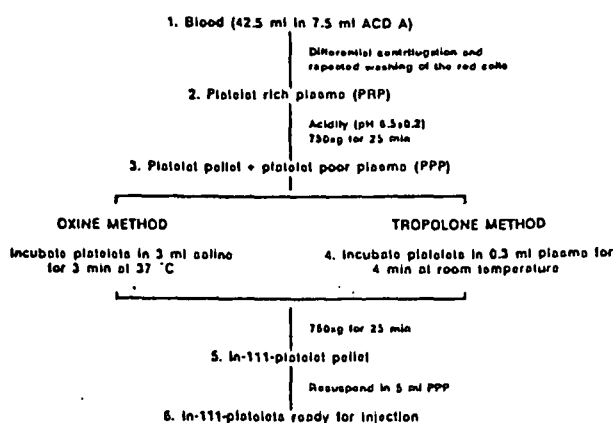


FIGURE 1
Methodology for labeling platelets with ^{111}In -oxine and ^{111}In -tropolone.

techniques that we used are appropriate for such a comparative study: they have been validated and provide quantitative data on the in vivo distribution of the labeled platelets.

MATERIALS AND METHODS

Subjects

Twelve normal human subjects (6 males) ranging in age from 18 to 35 yr, volunteered for a study approved by the Ethical Committee of the Provincial Administration and University of the Orange Free State. The blood platelet counts of the subjects were normal (range $200/l$ to $304 \times 10^9/l$). The subjects were instructed not to take any drugs, known to affect platelets, for at least 2 wk before and during the 7-day study period.

The volunteers were randomly divided into two groups of six. Autologous platelets were labeled either with ^{111}In -oxine (Amersham International plc, UK) or ^{111}In -tropolone (^{111}In -chloride supplied by Amersham International plc, UK, and tropolone by Sigma Chemical Corp., St. Louis, MO).

Platelet Labeling with ^{111}In

After discarding the first 2 ml, 42.5 ml blood were drawn from the antecubital vein with a 19-g Abbot "butterfly" needle directly into a polystyrene syringe containing 7.5 ml ACD A (Baxter Travenol, Baxter Laboratories). The blood was transferred to 2×50 ml polystyrene tubes (Falcon 2070, Falcon Labware, Oxnard, CA) and platelets were harvested from the blood by differential centrifugation and repeated washing of the red cells (7,8,14). The mean recovery of platelets from the blood was $95\% \pm 3\%$.

The platelets were labeled as outlined in Figure 1. In the

oxine method (8), the platelet pellet was gently resuspended in 3 ml saline and incubated with the radionuclide. In the tropolone method (15), the labeling medium was 0.3 ml plasma. Tropolone (40 nM final concentration), followed by ~ 17 MBq ^{111}In -chloride, were added. In both methods, unbound radionuclide was removed by withdrawing the supernatant from the centrifuged labeled platelets. The latter were resuspended in platelet poor plasma for reinjection.

Labeled platelets were considered viable if aggregates consisting of 20 or more platelets were induced by ADP (7). Contamination of the labeled platelets with red cells and plasma proteins, the labeling efficiency, and the radioactivity of the injected labeled platelets, were measured (16).

In Vivo Platelet Kinetic Studies

Mean Platelet Lifespan (MPLS). MPLS was estimated from the rate of disappearance of the labeled platelets from the circulation. The disappearance curves were constructed from the radioactivity in blood samples collected at 5 and 90 min after reinjection of the labeled platelets, and then daily for 6 days. The count rate of the blood samples was measured in a well-type scintillation counter. MPLS was estimated by fitting several mathematical models to the blood clearance curves (17).

The recovery of labeled platelets in the circulation at equilibrium was calculated from the radioactivity/ml injected, an estimate of the blood volume (18), and extrapolation of the survival curve back to zero time (8,14).

Blood platelets were counted with an electronic particle counter (19). Mean platelet turnover was calculated from the MPLS and the platelet count was corrected for the splenic platelet pool (20). The size of the latter was determined with quantitative imaging.

In Vivo Quantification of the Distribution of Labeled Platelets. The methods have been described in detail elsewhere (8, 21,22). Briefly, the distribution of platelets was imaged with a large field of view scintillation camera and the scintigrams were analyzed on an A³ MDS data processing system. Whole-body and region of interest (ROI) ^{111}In radioactivity was corrected for geometry with the geometric mean method. The ROI activity was expressed as a percentage of whole-body activity. The radioactivity not in the liver or the spleen, is referred to as the "remainder." At the end of platelet lifespan, most of this radioactivity is located in the bone marrow (8, 21). The initial (equilibrium) radioactivity was derived by back extrapolation of linear least-square regression analysis of data, and the final organ radioactivity was determined at the end of the MPLS.

Statistical Methods

Data were assessed for normal distribution with the Shapiro-Wilks test. Unpaired data were evaluated with the t-test.

TABLE 1
Platelet Labeling Data

	Oxine	Tropolone	t value	p value	95% confidence interval
Labeling efficiency (%)	92 ± 2	79 ± 10	3.1225	0.03	4-22
% Contamination with:					
red cells	1.2 ± 0.4	1.8 ± 2.5	-0.5805	0.59	-1.7-2.9
plasma proteins	2.2 ± 0.5	1.7 ± 1.1	0.0136	0.99	-1.4-2.4

TABLE 2
Survival Data of Platelets Labeled with ^{111}In -oxine and ^{111}In -tropolone

	Oxine	Tropolone	t value	p value	95% confidence interval
Recovery (%)	70 $\pm$ 10	58 $\pm$ 11	1.9772	0.11	-1-25
Platelet turnover ($\times 10^9/\text{hr}$)	1.41 $\pm$ 0.32	2.00 $\pm$ 0.49	2.4694	0.06	-0.026-1.20
"Hits"	44 $\pm$ 30	20 $\pm$ 20	1.6305	0.16	-8-56
Mean Platelet Lifespan (hr):					
Linear	242 $\pm$ 23	228 $\pm$ 13	1.2980	0.25	-14-42
Weighted mean	225 $\pm$ 33	221 $\pm$ 11	0.2817	0.79	-33-41
Multiple hit	230 $\pm$ 29	226 $\pm$ 13	0.3083	0.77	-24-32
Dornhorst	219 $\pm$ 25	222 $\pm$ 10	0.2729	0.80	-31-25
Alpha order	224 $\pm$ 37	221 $\pm$ 10	0.1917	0.94	-37-43

for different means, or the non-parametric Wilcoxon rank sum test where appropriate. The difference between sample means was compared by determining the 95% confidence intervals (23).

RESULTS

Platelet Labeling

The mean blood platelet count of the two groups did not differ significantly (oxine $249 \pm 39 \times 10^9/\text{l}$ versus tropolone $282 \pm 45 \times 10^9/\text{l}$; $p = 0.23$). The results are summarized in Table 1. Labeling efficiency with oxine was significantly ($p = 0.03$) higher than with tropolone. Contamination of the labeled platelet product with red cells and plasma proteins was slight and similar with both methods. There was less than 1 leukocyte per 1,000 platelets present in the ^{111}In -labeled platelet suspensions that were reinjected.

In Vivo Platelet Kinetics

Recovery of ^{111}In -oxine (70% $\pm$ 10%) and ^{111}In -tropolone (58% $\pm$ 11%) labeled platelets in the circulation did not differ significantly (Table 2).

Mean Platelet Lifespan. The mean curves describing the disappearance of the labeled platelets are illustrated

in Figure 2. The MPLS, calculated with the different mathematical models and number of "hits" of the multiple hit model, did not differ significantly for oxine- and tropolone-labeled platelets (Table 2). The large number of "hits" of the multiple hit model indicate a near linear platelet survival curve with both labels.

In Vivo Distribution and Sites of Sequestration at Equilibrium. The results are summarized in Table 3, and the initial postinjection phase is illustrated in Figure 3.

The time-radioactivity curves, illustrating the redistribution of oxine- and tropolone-labeled platelets in the spleen and the liver for the first 90 min after reinjection of the labeled platelets, are presented in Figure 3. The radioactivity in the spleen increased monoexponentially. The rate constant of the increase of oxine-labeled platelets in the spleen was 0.177 ± 0.041 per minute and that of tropolone-labeled platelets was 0.155 ± 0.041 per minute. This difference was not significant ($t = 0.7675$, $p = 0.46$; 95% confidence interval -0.052-0.096).

A transient accumulation of radioactivity in the liver was evident with both the labeling methods (Fig. 3); this represents the "collection injury" (24). The extent of the injury observed with the two labeling methods was compared by measuring the area under the respective time-activity curves. This did not differ significantly ($t = 0.797$, degrees of freedom = 10, $p = 0.44$).

At equilibrium, 90 min after reinjection of the ^{111}In -platelets, the size of the splenic platelet pool and the radioactivity in the liver were similar for the two methods (Table 3).

In Vivo Distribution and Sites of Sequestration at the End of Platelet Lifespan. The results are summarized in Table 3. The contributions of the various components of the reticuloendothelial system to the sequestration of senescent ^{111}In -oxine- and ^{111}In -tropolone-labeled platelets did not differ significantly.

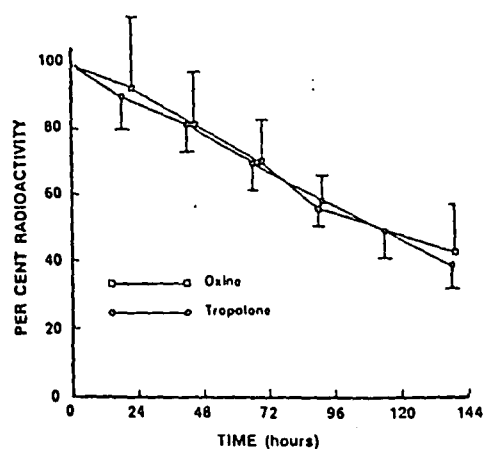


FIGURE 2

The curves demonstrate the disappearance of ^{111}In -oxine and ^{111}In -tropolone labeled platelets from the circulation. The values are expressed as a mean (± 1 s.e.m.) percent of the count rate of the blood sample collected at 5 min.

DISCUSSION

The methods to label platelets either with ^{111}In -oxine or ^{111}In -tropolone differ in one important aspect, namely the labeling medium which was used. A high

TABLE 3
In Vivo Distribution and Sites of Sequestration of Platelets Labeled with ^{111}In -oxine and ^{111}In -tropolone*

	Oxine	Tropolone	t value	p value	95% confidence interval
Equilibrium distribution					
Spleen	30.0 ± 6.3	30.1 ± 6.3	0.1442	0.89	-7.1-8.1
Liver	9.6 ± 1.2	9.5 ± 1.4	0.1328	0.90	-1.5-1.7
Distribution at MPLS					
Spleen	37.2 ± 8.8	45.3 ± 6.9	1.7743	0.14	-12.7-3.5
Liver	29.2 ± 9.6	31.3 ± 6.4	-0.4458	0.67	-12.4-8.2

* Values are given as a mean (± 1 s.d.) percent of whole-body radioactivity.

labeling efficiency is achieved with ^{111}In -oxine only if the platelets are suspended in an artificial medium with no plasma (3,8). In contrast, platelets may efficiently be labeled in plasma with ^{111}In -tropolone (4,15). This difference may be important because platelets are apparently better preserved in plasma than in artificial media (3,4,10). It is, therefore, conceivable that the tropolone method may be superior to the oxine method. Indeed, this has been reported (2,3,10), although the

advantage of tropolone is not evident when the mean platelet lifespan is measured (10).

It should, however, be noted that it is difficult to standardize the methods of isolating and labeling platelets. Differences observed with the two principal labeling methods may therefore be related to some aspect of one of the methods used in a particular laboratory. We have in this study compared our method of labeling of platelets with ^{111}In -oxine (8) with the recommended ^{111}In -tropolone method (1,15). We studied the influence of the two labeling methods on some aspects of platelet labeling and on the results of platelet kinetics.

The labeling efficiency was acceptable with both methods and the administered activity was sufficient to estimate the MPLS and perform quantitative imaging. Contamination with other blood cells was low with both methods. The labeled platelets aggregated well in response to ADP ex vivo. The result of our simple method to assess the in vitro aggregation response, was similar to that reported by others (10). The recovery of oxine- and tropolone-labeled platelets in the circulation did not differ. In essence, these results show that with either of the two methods, a viable platelet population can be labeled efficiently.

The measurement of the MPLS is an important clinical and research tool. In normal subjects, platelets disappear from the circulation in an age-dependent manner (20,24). Thus, labeling a random sample of the circulating platelet population, would result in a near-linear platelet survival curve (24). It is evident (Fig. 2, Table 2) that the estimate of the MPLS with ^{111}In -oxine and ^{111}In -tropolone labeling methods, correspond closely. The number of "hits" in the multiple hit model is substantial and the difference between the MPLS calculated by fitting the data to a linear function and the multiple hit model, was not significant. This and the fact that our estimate of the MPLS is at the upper limit of the accepted normal value (2,10,24) indicates that with both methods there is minimal random destruction of the labeled platelets. This shows that the platelets are not adversely affected by either of the labeling procedures.

Vallabhajosula et al. (11) found a slightly longer

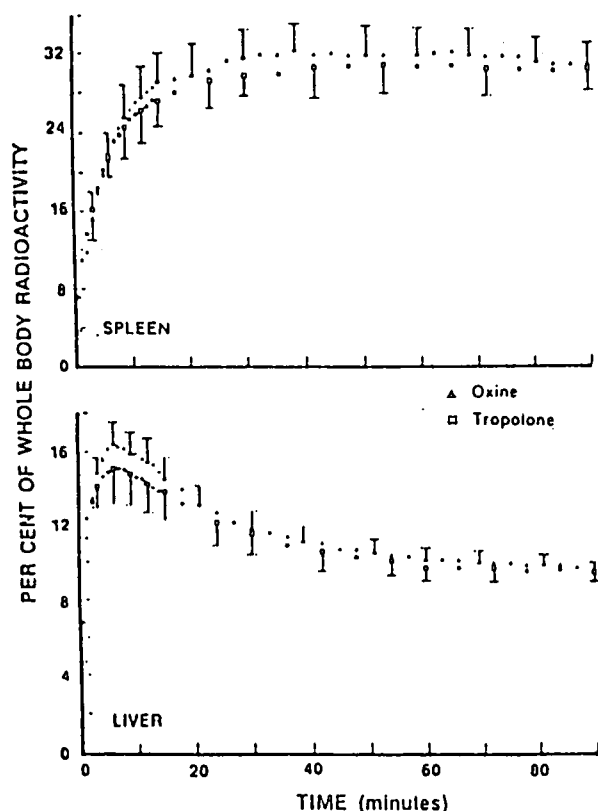


FIGURE 3
The redistribution of the labeled platelets in the spleen and liver in the first 90 min after their reinjection. Values are expressed as a mean ± 1 s.e.m. The accumulation of oxine- and tropolone-labeled platelets in the spleen was monoexponential and was not different. There was a measure of "collection injury" in the liver for both oxine- and tropolone-labeled platelets. The collection injury was not significantly different.

MPLS with the tropolone method than with oxine; however this was only evident with linear regression analysis. Using five mathematical models, we did not find such a difference. This discrepancy may be explained by a difference in methodology. Our method of labeling platelets with ^{111}In -oxine differs in one aspect from that of Vallabhajosula et al. (11). We do not include the extra washing step, which involves sedimenting of the platelets for 10 min at $2000 \times g$. Our method is, therefore, potentially less harmful to the platelets since additional mechanical stress to the platelets is avoided.

The influence of the "collection injury" on platelet kinetics may be assessed by quantifying the extent of the transient accumulation of labeled platelets in the liver immediately after their reinjection (8,14,25,26). It is evident from Figure 3 that both labeling methods slightly activate platelets. The accumulation of the platelets was transient in both instances, and the duration and the extent of the accumulation corresponded. Peters et al. (2,3) have suggested that ^{111}In -tropolone labeled platelets do not accumulate in the liver. However, they did not quantitate the hepatic radioactivity. It is, therefore, difficult to directly compare our and their results. It has been shown that the "collection injury" has no influence on the MPLS or the in vivo redistribution of platelets during their lifespan (25). The finding that the "collection injury" induced by ^{111}In -oxine labeling is reversible is supported by the results of the present study.

Our data demonstrate that the in vitro and in vivo function, MPLS, and the in vivo distribution of platelets labeled with ^{111}In -oxine in saline and ^{111}In -tropolone in plasma do not differ significantly. Our results, therefore, do not support the contention that platelets labeled with ^{111}In -tropolone are better preserved. Evidently, the methods used for the labeling, is crucial. Our results indicate that it is acceptable to compare results obtained with the two methods. It would, therefore, seem unwarranted to discard the established and widely accepted method of labeling platelets with ^{111}In -oxine for the tropolone method.

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NEW TECHNIQUES AND THE STANDARDIZATION OF PLATELET LABELLING

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ABSTRACT

In-111 is now accepted to be the platelet label of choice. The methods for isolating platelets from whole blood and the labelling procedures have however not been standardized. This makes it difficult to directly compare results of studies from different laboratories. Although it is theoretically possible that harvesting a select subpopulation of platelets from the blood by the usual techniques of differential centrifugation may give misleading results, our studies do not confirm this notion. Mean platelet life span, recovery in the circulation, in vivo distribution and the final sites of platelet sequestration of a fully representative platelet population do not differ from that harvested by the usual techniques. The influence of labelling platelets in plasma with tropolone as chelate was compared to the technique with oxine-labelling in saline. Although the extent of the "collection injury", reflected by the transient accumulation of platelets in the liver, was slightly less marked with tropolone, the difference was not significant. The other parameters of platelet kinetics were not influenced by the choice of chelate. There was however an unexplained loss of In-111 from the whole body with tropolone. We recommend that standardization should be with In-111-oxine as platelet label and that it is not necessary to isolate all the platelets from the blood in order to obtain an accurate picture of platelet kinetics.

INTRODUCTION

The International Committee for Standardization in Hematology (ICSH) in 1977 made recommendations for the isolation and labelling of blood platelets with Cr-51-sodium chromate (1). Although sound, these have largely been superseded because In-111, introduced as a platelet label by Mathew Thakur et al. in 1976 (2), is now generally the preferred radionuclide.

The labelling techniques with In-111 were developed in parallel in several countries. It is therefore not surprising that there has been a proliferation of protocols. This is unsatisfactory, especially since this precludes the comparison of results from different laboratories. Standardization of methods will also facilitate the successful and smooth introduction of these techniques into laboratories with little experience in the handling and labelling of blood cells. Also, at present various In-111 chelates are advocated as superior by different experts. It will be important to standardize on only one chelate, if possible. In the USA it is already uneconomic to license a product to replace

In-111-oxine for use in clinical studies. The recommendations for standard procedures should take this into account.

In this paper I am not going to attempt to address all the issues involved in the choice of methods for the standardization of the relevant procedures. I will focus on some of the problems that have been especially worrying to us. My final recommendations will therefore only be in broad terms, but may provide a reasonable basis for discussions on standardization.

RADIONUCLIDES

There are at present only two radionuclides that are suitable for general clinical use, Cr-51 and In-111. The physical characteristics and some relevant information on these are summarized in Table I. Some aspects deserve some comment.

TABLE I: RADIONUCLIDES USED FOR PLATELET LABELLING

Radionuclide	Half-life	Gamma emission (keV)	Gamma rays	Comments
Cr-51	27.7 days	320	10	Low labelling efficiency
In-111	67.3 hours	173 247	91 94	Quantitative imaging possible may be contaminated with In-113m
In-113m	99.5 min	392	64	Short half-life
In-114m	49.5 days	192	97	Radiation dose unacceptable

In-111 has now definitely been established as the platelet label of choice. It permits labelling of platelets with high specific activity; the binding to high molecular weight intracellular proteins is firm, thus limiting elution of label; and its physical characteristics allow quantitative imaging of the in vivo distribution of the labelled platelets (3). Also, the radiation exposure of the patients is well within acceptable limits (4). These characteristics make the study of platelet kinetics with In-111 more reliable than with Cr-51.

It is also possible to label platelets with the short half-life radionuclide In-113m. This label is useful to study diseases such as immune thrombocytopenic purpura where the platelet life span is very short. Studies with this radionuclide label may be repeated at short intervals. The kinetics of platelet populations labelled with In-111 and In-113m may also be studied simultaneously.

in the same patient.

Note that preparations of In-111 may be contaminated with In-114. This radionuclide increases the radiation dose significantly. Recently however, commercial suppliers have paid attention to this factor and the problem has therefore been minimized.

Chelation of In-111 to a lipid soluble agent is needed to carry the radionuclide into the cell. This is facilitated if the cells are labelled in a medium devoid of plasma. The removal of plasma prevents the binding of the In-111 to transferrin and other proteins; this form of the bound radionuclide does not enter the blood cell. Three candidates vie for the position of the chelate of choice: oxine, tropolone, and mercaptopyridine-1-oxide (Merc). Acetylacetone is somewhat toxic for platelets in the concentrations necessary for adequate labelling efficiency, and is therefore not popular.

In-111-oxine is the label most commonly used. It is poorly soluble in water, but commercial preparations containing solutions without alcohol (which may impair platelet function) are now readily available. Labelling of platelets with this complex must be suspended in saline. The chelate has been criticised because platelets subjected to a plasma-free medium are reported to be functionally impaired, and that they show evidence of an excessive "collection injury". These problems have initiated a search for better chelates especially with properties which will permit labelling of platelets in a plasma milieu.

Tropolone is water soluble and alcohol is therefore not required in the labelling medium. The major advantage of this complex is that In-111-tropolone permits labelling of platelets in plasma with an efficiency of about 80%.

Thakur favours Merc as the ligand of choice (5). Platelets may efficiently be labelled in a plasma milieu, and are viable. Despite these characteristics, I have personally not been convinced that this complex is clearly superior to In-111-tropolone.

I would like to draw attention to some important issues. I think that undue emphasis has been placed on the labelling efficiency. This is not critical since In-111 bound to plasma is easily removed, and it is simple to label platelets with the required specific activity by adding sufficient In-111 to the platelet suspension. Also, we have shown that with a method where platelets are labelled in saline, the isolation and labelling procedure does not affect results of platelet kinetics significantly (6). I therefore consider efforts to obtain a functionally viable labelled platelet population more productive than attempts to increase the labelling efficiency.

The important factors that may influence labelling efficiency are well known and will not be discussed: pH, temperature, cell concentration, incubation medium, and ligand concentration. These can easily be controlled by careful attention to technique.

ISOLATION OF PLATELETS FROM WHOLE BLOOD

Several methods for the isolation of platelets from whole blood are currently in use. The most important are separation of platelets by differential centrifugation, counterflow centrifugation (elutriation) and gel filtration. The only practical method for clinical studies is that of differential centrifugation. This basic method has had numerous modifications and some of these deserve comment.

Platelets tend to aggregate irreversibly during the isolation procedure especially when exposed to relatively high pH or to excessive centrifugal forces. This is generally overcome by an excess of the anticoagulant, acid citrate dextrose since the platelets are inactive at low pH. Some investigators especially in Britain and Europe, have also attempted to overcome the platelet activation by adding prostaglandins, especially PGE₁ (7) and PGI₂ (8). Although these may be useful, to my mind this manipulation introduces an unnecessary complicating factor. Prostaglandins are not necessary. Several studies have demonstrated this. Also, there may be ethical issues involved in justifying their clinical use. I would thus propose that the use of prostaglandins be avoided and certainly not included in any standardization protocol.

It should also be noted that the harvesting of platelets from whole blood by differential centrifugation is not complete and may be as low as 50%. This may have some implications on the study of platelet kinetics in health and disease. For instance, although results have been conflicting and confusing, it has been suggested that platelet density is related to platelet age and function. If this were important, the labelling of a selected platelet subpopulation will give misleading results of platelet kinetics.

We have modified the method of Corash (9) and have been able to harvest virtually total platelet population by repeatedly washing the platelets from the red cell layer. We have studied the kinetics of such a total platelet population and compared the results with that of platelets separated by the usual single step centrifugation method recommended by the ICSH. The results are summarized in Table II. It is clear that the mean platelet life span, recovery of labelled

TABLE II: INFLUENCE OF A TOTAL PLATELET POPULATION ON MPLS

	Single step Centrifugation	Total Population
MPLS (h)	21 ± 18	224 ± 23
Recovery (%)	68 ± 9.1	61 ± 12
Splenic pool (%)	25.9 ± 10	31.1 ± 6.1

platelets in the circulation and the size of the splenic platelet pool as measured with the two platelet populations are similar. Our results therefore support the view that platelet density and platelet size do not determine platelet age, at least not in humans (10). Although the isolation of the total platelet population is not absolutely necessary, this technique does have distinct advantages: it is possible to use smaller volumes of blood for the isolation of adequate numbers of platelets even in the presence of severe thrombocytopenia; and, there is some evidence that young platelets are functionally more effective. Therefore until the issue has been resolved, it may be prudent to label a fully representative platelet population in studies where optimal platelet functional effectivity is essential.

This raises the question of the assessment of the viability of a labelled platelet population. This may be done by in vitro or in vivo tests. Platelet aggregation response is the commonest in vitro test, usually measured in an aggregometer, or, by simple visual inspection. Although platelet aggregation is usually reported as being normal in platelet kinetic studies, I do not think that this finding necessarily correlates well with in vivo function.

The commonly performed in vivo tests of platelet function are: recovery of labelled platelets in the circulation, the shape of the platelet survival curve, and the rate and extent of the temporary accumulation of platelets in the liver after reinjection of the labelled preparation (11). These tests are recommended as the only valid assessment of platelet viability. All reports of platelet kinetic studies should therefore provide evidence that the investigator has employed a method that in normal reference subjects the recovery of the platelets in the circulation is acceptable, and that the disappearance of platelets from the circulation is age-dependent and not random. This will be reflected by a linear platelet survival curve.

The most sensitive indicator of the "collection injury", is the extent of the transient accumulation of an excess of platelets in the liver. Although this phenomenon has little effect on the results of platelet kinetic studies, it is an excellent monitor of the adequacy of the labelling procedure. It also provides a sensitive method to compare the functional effect of labelling platelet in saline, with In-111-oxine or in plasma, with In-111-tropolone. It has been suggested that the latter does not induce the temporary accumulation of platelets in the liver.

We have investigated this, and the results are illustrated in Fig 1 and given in Table III.

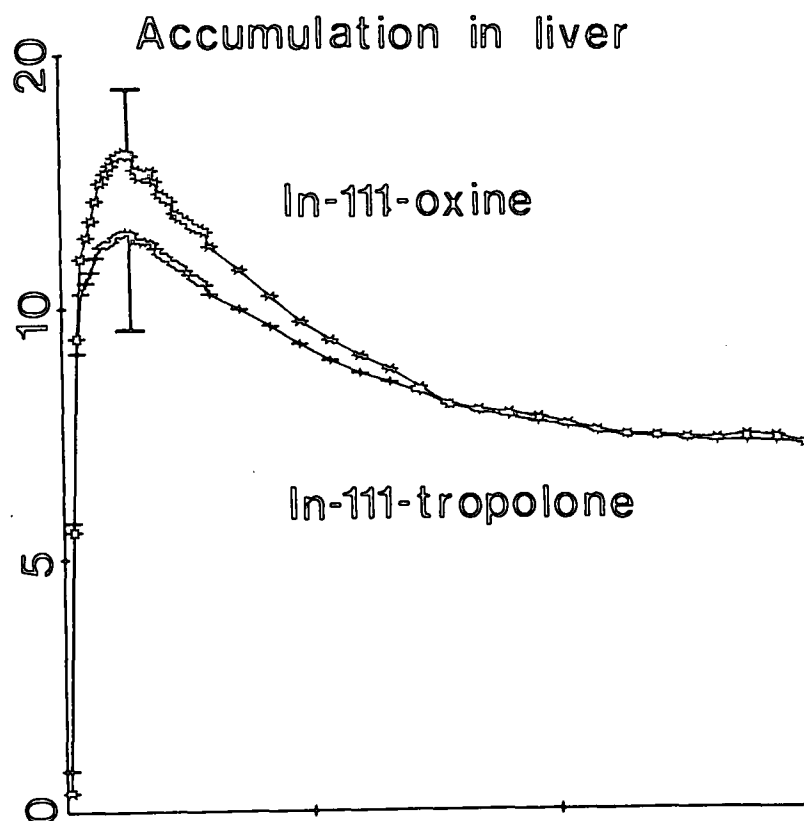


Fig 1: Kinetics of the accumulation of In-111 labelled platelets: the activity curves clearly indicate the transient accumulation of platelets in liver. Although the effect is somewhat more pronounced with the chelate oxine than with tropolone, the difference is not statistically significant.

TABLE III: IN VIVO DISTRIBUTION OF IN-111

	MPLS(h) RECOVERY (%)		EQUILIBRIUM (%)			FINAL (%)		
			Whole Body	Spleen	Liver	Whole Body	Spleen	Liver
OXINE	224 $\pm$ 23	61 $\pm$ 12	100	31 $\pm$ 5.6	13 $\pm$ 2	97 $\pm$ 5	35.6 $\pm$ 9	28.4
TROPOLONE	214 $\pm$ 10	66 $\pm$ 10	100	27 $\pm$ 7.2	9.6 $\pm$ 1	91 $\pm$ 7	34.6 $\pm$ 8	25.1

It is evident that both labelling techniques induce some degree of "collateral injury". Although the mean peak hepatic activity of In-111-tropolone is somewhat lower than that seen with In-111-oxine, these differences are not statistically significant. Also, the results of the MPLS estimated with the two methods are similar. We could also not detect significant differences in the equilibrium or final in vivo distribution of the labelled platelets. There was however an unexplained, somewhat worrying, constant feature of the results with In-

tropolone labelled platelets. After remaining near 100% for about 4 days, there was a relatively sharp and significant decrease in whole body radioactivity. We have not observed this with In-111-oxine labelled cells. This finding needs further investigation. It may be related to the relative affinity constants of tropolone and intracellular proteins for the In-111. This aberration may influence the results of platelet kinetic studies.

RECOMMENDATIONS

On the basis of these studies and also those reported in the literature, I would like to make the following recommendations related to the study of platelets with radionuclide labels:

- * In-111 is clearly the label of choice, and it will certainly eventually replace Cr-51. It will therefore be wise to standardize the In-111-labelling technique at this relatively early stage and before it is in general use.
- * Although it may be preferable to label platelets in plasma, there is no unequivocal evidence at this stage that the "collection injury" sustained by platelets labelled with In-111-oxine is significantly more severe than with In-111-tropolone or In-111-Merc. Since In-111-oxine will shortly be licensed for clinical use in the USA, it is practical and logical to standardize on In-111-oxine at this stage.
- * Platelet populations isolated from whole blood by differential centrifugation is satisfactory. The single step technique is adequate for clinical studies and probably also for most research projects. Harvesting of the representative total platelet population is useful for the study of the kinetics of autologous platelets in patients with severe thrombocytopenia. Also, there is some evidence that platelet function of different platelet subpopulations may vary. The influence of this on platelet kinetic studies requires further study before firm recommendations can be made.
- * All reports of platelet kinetic studies should include information substantiating that the labelled platelets are functionally viable. Although the in vitro aggregation response may be an useful indicator, in vivo tests of demonstrating viability are essential: recovery in the circulation, the shape of the platelet survival curve, and if possible to measure, also the extent of the transient accumulation of platelets in the liver should all be within acceptable limits.

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Indium-111-Labelled Human Platelets: A Method for Use in Severe Thrombocytopenia

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Key words

Indium-111-labelled platelets – Platelet labelling in thrombocytopenia – Platelet label method

Summary

We describe and evaluate a simple method for labelling autologous human platelets with Indium-111-oxine in patients with severe thrombocytopenia. Twenty patients with immune thrombocytopenia and platelet counts ranging from 5 to $119 \times 10^9/l$ were investigated. Platelets were isolated from blood by differential centrifugation, residual platelets were repeatedly washed from the red cell layer and buffy coat and labelled with In-111 in saline. A mean of $55\% \pm 21$ of platelets were harvested from the blood, labelled with $49\% \pm 24$ efficiency and 15.8×10^8 labelled platelets reinjected to the patients. Contamination of the platelets with red cells and plasma was low. The labelled platelets were viable as assessed by *in vitro* aggregation, recovery in the circulation and mean survival time. This method permits quantitative platelet imaging with autologous labelled platelets in patients with severe thrombocytopenia.

Introduction

Since its introduction by Thakur (1), In-111 complexed with oxine or other lipophilic compounds, has increasingly become the preferred platelet label. Its physical characteristics are superior to that of Cr-51-disodiumchromate, recommended in 1977 for this purpose by the International Committee for Standardization in Haematology (2). The major advantage of In-111 is that its high labelling efficiency for platelets and its high gamma photon emission results in high platelet specific activity. This permits quantitative imaging of the *in vivo* distribution of In-111 labelled platelets (3), and determination of platelet survival with platelets isolated from relatively small volumes of blood (1).

We have exploited these advantages of In-111-oxine to study the kinetics of autologous labelled platelets in immune thrombocytopenic purpura (ITP), and have reported our early experience (4). We now describe and evaluate our method for separating platelets from whole blood with a low platelet count, and labelling these with In-111-oxine. The method is simple, does not adversely affect platelet function and it is possible to harvest and label sufficient numbers of a fully representative population of platelets for kinetic studies in the presence of severe thrombocytopenia.

Materials and Methods

Patients

Twenty-one patients with chronic ITP were studied. Details of their peripheral blood platelet counts are given in Table 1.

Separation of Platelets and Labelling Procedure

The procedure for harvesting and labelling the platelets with In-111 is illustrated schematically in Fig. 1.

The method is based on those of several previous reports (2, 4-9) and has been adapted to enable harvesting of a "total" platelet population from the blood sample. The method is described in detail elsewhere (10).

A 19G "Butterfly" (Abbott Laboratories, North Chicago, Illinois, USA) was inserted by clean venipuncture into an antecubital vein. Seventeen ml blood was withdrawn into a polystyrene syringe containing 3 ml acid-citrate dextrose, NIH formula A (ACD-A) (Fenwal, Baxter-Travenol Laboratories Inc. Illinois, USA). The blood was centrifuged at $1000 \times g$ for 10 min and the supernatant platelet poor plasma (PPP) retained. Through the same needle 42.5 ml blood was withdrawn, with continuous gentle mixing, into each of four 50 ml plastic syringes each containing 7.5 ml ACD-A. The blood was transferred to 8×50 ml conical polystyrene tubes (Falcon 2070; Falcon Labware, Oxnard, California, USA) so that each tube contained 25 ml of blood. These were centrifuged for 15 min at $180 \times g$, platelet rich plasma (PRP) aspirated, and pooled. Platelets were then washed from the red cell layer. The cells were resuspended by adding Plasmalyte B (Baxter; Baxter-Travenol Laboratories Inc., Illinois, USA) to the original volume of the citrated blood. The suspension was centrifuged at $750 \times g$ for 3 min, the supernatant removed, and pooled with the PRP. The washing procedure was repeated four times. The pooled PRP was acidified to $pH 6.5 \pm 0.2$ by adding 0.5 ml ACD-A for each 10 ml of PRP. The PRP was centrifuged for 6 min at $180 \times g$ to remove contaminating red cells, if necessary, and supernatant PRP aspirated. The platelets were concentrated to a pellet by centrifuging for 25 min at $750 \times g$. The supernatant PPP was decanted and discarded. Traces of plasma was removed by layering and decanting twice, 2 ml of physiological saline. Care was taken not to disturb the platelet button.

The platelet pellet was resuspended gently in 2 ml physiological saline prewarmed to $37^\circ C$. The required volume of In-111-oxine (Radiochemical Centre, Amersham UK) was added: generally this was about 18.5 MBq (500 μCi). The suspension was incubated at $37^\circ C$ for 1 to 2 min, and 3 ml PPP added. The mixture was centrifuged for 25 min at $750 \times g$ and the supernatant decanted. The radioactivity associated with platelets and the supernatant was measured in a dose calibrator.

The labelled platelets were finally resuspended in 5 ml PPP, and a 0.3 ml aliquot removed for a platelet count and an *in vitro* aggregation study with ADP. The aggregation response was considered normal if platelet clumps consisting of more than 10 platelets were seen (8). The platelets were administered to the patients after allowing platelets to recover from the "collection injury" by leaving at room temperature for 1 hr after labelling.

Platelets were counted with an electronic particle counter (11) or, if less than $30 \times 10^9/l$, by phase microscopy (12). Platelet labelling efficiency with In-111, and determination of percentage recovery of platelets from the whole blood sample was determined as described elsewhere (10). Contamination of labelled platelets by plasma and red cells was measured as recommended by the ICSH (2).

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Platelet Survival in the Circulation

Platelet survival studies were performed on whole blood specimens as recommended by the ICSH (2). Blood samples were collected at 5, 15, 30, 45, 60, 90 and 180 min after reinjection of labelled platelets and thereafter as necessitated by the changes in blood radioactivity counts. Blood volume was estimated (13). Mean platelet survival was calculated by least-squares computer fitting to a gamma function (14). Platelet recovery in the circulation at equilibrium was derived by back extrapolation of the platelet survival curve to zero time.

Statistical Analysis

Student's *t*-test were used to test for differences between means. The strength of the linear relationship between two variables was tested with the sample correlation coefficient (*r*), and its significance determined with the *t*-test.

Results

Platelet Harvesting and Labelling

The results are summarized in Table 1. All the patients were thrombocytopenic. The mean blood platelet count was $24 \times 10^9/l \pm 31$ (range 5 to $119 \times 10^9/l$). Fifteen of the patients had platelet counts less than $20 \times 10^9/l$.

The repeated washing technique removed virtually all platelets from the blood: the mean percentage platelets harvested and present in the PRP was $98\% \pm 2$. Platelets were however lost in the concentration procedure and a mean of $55\% \pm 21$ of the total number of platelets present in the original blood sample were recovered in the platelet rich saline (PRS) in which platelets were labelled. This was significantly ($p < 0.0005$) less than the percentage platelets recovered from blood of normal subjects ($87\% \pm 7$).

The mean platelet labelling efficiency with In-111 was $49\% \pm 24$, and this was significantly ($p < 0.0005$) lower than that in

normal subjects, $90\% \pm 3$. Labelling efficiency correlated positively with the blood platelet count ($r = 0.8006$, $p < 0.0005$), and the number of platelets recovered for labelling ($r = 0.5338$, $p < 0.01$).

The platelets were labelled with $6.7 \text{ MBq} \pm 4.3$ In-111 (range 2.7 to 16.2). This radioactivity eventually available for kinetic studies did not differ ($p > 0.2$) from that utilized for this purpose in subjects with normal platelet counts.

Red cell contamination of the PRS was low, $1.4\% \pm 1.6$ (range 0 to 6%) and did not differ significantly from that found in subjects with normal platelet counts ($p > 0.15$). However, contamination of PRS with plasma was $7.3\% \pm 5.9$, which was significantly ($p < 0.005$) higher than that of normals. In a few instances (subjects 2, 4, and 6) contamination of labelled platelets with radioactive plasma was excessively high.

Kinetics of In-111-Oxine Labelled Platelets

A mean of $15.8 \times 10^8 \pm 16.1$ (range 2.3 to 52×10^8) labelled autologous platelets were reinjected. This was significantly less than the $160 \times 10^8 \pm 60$ labelled platelets reinjected in studies of normal subjects. There was a positive correlation between blood platelet count and number of labelled platelets available for reinjection ($r = 0.6841$, $p < 0.005$). The labelled platelets aggregated normally when exposed to ADP as judged under the microscope.

The mean percentage of labelled platelets recovered in the circulation at equilibrium was $55\% \pm 22$. This was significantly lower ($p < 0.0005$) than in the normal reference group.

As was expected, mean platelet survival in the circulation was short, $35 \text{ h} \pm 27.4$. Mean platelet survival time did not correlate significantly with the percentage recovery in the circulation ($r = -0.1385$, $p > 0.25$).

Table 1 Patient and In-111-oxine labelling data

Patient	Platelet count ($\times 10^9/l$)	Platelets harvested for labelling (%)	Labelling efficiency (%)	Contamination of platelets (%)		In-111-oxine administered MBq (μCi)	Platelets injected ($\times 10^8$)	Recovery in circulation (%)	Platelet survival (h)
				Red cells	Plasma				
1	24	23	44	1.8	7.8	4.1 (150)	26.5	47	20.1
2	15	64	34	0.0	24.0	4.1 (150)	44.9	60	24.1
3	35	42	51	0.0	5.2	3.3 (123)	6.9	51	30.7
4	15	100	49	0.0	14.0	2.9 (108)	25.9	30	93.0
5	8	34	41	2.6	3.7	8.4 (309)	7.3	47	64.9
6	8	100	21	3.4	19.0	3.5 (130)	41.1	30	91.4
7	20	40	66	6.0	8.0	2.7 (100)	28.4	72	31.9
8	10	55	23	0.0	3.8	3.5 (131)	11.0	92	40.1
9	66	49	74	0.0	2.0	18.9 (700)	4.5	81	16.2
10	13	52	40	3.1	4.1	5.4 (200)	2.3	40	10.4
11	18	29	39	0.5	6.0	5.4 (200)	4.0	51	5.0
12	8	52	31	0.7	5.2	6.8 (250)	3.5	62	12.9
13	4	59	29	0.8	3.5	5.4 (200)	2.7	88	4.3
14	119	66	74	0.0	2.3	10.3 (380)	52.0	58	37.6
15	88	60	82	0.8	1.7	16.2 (600)	4.0	76	20.8
16	5	91	30	2.1	10.7	4.1 (150)	4.5	15	37.6
17	12	50	37	0.1	0.2	9.7 (360)	3.4	79	35.6
18	8	38	50	2.6	8.0	4.6 (170)	4.4	59	81.5
19	5	53	35	0.6	6.2	6.8 (250)	4.0	40	7.2
20	7	50	38	2.3	10.7	8.1 (300)	5.2	16	35.6
Mean + 1SD	24 ± 31	55 ± 21	49 ± 24	1.4 ± 1.6	7.3 ± 5.9	6.7 ± 4.3 (248 $\pm$ 160)	15.8 ± 16.1	55 ± 22	35 ± 27
Reference ($\pm 1\text{SD}$; $n = 28$)	150–400	87 ± 7	90 ± 3	1.5 ± 1	2.2 ± 1	8.2 ± 3	160.0 ± 60	61 ± 12	224 ± 22

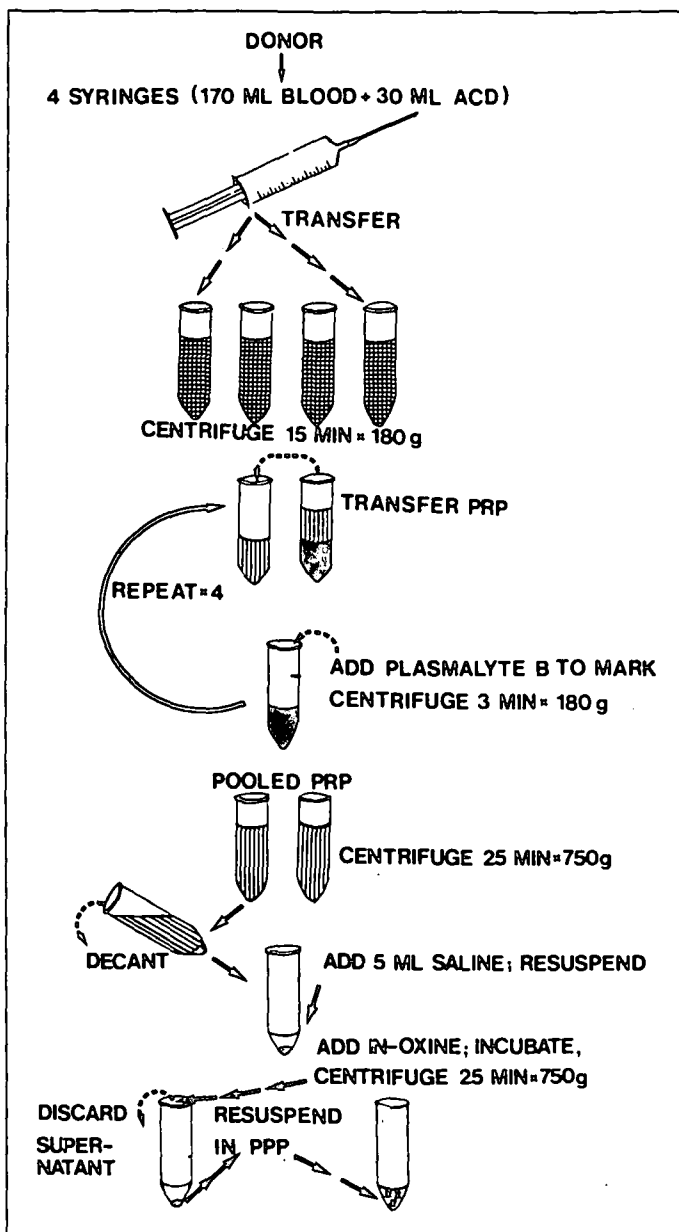


Fig. 1 Schematic presentation of the procedure for washing platelets from red cell layer and labelling human platelets with In-111-oxine (for details see Methods).

Discussion

Platelet survival studies have become an accepted investigative method for the clinical and experimental investigation of platelet and thrombotic disorders. A major problem has been the labelling of platelets in the face of severe thrombocytopenia, especially in ITP. This has also been the case with [Cr-51]-sodium dichromate, the platelet label recommended by the ICSH (2). This radionuclide labels platelets with a very low efficiency of 6 to 10% and it has only 9% gamma photon emission. Thus, large volumes of blood have to be withdrawn and blood platelet counts have to be greater than $25\text{--}50 \times 10^9/\text{l}$ for platelet kinetic studies with autologous Cr-51-labelled platelets in ITP (15, 16). If these requisites could not be satisfied, homologous platelets were labelled. Since patients may have platelet iso-antibodies with consequent shortened mean platelet survival times, and infections are a real risk, platelet kinetic studies with homologous labelled platelets are obviously far from ideal.

These problems have to a large extent been overcome by the introduction of In-111-oxine as platelet label (1). This radionuclide has superior physical characteristics, a high platelet labelling efficiency, and may be used for platelet survival studies and quantitative platelet imaging. The results of this study where autologous platelets could be labelled despite the severe thrombocytopenia, highlight the advantages of In-111-oxine.

We have adapted a method of Corash et al. (5) and washed the maximum number of platelets from the red cell layer of the whole blood. This was done because 14–31% of platelets may be lost in the red cell fraction and in the buffy coat (17–19). This is possibly important in ITP since it has been suggested that those platelets that are not harvested by conventional means are young and functional platelets (15, 20, 21), the typical platelet population of ITP (22, 23). We prefer to use the commercially available balanced solution of electrolytes, Plasmalyte B, for the washing procedure since it is nonpyrogenic and sterile. Aliquots may be sealed in ampoules and stored for later use, to reduce costs. It is however likely that solutions such as that of Tyrode (7, 9) will be as effective. In normal subjects $98 \pm 2\%$ of platelets are washed from the blood, but since some platelets are lost in the concentration and resuspension procedures, a mean of $87 \pm 7\%$ of platelets in the blood are eventually labelled (10). In these patients with ITP we harvested fewer platelets, $55 \pm 21\%$. The platelets which were lost probably did not represent a specific population since the mean platelet volume distribution did not change during the concentration steps (personal observation). However, in view of recent reports (24–26) which suggest that platelet density and volume are determined during thrombopoiesis, rather than platelet aging, the harvesting of a total platelet population might not be critical at all. The number of labelled platelets which were reinjected, $15.8 \times 10^8 \pm 16.1$, was adequate for the determination of mean platelet survival and quantitative imaging of the *in vivo* distribution of platelets even at the lower limits of approximately 2.3×10^8 labelled platelets. As expected there was a positive correlation between the blood platelet count and the number of labelled platelets available for reinjection.

The repeated cycles of washing of the platelets from the red cell layer had no adverse effect on *in vitro* platelet aggregation. Recovery in the circulation after reinjection of labelled platelets was variable, with a mean of $55\% \pm 22$. This is about 10% lower than that of normal subjects but the difference was not significant ($p < 0.2$). This is in agreement with the findings of Harker (15) who obtained normal recoveries in ITP. We have no explanation for the low recoveries reported by Branchög et al. (16).

Contamination of the labelled platelet population with red cells and plasma was acceptably low, although for unknown reasons some patients had high radioactivity associated with the plasma. It is possible that not all labelled platelets were sedimented by centrifugation when the standards were prepared (2). Since the platelet count of the pellet is relatively low, this contamination of plasma by a few platelets will give a false impression of contamination. Platelet survival was shortened in all the patients with ITP. We have previously noted that the mean survival of In-111-labelled autologous platelets in ITP is relatively long compared to studies reported with Cr-51-labelled homologous platelets (4). The reasons for this is not clear. It is possible that Cr-51 is more readily released than In-111 from labelled platelets by antiplatelet antibody (27). This will give a spuriously low estimate of mean platelet survival. It is thus probably more appropriate to label platelets with In-111 than Cr-51 in studies of ITP.

The simple method we describe for the harvesting and labelling of a representative population of autologous platelets in patients with severe thrombocytopenia may be recommended. Reagents are readily available, and there is not evidence of alteration of *in*

vitro or *in vivo* platelet function. The higher specific activity of labelled platelets, the low rate of elution, and the physical characteristics of In-111, make it possible to measure mean platelet life span accurately and to perform quantitative platelet imaging. There is little doubt that In-111 is now the preferred radionuclide for platelet labelling.

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Evaluation of Mathematic Models to Assess Platelet Kinetics

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Twelve mathematic methods used to calculate the mean platelet survival time were compared by determining the "goodness of fit" of the models to the platelet survival curves of 15 reference subjects and 54 patients. Platelets were labeled with [^{111}In]oxine. The linear (LN), exponential, weighted mean, multiple hit (MH), Dornhorst (DH), Meuleman (ML), alpha order (AO), and polynomial (PO) mathematic models were investigated. The goodness of fit for the exponential model was determined by the nonlinear least squares method (EP), and also by the linear least squares method on logarithmically transformed data (EX) as is recommended. The modified weighted mean (MWM) and the usual weighted mean method (WM) obtained with these exponential models were tested. The Dornhorst (DH10) and Meuleman (ML10) models, where the potential age-dependent platelet survival times were kept constant at 10 days, were also evaluated. The goodness of fit results, expressed as % s.d. indicated that the LN (5.2%), EX (5.0%), EP (4.4%), WM (3.7%), DH10 (3.7%), and ML10 (3.7%) models all fitted the data significantly worse than the MWM, MH, DH, ML, AO, and PO models (range 3.2–3.3%). The mean platelet survival time determined with the MH model differed significantly from the results with the DH, ML, and AO models. The results of mean platelet survival time calculated with different mathematic models cannot, therefore, be compared directly. The models that fitted the platelet survival curve well varied slightly in sensitivity to noise as is indicated by the coefficient of variation of the mean platelet survival time estimates for the reference subjects (range 7.9–12.0%). Fitting data to at least two mathematic models has definite advantages. Data on which the calculations are based are probably invalid if the following are true: (a) if the mean platelet survival time estimated with the alpha order model is shorter than that estimated with the EP, MWM, or MH models, or (b) the mean platelet survival time estimated with either the DH, ML, AO, or PO models, is longer than the LN, MWM, or MH estimate of the mean platelet survival time. We conclude that the mean platelet survival time can be reliably estimated by fitting the data to either the MWM method (if limited computing facilities are available) or the MH model. Confidence in the result will be increased if considered in conjunction with the finding obtained with one other model; in those cases where the platelet survival time is very short, the alpha order model is recommended. In other instances the results of the MWM method or MH model should be compared with that obtained with either the DH, ML, AO, or PO models.

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Shortening of the platelet life span is generally accepted as an important reflection of in vivo platelet activation. This measurement has been especially valuable for the understanding of the role of platelets in (a) various diseases characterized by thromboembolic phenomena or thrombocytopenia, (b) the evaluation of the efficacy of drug therapy in these diseases, and (c)

for the detection of some prethrombotic states. The accurate measurement of platelet life span is a prerequisite for the adequate investigation of these disorders.

The available methods for isolating or labeling a cohort of platelets of the same age are unsatisfactory and cannot be used in clinical studies. Therefore, a population of platelets representative of those in the circulation is isolated, labeled with a radionuclide, and reinjected. The mean survival time of the labeled platelets is indirectly estimated from the ratio of the

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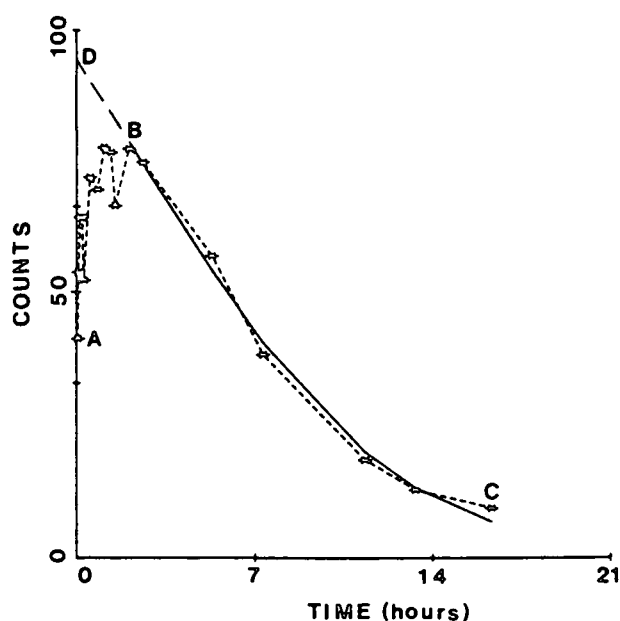


FIGURE 1
Calculation of mean platelet survival time is demonstrated. Period A to B represents equilibrium phase. Mathematic model is fitted from B to C and regression curve, indicated by solid line, is generated. This curve is back extrapolated from B to D as indicated by dotted line. At D y-intercept and initial slope can be determined

y-intercept and the initial slope of the platelet survival curve (1,2). This curve is constructed from the radioactivity of the labeled platelets remaining in the circulation. This is measured in blood samples taken at appropriate times after the reinjection of the labeled platelets. The y-intercept and the initial tangent of the survival curve cannot be directly measured because the stability of the early part of the curve is disturbed by the redistribution of platelets between the circulation and the splenic platelet pool, and by the temporary accumulation of platelets in the liver. The latter, ascribed to the "collection injury," varies in severity. The labeled and circulating platelets only equilibrate after 1-24 hr. This results in a plateau of the early phase of the platelet survival curve (3,4). Only the later part of the survival curve will reflect the true rate of clearance of platelets from the circulation. The y-intercept and slope of the curve can be determined only by back extrapolation of this part of the curve (Fig. 1). Although extrapolation may be performed visually, this method is subject to errors and bias. Mathematic models have therefore been developed to facilitate accurate fitting of the platelet survival curve to the data by using statistic methods. The regression constants generated by the mathematic models permit more precise estimation of the initial parameters. It should be noted that these mathematic models are not required to be based on valid physiologic assumptions. They solely function to

improve the "goodness of fit" of the platelet survival curve to the data points.

Although many such models have been applied to the study of platelet kinetics in health and disease, little information is available on their comparative value or on their suitability under specific pathologic conditions (5-9). The present study focuses on these questions. It also specifically provides information on the estimate of mean survival of autologous platelets labeled with indium-111- (^{111}In) oxine, a radionuclide complex which has specific advantages for the study of platelet kinetics (3,10).

MATERIALS AND METHODS

Subject Selection

Platelet survival was determined in 15 reference subjects and on 54 patients with a variety of disorders. The patient group consisted of four patients with valvular disease of the heart, 17 with previous renal transplantation, 14 with idiopathic thrombocytopenic purpura, two with diabetes mellitus, 14 with heterozygous Type IIa familial hypercholesterolemia, and three with aortic aneurysms. All subjects were in a steady state of platelet production and destruction during the course of investigation. This was reflected by a constant blood platelet count.

Platelet Labeling

Platelets were labeled with [^{111}In]oxine as previously described (9-11). Eighty-five milliliters of blood were collected in a polystyrene syringe containing 15 ml acid citrate dextrose and NIH formula A (ACD) as anticoagulant. Platelet rich plasma (PRP) was obtained by centrifugation of the blood at 180 g for 15 min. Platelets trapped in the red cell layer were harvested by washing four times: blood was centrifuged at 750 g for 3 min, the supernatant removed, and the cells resuspended in Plasmalyte B electrolyte solution. After acidifying to pH 6.2-6.5 with ACD, platelets were sedimented by centrifuging at 800 g for 30 min. The platelet pellet was resuspended in physiologic saline and incubated for 30 min with [^{111}In]oxine. The labeled platelets were washed with, and finally resuspended in, autologous platelet-poor plasma.

Labeled platelets were used for in vivo studies only if in vitro aggregation with ADP and collagen was normal (3,9,10). A dose not exceeding 500 μCi (18.5 MBq) of ^{111}In was administered.

Following injection of the labeled platelets, blood samples were obtained at 5, 10, 15, 30, 70, and 90 min, and daily thereafter. In patients with short mean platelet survival times, blood sampling was appropriately spaced so that at least eight valid data points were available for curve fitting. Blood samples of 3 ml were directly collected in the counting vials, the mass determined, and water added to a constant final volume of 4 ml. The samples were counted in a well scintillation counter with the pulse-height analyzer set to include the 172 and 247 keV peaks of ^{111}In . The measured count rate was corrected for background and mass. Plasma ^{111}In activity was determined in all cases, and was <5%.

MATHEMATIC FUNCTIONS AND METHODS TO FIT PLATELET SURVIVAL CURVES

The following mathematic functions (see full description in Appendix) were fitted to the data points reflecting the rate of clearance of platelets from the circulation:

Linear Function (LN) (11,12)

Exponential Function (11,12)

Two methods were used to fit the data to an exponential function. In the first method (EX) the regression constants for the exponential model were calculated as recommended by the ICSH; the data were logarithmically transformed and the curve fitted with the linear least squares method (11). In the second method (EP), the curves were fitted with the Newton-Gauss method, an iterative nonlinear least squares technique (13). The latter method was investigated because the ICSH recommends that the discrepancies between the fitted line and data points be calculated without logarithmic transformation of the data. The nonlinear least squares technique minimizes these discrepancies (11,13).

Weighted Mean Function (11,12)

This was calculated by two methods, dependent on the method used to fit the data to an exponential function. The first method (WM) was according to the recommendations of the ICSH where the exponential regression curve is constructed after transforming the data logarithmically (11). For the second, a modified weighted mean method (MWM), the exponential regression constants were obtained by fitting the data with a nonlinear least squares technique. The variance of the linear, exponential, and modified weighted mean methods was calculated. If the variance of either the linear or exponential models was less than that of the modified weighted mean method, the result of the method that fitted the data best was used as the estimate of the mean platelet survival time by the modified weighted mean method. This modification was introduced to minimize the influence of noise when data points were distributed perfectly linearly or exponentially.

Alpha Order Function (AO) (14)

This function was fitted to the survival curve data by the Newton-Gauss iterative nonlinear least squares technique (13). Initial estimates of regression constants for the iteration procedure were selected as follows. The initiation constant k_0 was set to unity and alpha, "a," replaced by the weighted mean estimate of the variance shape factor (12). The rate constant "k" was calculated substituting the alpha order mean platelet survival time, T_a , by the weighted mean estimate, T_w (see Appendix):

$$k = (1/T_w) [1 + (1 - a)k_0].$$

Polynomial Function (PO)

Only the second degree polynomial function was used. The higher degree polynomial functions were found to be too sensitive to noise and outliers.

Multiple Hit Function (MH) (11)

The curve fitting technique described by the ICSH was used. Initial values for iteration were obtained from the linear and exponential curve fitting procedures (11).

Dornhorst Function (11,15)

The curves were fitted with the Newton-Gauss iterative nonlinear least squares technique (13). Mean platelet lifespan was determined by two methods. First (DH10), the potential age-dependent platelet survival time was kept constant at the value determined for normal subjects, 10 days. In the second method (DH), the potential age-dependent platelet survival time as determined for the specific patient was used.

The iterative nonlinear curve fitting technique requires initial estimates of the potential age-dependent platelet survival time and the rate constant, "k." The first was taken to be 10 days, and the initial estimate of k was calculated by solving the following equation by the Newton-Raphson method (16) (see Appendix):

$$T_d = [1 - \exp(-kT)]/k.$$

The weighted mean estimate of the platelet survival time, T_w , was used in the initial value of T_d , the Dornhorst estimate of the mean platelet survival time.

Meuleman Function (17)

Curve fitting of the Meuleman function (ML and ML10) was performed as was described for the Dornhorst function.

CURVE FITTING PROCEDURE

A computer program in Fortran, executed on a nuclear medicine digital imaging system, was developed to fit survival curves to the mathematic models. The survival curves were entered by keyboard and stored on the system database. Curve fitting, with any of the above models, was menu driven. It allowed graphic display of the curves to select the range of points for curve fitting. The data points were selected visually as follows. The first point included was that reflecting the highest blood radioactivity after equilibrium had been reached. All subsequent data points with radioactivity >10% that of the initial counts were included. The goodness of fit of the data points to each of the mathematic models was visually evaluated by inspecting the graphic display of the survival and regression curves on the computer terminal. Print-out of results, including all relevant information such as regression constants, mean platelet survival time, and indication of goodness of fit could be selected by menu. A hard copy of measured and regression survival curves could then be recorded with a four-color plotter. A separate graphics package was developed for use with the system database.

COMPARISON OF MATHEMATIC MODELS

The different mathematic models were compared by calculating the root mean square of the difference between the measured and regression survival curve points. This value, reflecting the goodness of fit, was expressed as a percentage of the y-intercept and designated as the % s.d. The survival curves with different count rates could thus be compared.

The % s.d. and mean platelet survival time calculated for the different models were compared with Hotelling's multivariate T-squared statistic to test the hypothesis of equality of means (16,18). The patients were considered to be a random sample from a population and a log-transformation applied

TABLE 1
Percentage Standard Deviation (PSD) of Platelet
Survival Curve Points Around Fitted Curve

Model	Whole group (69)		Reference subjects (15)	
	PSD	s.d.	PSD	s.d.
Linear	5.2	3.4	2.9	1.3
Exponential (EP)	4.4	1.8	4.8	1.3
Exponential (EX)	5.0	2.4	6.1	2.6
Modified weighted mean	3.4	1.9	2.6	1.2
Weighted mean	3.7	2.3	2.6	1.2
Multiple hit	3.3	1.8	2.5	1.4
Dornhorst	3.3	1.9	2.5	1.3
Dornhorst (DH10)	3.7	1.8	2.9	1.3
Meuleman	3.4	1.9	2.5	1.3
Meuleman (ML10)	3.7	1.8	2.9	1.3
Alpha order	3.2	1.8	2.5	1.4
Polynomial	3.3	1.9	2.5	1.3

to the % s.d. and mean platelet survival time data. The multivariate model incorporates different observations that may be dependent, on the same patient. In those instances where the means differed significantly, multiple comparisons of the means were used to identify those differences which were responsible for the rejection of the hypothesis of equality of means.

The influence of experimental error ("noise") on the estimate of mean platelet survival was determined by the coefficient of variation (CV) in the normal subjects.

RESULTS

Evaluation of Mathematic Models: Goodness of Fit

Goodness of fit achieved by the different mathematic models were evaluated by calculating the % s.d. The results of the mean % s.d. for the reference subjects, and for the whole group (patients and reference subjects) determined for all the mathematic models are given in Table 1. The range was 3.2–5.2% for the whole group and 2.5–6.1% for the reference subjects. The differences in the % s.d. of data fitted

with the different models are given in Table 2. The % s.d. of the LN model and the fitting of the data to the exponential model both by a linear least squares fit of logarithmically transformed data (EX) or by the linear least squares method (EP) was significantly ($p < 0.01$) larger than that of the other models: the differences exceeded 0.6%. These models are therefore not suitable for the calculation of the mean platelet survival time. Also, the mean % s.d.s obtained by the Dornhorst model where the age-dependent survival time is held constant at the normal value (DH10), were significantly larger than the MH ($p < 0.01$), DH ($p < 0.01$), ML ($p < 0.05$), AO ($p < 0.05$), and PO ($p < 0.05$) models. The % s.d. associated with the Meuleman model, with a constant age-dependent survival time (ML10), was larger than that of the MH ($p < 0.01$), DH ($p < 0.05$), and AO ($p < 0.05$) models. The % s.d. obtained with the WM model was significantly larger ($p < 0.01$) than that obtained with the MH and DH models. The % s.d. of the MWM and DH models differed significantly ($p < 0.05$). The good correlation of the % s.d.s associated with those mathematic models which did not differ significantly is shown in Fig. 2.

In the reference group, the % s.d. of the exponential model, obtained by both methods of curve fitting (EX and EP), and the other models differed significantly (Table 3). The exponential models, therefore, fitted the data poorly.

Platelet Survival Time

We next investigated whether the mean platelet survival times estimated with those mathematic models that fitted the survival curves equally well differed. The mean platelet survival time (ST), standard deviation (s.d.), and coefficient of variation (CV) are presented in Table 4. The estimate of the mean platelet survival time for the reference subject group varied from 204.2 ± 24.5 hr for the DH model to 227.7 ± 17.7 hr for the MH model. The mean platelet survival time estimated by the MH model was significantly longer when compared with that measured with the DH ($p < 0.01$), ML ($p < 0.01$), and AO models ($p < 0.05$) (Table 5). The estimate with the WM method was also significantly longer ($p < 0.05$) than the DH and ML models. In the reference subjects there was also a significant difference ($p < 0.01$) between the mean platelet survival time calculated with the MH method com-

TABLE 2
Differences in PSD for Mathematic Models Investigated in Patient Group and Reference Subjects (N = 69)

Model	EP	EX	MWM	WM	MH	DH	DH10	ML	ML10	AO	PO
LF	0.9	0.2	1.8†	1.5†	1.9†	1.9†	1.6†	1.9†	1.6†	2.0†	2.0†
EP		-0.6†	1.0†	0.7†	1.1†	1.1†	0.7†	1.0†	0.7†	1.2†	1.1†
EX			1.6†	1.3†	1.7†	1.7†	1.3†	1.7†	1.4†	1.8†	1.7†
MWM				-0.3	0.1	0.1	-0.3	0.1	-0.2	0.2	0.1
WM					0.4†	0.4†	0.0	0.3	0.0	0.5	0.4
MH						-0.0	-0.4†	0.0	-0.3†	0.1	0.0
DH							-0.4†	-0.0	-0.3†	0.1	0.0
DH10								0.3†	0.0	0.5†	0.4†
ML									-0.3	0.2	0.1
ML10										0.4†	0.4
AO											-0.1

† Difference significant at $p < 0.05$.

‡ Difference significant at $p < 0.01$.

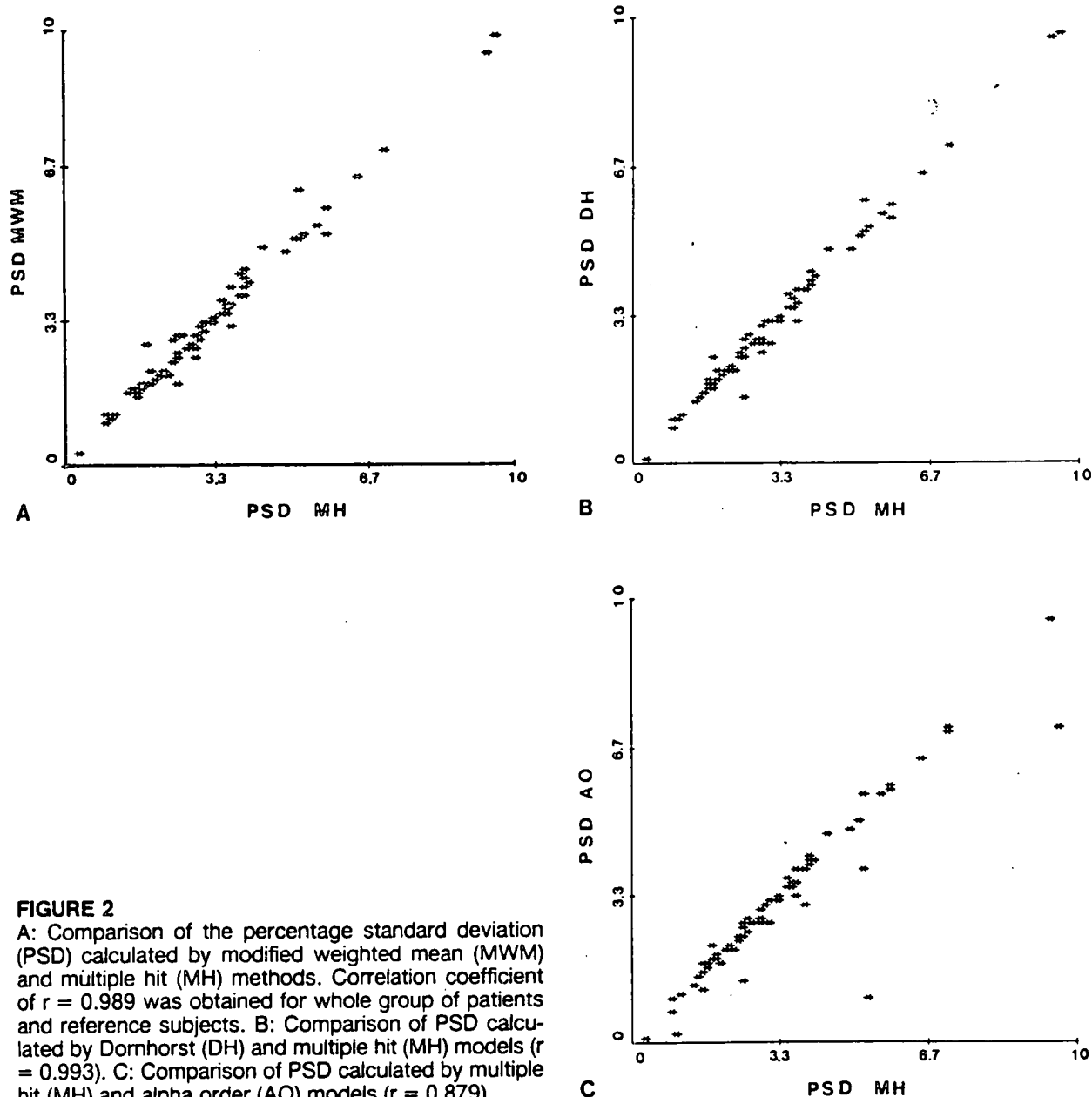


FIGURE 2

A: Comparison of the percentage standard deviation (PSD) calculated by modified weighted mean (MWM) and multiple hit (MH) methods. Correlation coefficient of $r = 0.989$ was obtained for whole group of patients and reference subjects. B: Comparison of PSD calculated by Dornhorst (DH) and multiple hit (MH) models ($r = 0.993$). C: Comparison of PSD calculated by multiple hit (MH) and alpha order (AO) models ($r = 0.879$)

pared to that with the DH, ML, and the PO models (Table 6). The estimated mean platelet survival time with the AO model also differed significantly ($p < 0.05$) from that with the DH and ML models. The estimated mean platelet survival time with the DH and ML models were nearly identical.

The range of the mean platelet survival time (CV) observed in the reference subjects was used as an approximate indicator of the sensitivity of the mathematic models to noise. The CV for the MH model was 7.9% (Table 4). The range of the CV for the MWM, DH, ML, PO, and AO models was 9.9–12%.

There was good correlation ($r = 0.986$) of mean platelet survival times calculated with MH and MWM models (Fig. 3). In addition, the MH and DH models correlated well ($r = 0.962$), despite a deviation from the linear regression line in some instances where the mean platelet survival time fell within the normal range (Fig. 4). This deviation was not observed when the DH, ML, AO, and PO models were cor-

related (Fig. 5). Deviations from the linear regression line within the normal range was accompanied by another finding: the estimate of the mean platelet survival time with the DH, ML, AO, and PO models was longer than that obtained by fitting the data to the LN model. This finding was reflected by a slightly concave shape of the platelet survival curve.

DISCUSSION

Although the method of labeling platelets with chromium-51 has been widely used, the estimation of platelet life span with ^{111}In -labeled platelets has been validated and is now generally regarded as superior (4,9,10). The latter radionuclide also permits quantitative imaging of the in vivo distribution of platelets. This has some important advantages when determining the

TABLE 3
Differences in PSD for Mathematic Models Investigated in Reference Subjects (N = 15)

Model	EP	EX	MWM	WM	MH	DH	DH10	ML	ML10	AO	PO
LF	-1.9*	-3.2†	0.3	0.3	0.4	0.4	-0.0	0.4	-0.0	0.4	0.4
EP		-1.3†	2.2†	2.3†	2.3†	2.3†	1.9†	2.3†	1.9†	2.3†	2.3†
EX			3.5†	3.5†	3.6†	3.6†	3.2†	3.6†	3.2†	3.6†	3.6†
MWM				0.0	0.1	0.1	-0.3	0.1	-0.3	0.1	0.1
WM					0.1	0.1	-0.3	0.1	-0.3	0.1	0.1
MH						0.0	-0.4	0.0	-0.4	0.0	0.0
DH							-0.4	0.0	-0.4	-0.0	-0.0
DH10								0.4	0.0	0.4	0.4
ML									-0.4	-0.0	-0.0
ML10										0.4	0.4
AO											0.0

* Difference significant at $p < 0.05$.

† Difference significant at $p < 0.01$.

mean platelet survival time. Monitoring of the extent of the early accumulation of labeled platelets in the liver permits more precise evaluation of the "collection injury." Also, the size of the exchangeable splenic platelet pool may be estimated accurately by quantitative imaging. These advantages were exploited in our study.

The confidence in the accuracy of the estimate of platelet life span is dependent on accepting a number of assumptions and satisfying certain basic prerequisites: platelet production and destruction must be in a steady state, the platelet population isolated for labeling must be fully representative of those in the circulation and a subpopulation of either younger or older platelets should not be harvested, and in vivo elution of the label must not be excessive. In the present study, platelets were labeled with a validated method (9,10). Only those patients in a steady state of platelet destruction and production were included. There was no evidence of severe in vitro damage to platelets: This was reflected by an adequate in vitro platelet aggregation response, normal recovery of labeled platelets in the circulation, and the transient excess accumulation of platelets in

the liver after reinjection of ^{111}In -labeled platelets was $<5\%$.

One of the major problems in estimating the mean platelet survival time is the decision to include or exclude initial data points on which the calculation of the rate of clearance of platelets from the circulation will be based. We did not use an automatic mathematic method but relied on visual inspection of the selected data points and correlating this with the data obtained from the in vivo quantification of platelet distribution. Excessive collection injury can readily be recognized by visual inspection and correlation with quantitative data of organ radioactivity. The signs of collection injury are as follows: The early plateau of the platelet survival curve is prolonged or fluctuates markedly, and radioactivity in the liver does not reach a plateau or remains higher than expected (9,10). Care was also taken to include a sufficient number of appropriately spaced data points for fitting the survival curve.

Although the ICSH has recommended the weighted mean, multiple hit, and Dornhorst models for the estimate of platelet life span (11), other mathematic models have their proponents (19-22). We have slightly modified the weighted mean method recommended by the ICSH (11). We determined the regression constants by

TABLE 4
Estimation of Mean Platelet Survival Time (hr) with Different Mathematic Models

Model	Whole group (69)		Reference subjects (15)		
	ST	s.d.	ST	s.d.	CV
Linear	176.9	72.5	232.6	15.2	6.5
Exponential (EP)	97.8	42.1	122.7	15.4	12.5
Exponential (EX)	87.5	37.1	99.9	15.9	15.8
Modified weighted mean	144.0	74.3	214.3	23.0	10.7
Weighted mean	145.0	69.0	215.4	19.1	8.9
Multiple hit	149.4	75.6	222.7	17.7	7.9
Dornhorst	142.4	78.2	204.2	24.5	12.0
Meuleman	143.1	78.5	204.7	24.1	11.7
Alpha order	146.2	87.6	212.4	21.0	9.9
Polynomial	147.3	78.4	206.8	22.6	10.9

TABLE 5
Differences in Mean Platelet Survival Time Obtained with Mathematic Models in Reference Subjects and Patient Group (N = 69)

Model	WM	MH	DH	ML	AO	PO
MWM	-1.0	-5.4	-1.6	0.9	-2.1	-3.2
WM		-4.4	2.6*	1.9*	-1.2	-2.3
MH			7.0†	6.3†	3.3†	2.2
DH				-0.7	-3.8	-4.9
ML					-3.0	-4.1
AO						-1.1

* Difference significant at $p < 0.05$.

† Difference significant at $p < 0.01$.

Table 6
Differences in Mean Platelet Survival Time of
Reference Subjects Estimated with Mathematic
Models (n = 15)

Model	WM	MH	DH	ML	AO	PO
MWM	-1.1	-8.4	10.1	9.6	2.0	7.5
WM		-7.3	11.2	10.7	3.0	8.6
MH			18.5†	18.0†	10.3	15.9†
DH				-0.5	-8.1*	-2.5
ML					-7.6*	-2.0
AO						5.6

* Difference significant at $p < 0.05$.

† Difference significant at $p < 0.01$.

nonlinear least squares calculation rather than by a linear least squares fit of logarithmically transformed data. The nonlinear curve fitting technique improved the goodness of fit for the exponential model significantly. We also preferred to adopt the result with either the linear or the exponential models rather than that of the modified weighted mean model if the % s.d. of data points from the fitted line of the latter was greater than that of either of the former. These modifications, which are logical and do not increase the complexity of the calculations, improved the goodness of fit of the modified weighted mean method (Table 2). It is, therefore, preferred over the ICSH weighted mean method.

On the basis of our results we could divide the ability of the models to fit the data into three groups. The linear and exponential models fitted the platelet survival

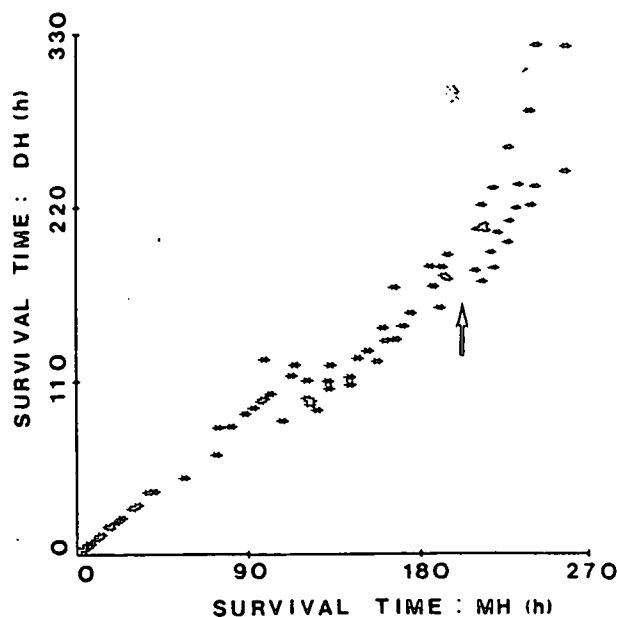


FIGURE 4
Comparison of mean platelet survival time estimated by Dornhorst (DH) and multiple hit (MH) models ($r = 0.962$). There was deviation of some points from regression line for survival times exceeding 200 hr

curves of the patients badly. The poor fit of the data by the regression curves resulted in an inaccurate estimate of the mean platelet survival time. This finding is in agreement with results reported by Paulus (20). Estimates of the mean platelet survival time were in many cases in error by 50–300%. These methods can obviously not be recommended. Our results also indicate

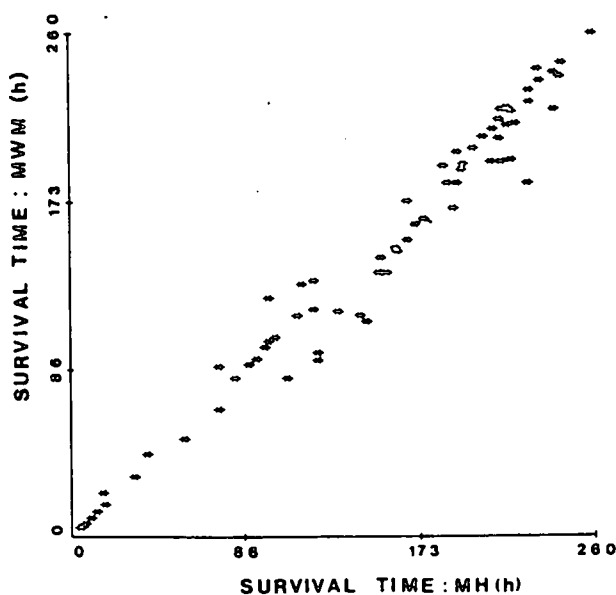


FIGURE 3
Comparison of mean platelet survival time estimated by modified weighted mean (MWM) and multiple hit (MH) models ($r = 0.986$). Regression curve close to line of identity was obtained

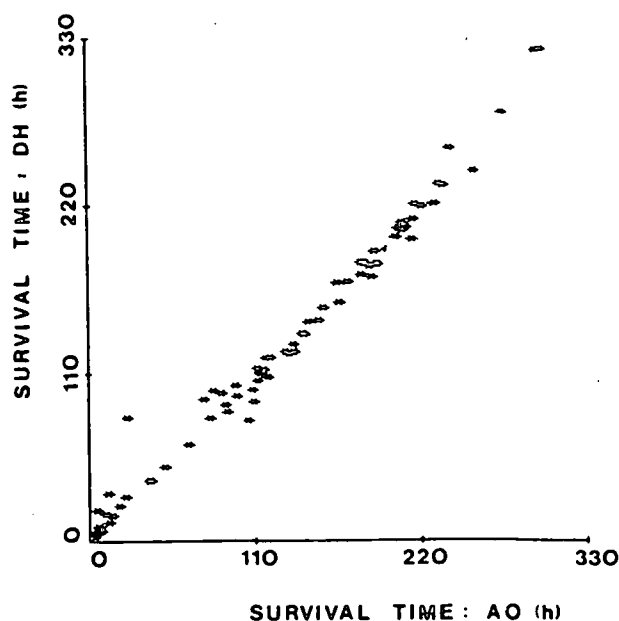


FIGURE 5
Comparison of mean platelet survival time estimated by Dornhorst (DH) and alpha order (AO) models ($r = 0.949$)

that the weighted mean method applied as recommended by the ICSH, and the variations of the Dornhorst and Meuleman models where the potential age-dependent platelet life span is kept constant at 10 days (the normal platelet survival time), also fit the data fairly poorly. Our modification of the weighted mean model, multiple hit, Dornhorst, Meuleman, alpha order, and polynomial models all performed well and there was no evidence that any one of these is definitely superior.

Although these mathematic models fitted the curves equally well, their estimates of the mean platelet survival times differed. In the whole group, the mean platelet survival time estimated with the multiple hit model differed significantly from the results with the Meuleman, Dornhorst, and alpha order models. Also, in the normal subjects, there were significant differences between results of mean platelet life span estimated with the Dornhorst or Meuleman models compared with the estimate when the curve was fitted with the multiple hit or alpha order models. The mean platelet survival time obtained with the multiple hit and polynomial models also differed significantly. There is no obvious physiologic basis for these differences and it is obvious that the application of, or preference for, any specific model is arbitrary. The major implication of this finding is, however, that it may be hazardous to directly compare results of studies where the platelet life spans were not estimated with the same mathematic model.

The use of more than one mathematic model for the assessment of the data has definite advantages. This is illustrated by our findings. The shape of the platelet survival curve is concave if the basic requirement of a steady state is not satisfied and the rate of platelet production exceeds that of platelet destruction. Although this may be detected by visual inspection of the shape of the platelet survival curve, doing this with the mathematic models is more sensitive and less subjective. A concave survival curve is present when the mean platelet survival time estimated with either the Dornhorst, Meuleman, alpha order, or polynomial models' exceeds that obtained by fitting the data to a linear function. Other indicators of a concave platelet survival curve are that the Dornhorst and Meuleman models' estimates of the platelet life span are longer than the potential age-dependent platelet life span, or, if "alpha" determined in the alpha order model has a negative value. It should be noted, as predicted by theory, that the mean platelet survival time estimated with either the multiple hit or weighted mean methods never exceeded that determined by fitting the data to a linear function.

In patients with immune thrombocytopenic purpura, where the mean platelet survival time was very short, results with the alpha order model differed from those

of the other models. In some instances the value of alpha was greater than unity and the estimate of mean platelet life span shorter than that estimated with an exponential function. This indicates that a higher order function is operative. In these cases the other mathematic models, in contrast to the alpha order model, simply fitted the curves to an exponential function. The investigator may, however, be alerted to the presence of such a higher order function by another finding: The estimate of the potential age-dependent platelet survival time will be greater than normal. It is therefore evident that certain patterns of results obtained by fitting the data to several mathematic models may alert the investigator to the possibility that the data on which the estimates were based were invalid, or that the assumptions on which the mathematic model are based are not operative in the particular case.

The usefulness of a mathematic model will also be determined by its sensitivity to "noise." "Pure" noise is introduced by experimental error and is related either to the sampling procedure or to the statistics of counting the radioactivity of the blood sample. "Physiologic" noise could also be introduced by the redistribution of platelets owing to their pooling in organs, and the influence of exercise and posture. Sensitivity to noise will decrease the precision of the measurement of the mean platelet survival time. The sensitivity to noise cannot be directly determined because the "true" mean platelet survival time, represented by the platelet clearance curve under investigation, is not known. We have therefore used the range of the mean platelet survival time observed in normal subjects (where the platelet survival curve is effectively linear) as an approximate indicator of sensitivity to noise. The multiple hit model is relatively insensitive to noise. This is reflected by the coefficient of variation: In normal subjects it was 7.9% for the multiple hit, compared with 6.5% obtained with the linear model. The other models that fitted the data well (modified weighted mean, Dornhorst, alpha order, Meuleman and polynomial function) are somewhat more sensitive to noise: In normal subjects their coefficients of variation varied from 9.9–12.0%. The good correlation of the mean platelet survival times calculated with these models that fitted the data well also indicates that the sensitivity to noise of these models is acceptable.

On the basis of the results of this study, we would make the following recommendations for estimating the mean platelet survival time. The multiple hit model is recommended, but if limited computing facilities are available, our modified weighted mean method is satisfactory. These models are insensitive to noise, but do not indicate the presence of invalid physiologic mechanisms. Therefore, it is always necessary to inspect the platelet survival curve visually. This will provide information on the influence of the collection injury, the

presence of obvious outliers, and the shape of the platelet survival curve. This will alert the investigator to the possibility of an imbalance between platelet production and destruction, to problems with the labeling and harvesting of the platelets, and to the possibility of faulty techniques of blood sampling or counting of radioactivity. It is advisable to use at least one other mathematic model to facilitate the recognition of above mentioned pitfalls. The Dornhorst, Meuleman, alpha order, or polynomial models are equally satisfactory for the detection of a concave shaped platelet survival curve, while the alpha order is recommended for the detection of higher order functions in patients with a very short mean platelet survival time.

APPENDIX

MATHEMATIC FUNCTIONS TO FIT PLATELET SURVIVAL CURVES

The following mathematic functions were fitted to the platelet survival curves.

Linear Function (11,12)

The linear function was calculated by:

$$H_l(t) = H_l(0) - at,$$

where $H_l(t)$ represented the linear regression line, $H_l(0)$ the y-intercept, and "a" the rate of platelet disappearance. The mean platelet survival time, T_l , was given by:

$$T_l = H_l(0)/a.$$

Exponential Function (10,11)

The exponential regression curve, $H_e(t)$, was calculated by:

$$H_e(t) = H_e(0) \exp(-kt),$$

where $H_e(0)$ was the y-intercept and "k" the relative rate of platelet disappearance. The mean platelet survival time was given by:

$$T_e = 1/k.$$

Weighted Mean Function (11, 12)

The weighted mean regression curve, $H_w(t)$, was calculated by:

$$H_w(t) = [S_e H_l(t) + S_l H_e(t)] / [S_e + S_l],$$

where S_l and S_e were the residual sum of squares associated with linear and exponential regression curves. The residual sum of squares was calculated by:

$$S_l = \sum [(H_l)_i - N_i]^2 \text{ and}$$

$$S_e = \sum [(H_e)_i - N_i]^2,$$

where $(H_l)_i$ and $(H_e)_i$ were linear and exponential regression curve points corresponding to data points N_i .

The weighted mean survival time was calculated by (10):

$$T_w = [S_e T_l + S_l T_e] / [S_e + S_l].$$

Alpha Order Function (14)

The alpha order function was calculated by:

$$H_a(t) = H_w(0) [1 + (1 - a)(k_0 + kt)]^{1/(1-a)},$$

where $H_w(0)$ was the y-intercept calculated by the modified weighted mean method, "k" the rate constant, and k_0 an initiation constant. The value of "a" or alpha determined the shape of the function; when $a = 0$, a linear and when $a = 1$, an exponential function was obtained. The y-intercept was calculated by:

$$H_a(0) = [H_w(0)][1 + (1 - a)k_0]^{1/(1-a)}.$$

The mean platelet survival time was calculated by:

$$T_a = \frac{1}{k} [1 + (1 - a)k_0].$$

Polynomial Function

The polynomial function, $H_p(t)$ was calculated by:

$$H_p(t) = a_0 + a_1 t + a_2 t^2 + \dots + a_n t^n,$$

where $a_0, a_1, \dots, a_n$ were constants. The mean platelet survival time was given by:

$$T_p = a_0/a_1.$$

Multiple Hit Function (11)

The multiple hit function, $H_n(t)$ was calculated by:

$$H_n(t) = (c/n) \sum_{i=0}^{n-1} \{[(n-1)/i!]\exp(-at)(at)^i\},$$

where "n" was the number of hits, "c" the normalizing constant, and "a" the mean waiting time between hits. The mean platelet survival time was given by:

$$T_n = n/a.$$

The number of hits, "n" reflected the mechanism of platelet destruction; when $n = 1$ it indicated exponential survival and when "n" became large it reflected linear survival.

Dornhorst Function (11,15)

The Dornhorst function, $H_d(t)$, was calculated by:

$$H_d(t) = H_d(0) \{[\exp(-kt) - \exp(-kT)]/[1 - \exp(-kT)]\},$$

where $H_d(0)$ was the y-intercept, "k" the relative random rate of platelet destruction, and "T" the potential age dependent platelet survival time. The mean platelet survival time was given by:

$$T_d = [1 - \exp(-kT)]/k.$$

Meuleman Function (17)

The Meuleman function, $H_m(t)$, was calculated by:

$$H_m(t) = H_m(0) (1 - t/T) \exp(-kt),$$

where $H_m(0)$ was the y-intercept, "k" the random rate of platelet destruction, and "T" the potential age dependent survival time. The mean platelet survival time is given by:

$$T_m = T/(1 + kT).$$

FOOTNOTE

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A COMPUTER PROGRAM IN COMPILED BASIC FOR THE IBM PERSONAL COMPUTER TO CALCULATE THE MEAN PLATELET SURVIVAL TIME WITH THE MULTIPLE-HIT AND WEIGHTED MEAN METHODS

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Abstract—We developed an easy-to-operate computer program for the IBM personal computer to calculate, display and store in a database platelet kinetic data determined by analysis of the rate of clearance of radiolabeled blood platelets from the circulation. This was done by curve fitting using the weighted mean method and multiple-hit model. These models are complementary and calculating the mean platelet survival time with both is recommended. Improvement of the weighted mean method was investigated. The optimized weighting and fitting the exponential function with the Marquardt non-linear least squares method improved the weighted mean method. The weighted mean and multiple-hit models fit the survival curve data equally well. The calculation of the mean platelet survival time with the weighted mean method was very fast. The duration of calculation with the multiple-hit model could take up to 2 minutes. Calculation of the mean platelet survival time using both models has the advantage that conditions when calculation of the mean platelet survival time would be invalid, can be detected. The computer program will promote the valid comparison of results obtained at different institutions.

Blood platelets	kinetics	mean platelet survival time	radionuclide	¹¹¹ In
computer program	IBM personal computer	mathematical models	multiple-hit model	
weighted mean model				

INTRODUCTION

The estimate of the mean platelet survival time, which has become feasible in general hematology practice since the introduction of satisfactory radionuclide labels, is regarded as the most useful test for *in vivo* platelet activation [1]. It would therefore clearly be advantageous to make the calculation of mean platelet life-span available in such a format that it will be acceptable to all investigators. This will also promote standardization of these methods and facilitate the comparison of results obtained in different laboratories.

The mean platelet survival time is estimated as the ratio of the initial y-intercept and the slope of the survival curve obtained by measuring the rate of clearance of radiolabeled blood platelets from the circulation [2,3]. This is a deceptively simple procedure which may be influenced by several factors. First, the distribution of the data points around the regression line may be affected by damaging or activating the platelets during the collection and isolation of platelets from the blood. The labeling procedure may also be toxic to the platelets, and other, as yet undefined, mechanisms may also lead to the "collection injury". Second, there may be bias in selecting and weighting the respective importance of the data points used for the estimate of the mean platelet survival time.

The International Committee for Standardization in Hematology (ICSH) has proposed methods for the isolation and labeling of a representative population of platelets and, to eliminate bias, recommends fitting the survival curve to the data points with one of a number of mathematical models [2,4]. Their original weighted mean method, which could be applied with an unsophisticated calculator, solved the problem of fitting the data points to a survival

curve simply by weighting the linear and exponential estimate of the mean platelet survival time inversely with the residual sum of squares. Another recommended method, which is now generally accepted as the gold standard for analysis of the platelet survival curve, is the multiple-hit model [5]. In 1977, when the ICSH made their recommendations, the procedure for estimating the mean platelet survival time with the multiple-hit model, required a mainframe computer, which was beyond the resources of most hematology laboratories.

Personal computers are now widely available in laboratories. It will therefore be useful to have access to a program which will allow easy and accurate estimation of the mean platelet survival time with such a computer. Thus the major aim of this study was to develop a user-friendly computer program for an IBM personal computer (PC), or compatible computer, for the analysis of platelet survival data. The multiple-hit model is generally accepted as the gold standard for the analysis of platelet survival curves. Because the multiple-hit model does not detect all conditions which may invalidate the theoretical assumptions on which the accurate calculation of platelet survival are based, it is advisable to use two models when estimating the mean platelet survival time [6,7]. We selected the multiple-hit and weighted mean models for our computer program.

The goodness of fit of the weighted mean method may be improved by fitting the data to the exponential function by a non-linear least square method, rather than the logarithmic method advocated by the ICSH [6,7]. The weighted mean method may also be improved by optimizing the weighting of the linear and the exponential functions [8]. The second purpose of this investigation was to evaluate the improvement of the new weighted procedure and non-linear least squares method of fitting the data to the exponential function in relation to the ICSH proposed weighted mean method.

The third aim of this study was to compare the new modified weighted mean method with the multiple-hit model as an estimator of the mean platelet survival time. This was done in 15 normal reference subjects and 54 patients with a variety of disorders affecting platelet survival and platelet turnover.

METHODS

Patient selection

The mean platelet survival time was estimated in 15 reference subjects and 54 patients with a variety of disorders. All were in a steady state of platelet production and destruction as reflected by a stable blood platelet count during the course of the study. Platelets were labeled with In-111 and re-injected [9]. Blood was sampled at regular intervals, the radioactivity of the samples measured by scintillation counting, and the platelet survival curves constructed from the data.

Curve fitting methods

The weighted mean regression curve, at time t , was calculated by:

$$W(t) = kE(t) + (1 - k)L(t).$$

The constant k is the weight of the linear, $L(t)$, and exponential, $E(t)$, regression curves fitted to the data and

$$L(t) = L_0 - at$$

$$E(t) = E_0 \exp(-rt)$$

where L_0 and E_0 is the y -intercept of the linear and exponential functions, a the linear slope and r the exponential rate constant.

The weighted mean estimate of the mean platelet survival time was calculated by:

$$T_w = kT_E + (1 - k)T_L.$$

The linear estimate of the mean platelet survival time, T_L , was calculated by the linear least squares method [2,4].

We investigated methods to improve the calculation of the weighted mean estimate of the

Table 1. Weighted mean methods

Method name	Weighting method	Curve fitting method
WMEX	ICSH	Logarithmic
WMEXZ	Van Zyl	Logarithmic
WMEP	ICSH	Marquardt
WMEPZ	Van Zyl	Marquardt

mean platelet survival time (Table 1). In the WMEX method, proposed by the ICSH, the data is transformed logarithmically and the exponential function fitted with a linear least squares method [4]. The weight k for this method is calculated by:

$$k = S_{LL}/(S_{LL} + S_{EE})$$

$$S_{LL} = \sum_{i=1}^n (L(t_i) - Y_i)^2$$

$$S_{EE} = \sum_{i=1}^n (E(t_i) - Y_i)^2$$

where Y_i is the observation at time t_i and $i = 1, 2, \dots, n$.

We performed curve fitting for the WMEXZ method similarly, but we optimized the weight of the linear and exponential functions according to van Zyl [8]:

$$k = (S_{LL} - S_{LE})/(S_{LL} + S_{EE} - 2S_{LE})$$

where

$$S_{LE} = \sum_{i=1}^n (L(t_i) - Y_i)(E(t_i) - Y_i).$$

Note that k will be negative when $S_{LL} < S_{LE} < S_{EE}$. When a negative k is substituted in the equation for T_w we find that $T_w > T_L$ since T_E is always less than T_L . Also the second derivative of $W(t)$ is:

$$W''(t) = r^2 k E_0 \exp(-rt).$$

$W''(t)$ will be negative for $k < 0$ and the survival curve $W(t)$ will be concave downwards. When $S_{LL} > S_{LE} > S_{EE}$ we find that $k > 1$ and $T_w < T_E$.

The shape of the platelet survival curve should vary between the shapes of the linear and exponential functions. The corresponding value of the weight k , should be between 0 and 1. If the value of k was larger than 1, the weighted mean method mean platelet survival time was replaced by the exponential estimate; if k were negative, the linear estimate was used.

When the weight k was outside the range 0–1 we considered it an indication that the measured platelet population may not comply with the underlying theoretical considerations for calculation of the mean platelet survival time.

The weights for the WMEP and WMEPZ methods were calculated as for the WMEX and WMEXZ methods respectively, but we fitted the exponential function to the survival curve with the Marquardt non-linear least squares method [10].

The multiple-hit mean platelet survival time was estimated according to the method of Murphy [5]. Curve fitting was with the Marquardt non-linear least squares method. This method is not so dependent on the closeness of the initial constants to the true values to ensure convergence.

Platelet turnover

The platelet turnover was calculated by [11]:

$$P = \frac{90C}{RT}$$

Table 2. Program Menus

Patient group	
F1—Normal	F2—Diabetics
F3—Hypercholesterolaemia	F4—Renal transplant
F5—Valvular disease	F5—Immune thrombocytopenia
F7—Display and print menu	F8—Exit
Data input	
F1—Display and print menu	F2—Curve fitting menu
F3—Append patient	F4—Edit curve data
F5—Delete patient	F6—Delete curve data
	F8—Exit
Curve fitting	
F1—Select patient	F2—Select fitting range
F3—Fit all models	F4—Fit weighted mean
F5—Select models	F6—Display and print menu
	F8—Exit
Mathematical models	
F1—Linear (LN)	F2—Exponential (EP)
F3—Weighted mean	F4—Multiple hit (MH)
Display and print	
Display	Print
	F2—Selected patient:results
	F4—Survival times or mean estimate of error
F1—Survival times	
F3—Mean estimate of error	
	F6—Survival times or mean estimate of error
F5—Survival times or mean estimate of error	F8—Exit
F7—All results	

where C is the platelet count and T the mean platelet survival time. The percentage recovery of platelets in the circulation, R , is given by:

$$R = 100F(t_i)/A$$

where $F(t_i)$ is the y -intercept of the regression curve constructed from the In-111-radioactivities per ml blood, and A the total radioactivity injected, expressed per ml of the subject's blood volume.

Statistical methods

We used the mean error of the estimate (MEE) as the indicator of the goodness of fit of the regression curves to the survival curve data. The MEE was calculated as the standard deviation of the curve data points from the regression curve. The MEE and the estimates of the mean platelet survival times for the different methods were compared by the nonparametric Wilcoxon signed rank test for matched pairs [12]. We have assumed that the population medians of differences are equal between patients. The MEE was normalized, for inter-comparison, to the Y -intercept calculated with the WMEPZ method.

Computer program

We developed a computer program, written in compiled BASIC, for the IBM Personal Computer to calculate the mean platelet survival time with the weighted mean and multiple-hit methods. The minimum hardware requirements are 256 KB memory, graphic

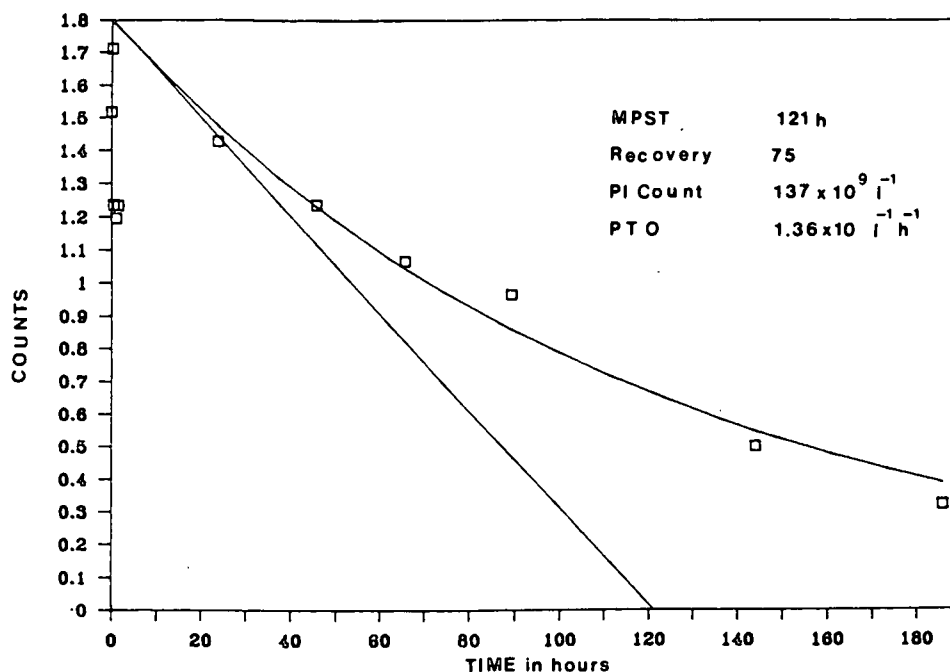


Fig. 1. Graphical display to evaluate curve fitting. The solid line is the regression curve fitted to the survival curve data (open square symbols). The mean platelet survival time is the intercept of the tangent (+ symbols) and the x-axis.

display and two floppy disc drives. The program is menu driven. The menus are presented in Table 2. The patients are divided into groups according to disease with the "Patient Group" menu. Patient data are entered and edited with the "Data Input" menu. The information is stored on disc and may be retrieved at any time.

The curve fit range is selected with the "Patient Selection and Curve Fitting" menu. The survival curve is displayed and the position keys used for the visual selection of the curve fit range. After fitting the curve, the platelet survival and regression curves are displayed. This permits visual evaluation of the goodness of fit of the regression curve to the data. The curve fitting procedure may be repeated if necessary. The initial tangent, which indicates the platelet survival time, is displayed with the regression curve (Fig. 1). The mean platelet survival time, MEE, recovery of platelets in the circulation, and platelet turnover are also displayed on the screen. Hard copies may be obtained with a screen dump to a dot matrix printer with graphics capabilities.

The linear, exponential, weighted mean and multiple-hit models may be selected for the curve fitting procedure with the "Mathematical Models" menu. The program also makes provision for the incorporation of additional or other, models, if desired.

The mean platelet survival time, MEE and regression constants may be selected for display with the "Results: Print or Display" menu. All the data are stored on disc and may be incorporated into a spreadsheet or database.

RESULTS

Curve fitting

The goodness of fit for the methods used to calculate the mean platelet survival time, were evaluated by comparing their respective MEE. It should be noted that it can be shown theoretically that the MEE using the optimized weights for the weighted mean method is always less than or equal to that of a weighted mean method using the weights proposed by the ICSH [8]. The MEE of an exponential curve fitted using a logarithmic transformation is larger than or equal to that fitted by minimizing the sum of squares directly. Therefore the

MEE of the WMEXZ is less than or equal to that of the WMEX, the MEE of WMEP is less than or equal to that of the WMEPZ and the WMEPZ method has the smallest MEE of the weighted mean methods. The average values of the MEE for the different models and groups are given in Table 3. The MEE for the multiple-hit (MH) method ($3.32 \pm 1.83\%$) and the weighted method, WMEPZ ($3.34 \pm 1.78\%$) were the smallest.

Differences in average values and results of statistical tests are given in Table 4 and 5. Analyzing all the subjects, the MEE of the optimized weighted mean methods, WMEXZ and WMEPZ, were significantly smaller ($p < 0.01$) than that of the corresponding ICSH weighted methods, WMEX and WMEP (Table 4). In the whole group of patients, the optimized WMEPZ method fitted the survival curves better than the optimized WMEXZ method ($P < 0.01$). The goodness of fit with the WMEPZ and MH methods did not differ significantly. In the reference subjects, the optimized weighted mean methods (WMEXZ and WMEPZ) fitted the survival curves significantly better ($p < 0.01$) than with the ICSH methods (WMEX and WMEP) (Table 5). In the reference subjects, the method of exponential curve fitting did not influence the goodness of fit for the optimized weighted mean methods; the MEE for the WMEXZ and WMEPZ methods did not differ significantly. The goodness of fit with the WMEPZ and the multiple-hit (MH) method were also similar.

The WMEPZ method, fitting an exponential function to the data by the Marquardt non-linear least squares method and optimizing the weighting, was the best of the weighted mean methods. This explains why this method was chosen for the computer program. The WMEXZ method, based on linear functions, could be selected if convergence is not reached.

Mean platelet survival time

We then determined whether the mean platelet survival times estimated with the various methods differed. The mean and standard deviation (SD) for the reference subjects and the whole group are presented in Table 6.

The WMEXZ estimate of the mean platelet survival time of the whole group differed

Table 3. Mean error of estimate

Method	Patients $n = 69$	Reference subjects $n = 15$
WMEX	3.62 ± 1.98	2.60 ± 1.22
WMEXZ	3.48 ± 1.91	2.54 ± 1.22
WMEP	3.42 ± 1.81	2.57 ± 1.22
WMEPZ	3.34 ± 1.78	2.53 ± 1.23
MH	3.32 ± 1.83	2.49 ± 1.30

Table 4. Differences in mean error of the estimate for patients ($n = 69$)

	WMEX	WMEXZ	WMEP	WMEPZ
MH	0.30°	0.16°	0.10°	0.02
WMEPZ	0.28°	0.14°	0.07°	
WMEP	0.20°	-0.06		
WMEXZ	0.14°			

° $p < 0.01$.

Table 5. Differences in mean error of the estimate for reference subjects ($n = 15$)

	WMEX	WMEXZ	WMEP	WMEPZ
MH	0.11	0.05	0.08	0.04
WMEPZ	0.07°	0.00	0.04†	
WMEP	0.03	-0.03†		
WMEXZ	0.06†			

° $p < 0.05$.

† $p < 0.01$.

Table 6. Mean platelet survival time

Method	Patients <i>n</i> = 69	Reference subjects <i>n</i> = 15
WMEX	145 ± 69	215 ± 18
WMEXZ	146 ± 73	217 ± 19
WMEP	144 ± 74	214 ± 22
WMEPZ	144 ± 74	214 ± 20
MH	149 ± 75	223 ± 17

Table 7. Differences in mean platelet survival time (hours) for patients (*n* = 69)

	WMEX	WMEXZ	WMEP	WMEPZ
MH	-4.4*	-3.4*	-5.4*	-5.7*
WMEPZ	1.3	2.3*	0.3	
WMEP	1.0	2.0		
WMEXZ	-1.0			

* *p* < 0.01.Table 8. Differences in mean platelet survival time (hours) for reference subjects (*n* = 15)

	WMEX	WMEXZ	WMEP	WMEPZ
MH	-7.3*	-5.7*	-8.4*	-8.6†
WMEPZ	1.3	2.8†	0.2	
WMEP	1.1	2.7		
WMEXZ	-1.6			

* *p* < 0.05.† *p* < 0.01.

significantly from the estimates with the WMEPZ (*p* < 0.01) (Table 7). The multiple-hit estimate of the mean platelet survival time was significantly different from all the weighted mean method estimates (*p* < 0.01).

For the reference subjects (Table 8) and weighted mean estimates only the WMEXZ and WMEPZ estimates were significantly different (*p* < 0.01). The multiple-hit estimate of the mean platelet survival time was significantly different from the weighted mean estimates.

The average value of the weight factor *k* for the WMEPZ method and the reference subjects was 0.14 ± 0.13.

Computer program

The curve fitting procedure and calculation of the mean platelet survival time is instantaneous for the weighted mean method. The calculation for the multiple-hit model is very fast if the survival curve is exponential, and increases to 2 min for 12 data points and 25 hits.

DISCUSSION

The reasons for our decision to select both the multiple-hit and weighted mean methods for inclusion in the computer program become clear after considering some of the basic concepts on which the measurement of the mean platelet survival time are based, and the limitations of the multiple-hit model.

The measurement of mean platelet survival time may be based on the analysis of cohort survival curves, where the labeled cell population is of the same age; or population survival curves derived from the circulating radioactivity of a random, uniformly labeled sample of the circulating platelet population. A steady state is an essential requirement for the accurate determination of mean platelet survival time from such a survival curve.

Population survival curves permit valid analyses even when the mode of the disappearance of the cells is unknown. This is based on the assumption that the age-distribution of the circulating platelets is identical with the cohort survival curve. It should be noted that, in a steady state, the population curve is non-convex upwards. Since the survival curve is generally curvilinear, it follows that the population curve will generally be concave upwards.

The parameters of the multiple-hit model are based on "physiological" principles, although this is not a prerequisite for its validity to calculate the mean platelet survival time. This model assumes that platelets are removed from the circulation after sustaining a number of insults or "hits". The number of "hits" reflect the mechanism of platelet destruction and the shape of the survival curve. One "hit" will result in an exponential shaped survival curve. As the shape of the survival curve becomes linear the number of "hits" increases. The shape of the survival curve only varies between linear and exponential. Therefore, with the multiple-hit model the investigator will not be aware when the survival curve lies outside the linear exponential range. This does not permit the detection of conditions which invalidate the theoretical assumptions on which the accurate calculation of the mean platelet survival time are based [6,7]. For example, platelet production and destruction will not be in equilibrium if a large proportion of the circulating platelet population are wiped out. The platelets labeled under such conditions will be newly formed and of a more uniform age. The survival curve will therefore not be that of a population curve, but a "cohort type" curve, shaped concave downwards, will result. Analysis of such a curve with the multiple-hit model will give the result of a large number of hits, incorrectly indicating a linear curve and overestimating the mean platelet survival time. This is because the parameter of the multiple-hit model that represents the shape of the curve, the value of n indicating the number of hits, does not distinguish between a truly linear and a concave downwards shaped platelet survival curve.

This limitation to detect possible non-valid conditions may be overcome by analyzing the platelet survival curve with two different models and comparing the results. Since an optimized weighted mean method does permit the detection of a concave survival curve [8], we propose that this method be used in conjunction with the multiple-hit model. Although some of the simplicity of the weighted mean method is sacrificed, the effective time of calculation with an IBM PC is not increased. Furthermore, the same non-linear curve fitting method is now used for the weighted mean and multiple-hit functions.

In this study we estimated the mean platelet survival time both with the improved weighted mean method and the standard multiple-hit model. Both the models fitted the survival curves equally well and either of these methods may therefore be used. However, the estimates of the mean platelet survival time calculated with these two methods did differ significantly and the results obtained with the two methods may therefore not be directly compared, especially if small differences in the mean platelet survival time are to be detected. On the other hand, this difference is so small that in clinical practice preference for either of these methods should have no influence on the estimate of the mean platelet survival time in individual patients.

The modified weighted mean method, especially in conjunction with the multiple-hit model, does have some advantages. The calculation of the survival time is simple and rapid. The weighting factor, k , also gives an indication of the shape of the survival curve. Thus, a value of 1 suggests exponential removal of platelets from the circulation. This reflects predominantly random destruction of platelets and will correspond to a single hit with the multiple-hit model. On the other hand, a weighting factor, k , of zero indicates a linear platelet survival curve, and that clearance of platelets from the blood is age-dependent. The number of hits for the multiple-hit model is large for age-dependent survival. If the weighting factor, k , is larger than 1 or negative, the results of survival curves should be interpreted with caution [2, 6]. A negative weighting factor, k , was associated with a weighted mean platelet survival estimate that exceeded that of the linear estimate. A weighting factor, k , larger than 1, was associated with the observation that the weighted mean estimate of the mean platelet survival time was shorter than that obtained by fitting the data to an exponential function. The multiple-hit function only varies between exponential and linear and with this model it is not

possible to detect curve shapes that does not lie between the linear and exponential functions.

The computer program described here, especially if it were generally available, does offer several advantages. The function of the computer program is reliable and it can be used by staff with no specialized computer training. The facility to calculate the mean platelet survival time with two independent models, permits evaluation of noise and detection of conditions when the method of calculation of the mean platelet survival time is not valid. More important, standardization of mathematical methods used for the estimate of the mean platelet survival time offers clear advantages. Results of different laboratories may also be compared directly and the interpretation of the results of their studies will be much easier. Also, the flexibility of the program does make provision for those investigators who prefer, wish to include, or evaluate, other mathematical models for the measurement of the mean platelet survival time.

SUMMARY

The estimate of the mean platelet life-span is regarded as the most useful test for *in vivo* platelet activation. It would therefore be advantageous to make the calculation of the mean platelet survival time available in such a format that it will be acceptable to all investigators. The major aim of this study was to develop a user-friendly computer program for an IBM personal computer for analysis of platelet survival data. The mean platelet survival time is determined by measuring the initial rate of clearance of radiolabeled blood platelets from the circulation by fitting a mathematical model to the survival curve. We used the multiple-hit model, and the weighted mean estimate of the mean platelet survival time for this purpose. Calculation of the mean platelet survival time using two independent models has the advantage that it permits detection of conditions when the calculation of the mean platelet survival time may be invalid.

The second aim of this investigation was to optimize the weighted mean method and to compare the new weighted mean method with the multiple-hit model as an estimator of the mean platelet survival time.

Since IBM personal computers are widely available a computer program was developed for the analysis of platelet survival data. We required the program to permit analysis, storage and retrieval of data. Also, the survival curve should be displayed graphically, thus aiding the selection of the curve fitting range and permitting visual judgment of the goodness of fit of the generated curve to the data. The recovery of the labeled platelets in the circulation and the platelet turnover should also be calculated.

To optimize the weighted mean method we compared the non-linear and the logarithmic methods of exponential curve fitting, with and without optimal weighting, by analyzing the survival curves of 15 reference subjects and 54 patients. In the WMEX method, proposed by the ICSH, the data are transformed logarithmically and the exponential function fitted with a linear least squares method. We performed curve fitting for the WMEXZ method similarly, but we optimize the weight of the linear and exponential functions. The weights for the WMEP and WMEPZ methods were calculated as for the WMEX and WMEXZ methods respectively, but the exponential function was fitted to the survival curve with the Marquardt non-linear least squares method.

The multiple-hit mean platelet survival time was estimated according to the method by the International Committee for Standardization in Hematology.

We used the mean error of the estimate (MEE) as the indicator of the goodness of fit of the regression curves to the survival curve data. Analyzing all the subjects, the MEE of WMEPZ, was significantly smaller ($p < 0.01$) than that of the other weighted methods.

The WMEPZ method, fitting an exponential function to the data by the Marquardt non-linear least squares method and optimizing the weighting, was the best of the weighted mean methods and chosen for the computer program. In addition, the weight factor also reflects curve shape.

The goodness of fit with the WMEPZ and MH methods did not differ significantly. The

multiple-hit estimate of the mean platelet survival time was however significantly different from WMEPZ ($p < 0.01$).

The curve fitting procedure and calculation of the mean platelet survival time is instantaneous for the weighted mean method, but could increase to 2 min for 12 data points and 25 hits for the multiple-hit model. The ease of use of the computer program permit calculation of the mean platelet survival time by both these models, which is a distinct advantage. Conditions when the calculation of the mean platelet survival time is not valid may be detected with the weighted mean method.

The program for the IBM PC met our specifications and its use should promote valid comparison of results between different institutions.

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Comment on 'A biological approach to the platelet survival curve'

The Editor,
Sir,

The work reported by Trowbridge and Martin (1983) described a method of platelet survival curve analysis based on a biological approach to avoid underinterpretation of the experimental information. We agree that methods based on the true mechanism of platelet destruction should be investigated, but we disagree that the information so obtained can replace the mean platelet survival time as a parameter to evaluate platelet kinetics (Fuster *et al* 1983). It should be seen as additional information complementary to the mean platelet survival time. The calculation of the mean platelet survival time is based on sound theory which is independent of the mechanism of platelet destruction and is calculated by the ratio of the initial y-intercept and the initial slope of the platelet survival curve (Murphy and Francis 1969). Measurements for the calculation of the mean platelet survival time cannot be accurately performed due to an initial plateau as a result of spleen pooling, temporary accumulation of platelets in the liver due to the 'collection injury' of platelets, and other factors not well understood (Peters and Lavender 1983, Heyns *et al* 1983). The initial parameters for calculation of mean platelet survival time are obtained by fitting a mathematical function to the data. The mathematical model has to fit the experimental data well, but does not have to be based on the true mechanism of platelet destruction (ICSH 1977). We have evaluated six mathematical models proposed in the literature and could not show a statistical difference in the 'goodness of fit' or mean survival time in 64 patients.

The method of platelet survival curve analysis described by Trowbridge and Martin (1983) requires the accurate estimation of the half-life and survival time, which correspond to two points only on the platelet survival curve. The method of accurately measuring these points is not described. Estimation of the half-time requires measurement of the initial y-intercept of the survival curve and direct measurement of this point is subject to error. This is evident in their figure 4 where the theoretical data points at day 1 and 2 do not fit the experimental data as well as the other points. Estimation of the survival time in patients with a short mean platelet survival time is also subject to error. In figure 7 of the paper the theoretical data at days 3 to 5 do not fit the experimental data well due to the value of the survival time selected.

Trowbridge and Martin (1983) did confirm the validity of their theory by experimental evidence obtained from the platelet survival curve and could then identify the type of platelet destruction. Wichman and Gerhardt (1981) showed that the non-linear shape of the normal survival curve is due to the selective pooling of younger platelets in the spleen rather than random destruction. Confirming the validity of theory requires a multidisciplinary approach to support evidence based on the survival curve.

We feel that the method proposed by Trowbridge and Martin (1983) can potentially provide additional information, but also needs further verification.

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Platelet survival curve analysis

The Editor,
Sir,

We support the assertion that mean platelet lifetime (MPL) is an important parameter in the evaluation of platelet kinetics. Our study, Trowbridge and Martin (1983), never suggested that information about random and constant destruction of platelets should replace this parameter. Indeed MPL was evaluated, together with random and constant destruction rate, and presented in table 1, p. 1366.

The main message which we hoped to convey in this work was the inadequacy of fitted functional forms as a determination of platelet destructive mechanisms. Our statement, p. 1365, 'Evidence that there is no unique curve fitting procedure' is confirmed by your correspondents' statement that six different functional forms showed no statistical difference in the MPL of 64 patients.

A sensible mathematical model attempts to identify those dominant mechanisms which lead to the experimental observations. It should possess predictive capabilities. For this reason evaluation of the parameters K_2 and C from the half-life $t_{1/2}$ and survival time t_s enabled the experimental observations to be predicted by our model (not fitted) at any other time. Accurate evaluation of $t_{1/2}$ and t_s can be obtained from a least square error best polynomial fit to the experimental data (p. 1364).

The prediction, by our mathematical model, of a random-to-constant destruction rate of platelets, which is likely to be of the order of one-to-one in man and three-to-one

EVALUATION OF MATHEMATICAL MODELS TO ASSESS PLATELET KINETICS.

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ABSTRACT

Twelve mathematical methods used to calculate the mean platelet survival time were compared by determining the 'goodness of fit' of the models to the platelet survival curves of fifteen reference subjects and 54 patients. Platelets were labelled with indium-oxine (In-111). The linear (LN), exponential, weighted mean, multiple hit (MH), Dornhorst (DH), Meuleman (ML), alpha order (AO) and polynomial (PO) mathematical models were investigated. The 'goodness of fit' for the exponential model was determined by the nonlinear least squares method (EP), and also by the linear least squares method on logarithmically transformed data (EX) as is recommended. The modified weighted mean (MWM) and the usual weighted mean method (WM) obtained with these exponential models were tested. The Dornhorst (DH10) and Meuleman (ML10) models, where the potential age-dependent platelet survival times were kept constant at 10 days, were also evaluated. The 'goodness of fit' results, expressed as percentage standard deviation, indicated that the LN (5.2 %), EX (5.0 %), EP (4.4 %), WM (3.7 %), DH10 (3.7 %) and ML10 (3.7 %) models all fitted the data significantly worse than the MWM, MH, DH, ML, AO and PO models (Range 3.2 % to 3.3 %). The mean platelet survival time determined with the MH model differed significantly from the results with the DH, ML and AO models. The results of mean platelet survival time calculated with different mathematical models can therefore not be compared directly. The models that fitted the platelet survival curve well varied slightly in sensitivity to noise as is indicated by the coefficient of variation of the mean platelet survival time estimates for the reference subjects (Range 7.9 % to 12.0 %). The sensitivity of the mathematical models to noise was also evaluated by calculating the change in the mean platelet survival time when the initial point and points over the curve were deleted. The differences in mean platelet survival time estimated with the different mathematical models were not significant. This result indicates that the models that fit the data well were equally sensitive to noise. Fitting data to at least two mathematical models have definite advantages. If the mean platelet survival time estimated with the alpha order model, is shorter than that estimated with the EP, MWM or MH models, or, the mean platelet survival time estimated with either the DH, ML, AO or PO models, are longer than the LN, MWM or MH estimate of the mean platelet survival time, the data on which the calculations are based, are probably invalid. We conclude that the mean platelet survival time can be reliably estimated by fitting the data to either the MWM method (if limited computing facilities are available) or the MH model. The confidence in the result will be increased if it is considered in conjunction with the finding obtained with one other model: In those cases where the platelet survival time is very short, the alpha order model is recommended. In other instances the results of the MWM method or MH model should be compared to that obtained with either the DH, ML, AO or PO models.

INTRODUCTION.

Shortening of the platelet life span is generally accepted as an important reflection of in vivo platelet activation. This measurement has been especially valuable for the understanding of the role of platelets in various diseases characterized by thromboembolic phenomena or thrombocytopenia, for the evaluation of the efficacy of drug therapy in these diseases, and for the detection of some prethrombotic states. The accurate measurement of platelet life span is a prerequisite for the adequate investigation of these disorders.

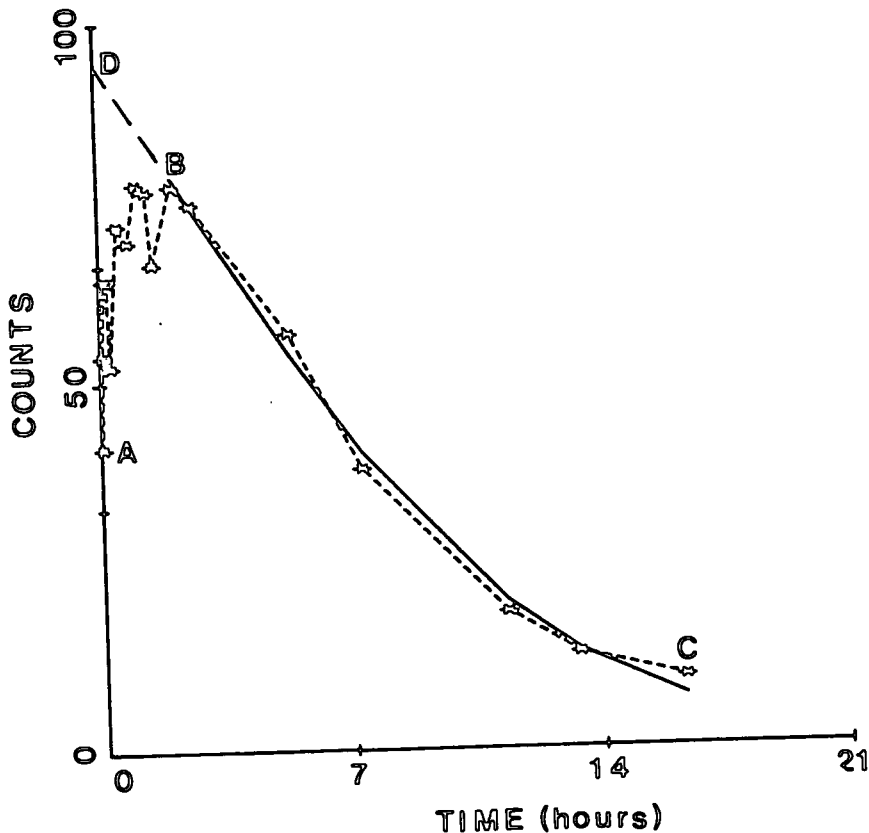


Figure 1: Calculation of the mean platelet survival time is demonstrated. Period A to B represents the equilibrium phase. A mathematical model is fitted from B to C and a regression curve, indicated by the solid line is generated. This curve is back extrapolated from B to D. The y-intercept and initial slope is determined at D.

The available methods for isolating or labelling a cohort of platelets of the same age are unsatisfactory and can not be used in clinical studies. Therefore, a population of platelets representative of those in the circulation is isolated, labelled with a radionuclide and reinjected. The mean survival time of the labelled platelets is indirectly estimated from the ratio of the y-intercept and the initial slope of the platelet survival curve (1,2). The y-intercept and the initial tangent of the survival curve cannot be directly

measured because the stability of the early part of the curve is disturbed by the redistribution of platelets between the circulation and the splenic platelet pool, and by the temporary accumulation of platelets in the liver. The latter, ascribed to the 'collection injury', varies in severity. The labelled and circulating platelets only equilibrate after 1 to 24 hours (3,4). Only the later part of the survival curve will reflect the true rate of clearance of platelets from the circulation. The y-intercept and slope of the curve can only be determined by back extrapolation of this part of the curve (Figure 1). Although extrapolation may be performed visually, this method is subject to errors and bias. Mathematical models have therefore been developed to facilitate accurate fitting of the platelet survival curve to the data, by using statistical methods. The regression constants generated by the mathematical models permit more precise estimation of the initial parameters. It should be noted that these mathematical models are not required to be based on valid physiological assumptions. They solely function to improve the 'goodness of fit' of the platelet survival curve to the data points.

Although many such models have been applied to the study of platelet kinetics in health and disease, little information is available on their comparative value, or, on their suitability under specific pathological conditions (5-9). The present study focuses on these questions.

MATERIALS AND METHODS.

Subject Selection

Platelet survival was determined in 15 reference subjects and on 54 patients with a variety of disorders. The patient group consisted of 4 patients with valvular disease of the heart, 17 with previous renal transplantation, 14 with idiopathic thrombocytopenic purpura, 2 with diabetes mellitus, 14 with heterozygous Type IIa familial hypercholesterolemia, and 3 with aortic aneurysms. All subjects were in a steady state of platelet production and destruction during the course of investigation. This was reflected by a constant blood platelet count.

Platelet Labeling

Platelets were labelled with In-111-oxine as previously described (9-11). Labeled platelets were used for in vivo studies only if in vitro aggregation with ADP and collagen was normal (3,9,10). A dose not exceeding 18.5 MBq (500 microcuries) of In-111 was administered.

Following injection of the labelled platelets, blood samples were obtained at 5, 10, 15, 30, 60 and 90 minutes, and thereafter daily. In patients with short mean platelet survival times, blood sampling was appropriately spaced so that at least 8 valid data points were available for curve fitting. Blood samples of 3 ml were directly collected in the counting vials, the mass determined, and water added to a constant final volume of 4 ml. The samples were counted in a well scintillation counter with the pulse-height analyzer set to include the 172 keV and 247 keV peaks of In-111. The measured count rate was corrected for background and mass. Plasma In-111 activity was determined in all cases, and was less than 5 %.

Mathematical Functions and Methods to Fit Platelet Survival Curves.

The following mathematical functions were fitted to the data points

reflecting the the rate of clearance of platelets from the circulation:

1. Linear Function (LN) (11,12).
2. Exponential Function (11,12). Two methods were used to fit the data to an exponential function. In the first method (EX), the regression constants for the exponential model were calculated as recommended by the ICSH: the data were logarithmically transformed and the curve fitted with the linear least squares method (11). In the second method (EP), the curves were fitted with the Newton-Gauss method, an iterative nonlinear least squares technique (13). The latter method was investigated because the ICSH recommends that the discrepancies between the fitted line and data points should be calculated without logarithmic transformation of the data. The nonlinear least squares technique minimizes these discrepancies (11,13).
3. Weighted Mean Function (11,12). This was calculated by two methods, dependent on the method used to fit the data to an exponential function. The first method (WM) was according to the recommendations of the ICSH where the exponential regression curve is constructed after transforming the data logarithmically (11). For the second, a modified weighted mean method (MWM), the exponential regression constants were obtained by fitting the data with a nonlinear least squares technique. The variance of the linear, exponential and modified weighted mean methods was calculated. If the variance of either the linear or exponential models was less than that of the modified weighted mean method, the result of the method that fitted the data best was used as the estimate of the mean platelet survival time by the modified weighted mean method. This modification was introduced to minimize the influence of noise when data points were distributed perfectly linearly or exponentially.
4. Alpha Order Function (AO) (14). This function was fitted to the survival curve data by the Newton-Gauss iterative nonlinear least squares technique (13).
5. Polynomial Function (PO). Only the second degree polynomial function was used. The higher degree polynomial functions were found to be too sensitive to noise and outliers.
6. Multiple Hit Function (MH) (11). The curve fitting technique described by the ICSH was used. Initial values for iteration was obtained from the linear and exponential curve fitting procedures (11).
7. Dornhorst Function (11,15). The curves were fitted with the Newton-Gauss iterative nonlinear least squares technique (13). Mean platelet lifespan was determined by two methods. First (DH10), the potential age-dependent platelet survival time was kept constant at the value determined for normal subjects, 10 days. In the second method (DH), the potential age-dependent platelet survival time as determined for the specific patient was used.
8. Meuleman Function (17). Curve fitting of the Meuleman function (ML and ML10) was performed as was described for the Dornhorst function.

Curve Fitting Procedure.

A computer program in Fortran, executed on a Nuclear Medicine digital imaging system, was developed to fit survival curves to the mathematical models. The survival curves were entered by keyboard and stored on the system database. Curve fitting, with any of the above models, was menu driven. It allowed graphical display of the curves to select the range of points for curve fitting. The data points were selected visually as follows. The first point included was that reflecting the highest blood radioactivity after equilibrium had been reached. All subsequent data points with radioactivity greater than 10 % that of the initial counts were included. The 'goodness of

fit' of the data points to each of the mathematical models , was visually evaluated by inspecting the graphical display of the survival and regression curves on the computer terminal.

Comparison of Mathematical Models.

The different mathematical models were compared by calculating the root mean square of the difference between the measured and regression survival curve points. This value, reflecting the 'goodness of fit ', was expressed as a percentage of the y-intercept and designated as the percentage standard deviation. The survival curves with different count rates could thus be compared.

The percentage standard deviation and mean platelet survival time calculated for the different models were compared with Hotteling's multivariate T-squared statistic to test the hypothesis of equality of means (16, 18). The patients were considered to be a random sample from a population and a log-transformation applied to the percentage standard deviation and mean platelet survival time data. The multivariate model incorporates different observations that may be dependent, on the same patient. In those instances where the means differed significantly, multiple comparisons of the means were used to identify those differences which were responsible for the rejection of the hypothesis of equality of means.

The influence of error ('noise') on the estimate of mean platelet survival time was determined by two methods. The coefficient of variation (CV) in the normal subjects was used as an approximate indicator of sensitivity to noise. The resultant differences on the mean platelet survival time when curve points were deleted were also used to evaluate the sensitivity to noise of the mathematical models. For this purpose 16 survival curves were used, each consisting of at least 6 pairs of blood samples. Differences in the mean platelet survival time were obtained firstly by deleting only the initial point and secondly by deleting a point of each of the pairs of data points.

TABLE 1
PERCENTAGE STANDARD DEVIATION (PSD) OF PLATELET SURVIVAL
CURVE POINTS AROUND THE FITTED CURVE

SUBJECTS	WHOLE GROUP		REFERENCE SUBJECTS	
NUMBER	69		15	
MODEL	PSD	SD	PSD	SD
LINEAR	5.2	3.4	2.9	1.3
EXPONENTIAL (EP)	4.4	1.8	4.8	1.3
EXPONENTIAL (EX)	5.0	2.4	6.1	2.6
MODIFIED WEIGHTED MEAN	3.4	1.9	2.6	1.2
WEIGHTED MEAN	3.7	2.3	2.6	1.2
MULTIPLE HIT	3.3	1.8	2.5	1.4
DORNHORST	3.3	1.9	2.5	1.3
DORNHORST (DH10)	3.7	1.8	2.9	1.3
MEULEMAN	3.4	1.9	2.5	1.3
MEULEMAN (ML10)	3.7	1.8	2.9	1.3
ALPHA ORDER	3.2	1.8	2.5	1.4
POLYNOMIAL	3.3	1.9	2.5	1.3

RESULTS.

Evaluation of Mathematical Models: Goodness of Fit.

"Goodness of fit" achieved by the different mathematical models were evaluated by calculating the percentage standard deviation. The results of the mean percentage standard deviation for the reference subjects, and for the whole group (patients and reference subjects) determined for all the mathematical models are given in Table I. The range was 3.2% to 5.2% for the whole group and 2.5% to 6.1% for the reference subjects. The differences in the percentage standard deviation of data fitted with the different models are given in Table II. The percentage standard deviation of the LN model and the fitting of the data to the exponential model both by a linear least squares fit of logarithmically transformed data (EX) or, by the linear least squares method (EP), was significantly ($p < 0.01$) larger than that of the other models: the differences exceeded 0.6 %. These models are therefore not suitable for the calculation of the mean platelet survival time. Also, the mean percentage standard deviations obtained by the Dornhorst model where the age-dependent survival time is held constant at the normal value (DH10), were significantly larger than the MH ($p < 0.01$), DH ($p < 0.01$), ML ($p < 0.05$) AO ($p < 0.05$) and PO ($p < 0.05$) models. The percentage standard deviation associated with the Meuleman model, with a constant age-dependent survival time (ML10), was larger than that of the MH ($p < 0.01$), DH ($p < 0.05$) and AO ($p < 0.05$) models. The percentage standard deviation obtained with WM model was significantly larger ($p < 0.01$) than that obtained with the MH and DH models. The percentage

standard deviation of the MWM and DH models differed significantly ($p < 0.05$). The correlation coefficients of the percentage standard deviations associated with those mathematical models which did not differ significantly, were larger than 0.88.

In the reference group the percentage standard deviation of the exponential model, obtained by both methods of curve fitting (EX and EP), and the other models differed significantly. The exponential models therefore fitted the data poorly.

TABLE II
DIFFERENCES IN PERCENTAGE STANDARD DEVIATION FOR MATHEMATICAL
MODELS INVESTIGATED IN PATIENT GROUP AND REFERENCE SUBJECTS (N=69)

MODEL	EP	EX	MWM	WM	MH	DH	DH10	ML	ML10	AO	PO
LF	0.9	0.2	1.8**	1.5**	1.9**	1.9**	1.6**	1.9**	1.6**	2.0**	2.0**
EP		-0.6**	1.0**	0.7**	1.1**	1.1**	0.7**	1.0**	0.7**	1.2**	1.1**
EX			1.6**	1.3**	1.7**	1.7**	1.3**	1.7**	1.4**	1.8**	1.7**
MWM				-0.3	0.1	0.1*	-0.3	0.1	-0.2	0.2	0.1
WM					0.4**	0.4**	0.0	0.3	0.0	0.5	0.4
MH						-0.0	-0.4**	0.0	-0.3**	0.1	0.0
DH							-0.4**	-0.0	-0.3*	0.1	0.0
DH10								0.3*	0.0	0.5*	0.4*
ML									-0.3	0.2	0.1
ML10										0.4*	0.4
AO											-0.1

* DIFFERENCE SIGNIFICANT AT $P < 0.05$

** DIFFERENCE SIGNIFICANT AT $P < 0.01$

TABLE III
THE ESTIMATION OF THE MEAN PLATELET SURVIVAL TIME
(HOURS) WITH DIFFERENT MATHEMATICAL MODELS.

SUBJECTS	WHOLE GROUP		REFERENCE SUBJECTS		
	ST	SD	ST	SD	CV
MODEL					
LINEAR	176.9	72.5	232.6	15.2	6.5
EXPONENTIAL (EP)	97.8	42.1	122.7	15.4	12.5
EXPONENTIAL (EX)	87.5	37.1	99.9	15.9	15.8
MODIFIED					
WEIGHTED MEAN	144.0	74.3	214.3	23.0	10.7
WEIGHTED MEAN	145.0	69.0	215.4	19.1	8.9
MULTIPLE HIT	149.4	75.6	222.7	17.7	7.9
DORNHORST	142.4	78.2	204.2	24.5	12.0
MEULEMAN	143.1	78.5	204.7	24.1	11.7
ALPHA ORDER	146.2	87.6	212.4	21.0	9.9
POLYNOMIAL	147.3	78.4	206.8	22.6	10.9

Platelet Survival Time.

We next investigated whether the mean platelet survival times estimated with those mathematical models that fitted the survival curves equally well, differed. The mean platelet survival time (ST), standard deviation (SD) and coefficient of variation (CV) are presented in Table III.

TABLE IV
DIFFERENCES IN MEAN PLATELET SURVIVAL TIME OBTAINED
WITH MATHEMATICAL MODELS IN THE REFERENCE SUBJECTS
AND PATIENTS (N=69)

MODEL	WM	MH	DH	ML	AO	PO
MWM	-1.0	-5.4	-1.6	0.9	-2.1	-3.2
WM		-4.4	2.6*	1.9*	-1.2	-2.3
MH			7.0**	6.3**	3.3*	2.2
DH				-0.7	-3.8	-4.9
ML					-3.0	-4.1
AO						-1.1

* DIFFERENCE SIGNIFICANT AT $P < 0.05$

** DIFFERENCE SIGNIFICANT AT $P < 0.01$

The estimate of the mean platelet survival time for the reference subject group varied from 204.2 + 24.5 hours for the DH model to 227.7 + 17.7 hours for the MH model. The mean platelet survival time estimated by the MH model was

significantly longer when compared to that measured with the DH ($p < 0.01$), ML ($p < 0.01$), and AO models ($p < 0.05$) (Table IV). The estimate with the WM method was also significantly longer ($p < 0.05$) than the DH and ML models. In the reference subjects there was also a significant difference ($p < 0.01$) between the mean platelet survival time calculated with the MH method compared to that with the DH, ML and the PO models (Table V). The estimated mean platelet survival time with the AO model also differed significantly ($p < 0.05$) from that with the DH and ML models. The estimated mean platelet survival time with the DH and ML models were nearly identical.

TABLE V
DIFFERENCES IN MEAN PLATELET SURVIVAL TIME OF THE
REFERENCE SUBJECTS ESTIMATED WITH THE MATHEMATICAL
MODELS (n=15)

MODEL	WM	MH	DH	ML	AO	PO
MWM	-1.1	-8.4	10.1	9.6	2.0	7.5
WM		-7.3	11.2	10.7	3.0	8.6
MH			18.5**	18.0**	10.3	15.9**
DH				-0.5	-8.1*	-2.5
ML					-7.6*	-2.0
AO						5.6

* DIFFERENCE SIGNIFICANT AT $P < 0.05$

** DIFFERENCE SIGNIFICANT AT $p < 0.01$

There was good correlation ($r = 0.986$) of mean platelet survival times calculated with MH and MWM models. Also, the MH and DH models correlated well ($r = 0.962$), despite a deviation from the linear regression line in some instances where the mean platelet survival time fell within the normal range (Figure 2). This deviation was not observed when the DH, ML, AO and PO models were correlated. Deviations from the linear regression line within the normal range was accompanied by another finding: the estimate of the mean platelet survival time with the DH, ML, AO and PO models was longer than that obtained by fitting the data to the LN model. This finding was reflected by a slightly concave shape of the platelet survival curve.

The range of the mean platelet survival time, expressed as the CV, observed in the reference subjects, was used as an approximate indicator of the sensitivity of the mathematical models to noise. The CV for the MH model was 7.9 % (Table III). The range of the CV for the MWM, DH, ML, PO and AO models was 9.9 % to 12 %. Sensitivity of the mathematical models was also determined by computing changes in the mean platelet survival time estimates with the initial and with a point of the paired points deleted. The resultant differences in the mean platelet survival time for the different mathematical models were not statistically significant (Fig. 3).

DISCUSSION

On the basis of our results we could divide the ability of the models to fit the data into three groups. The linear and exponential models fitted the

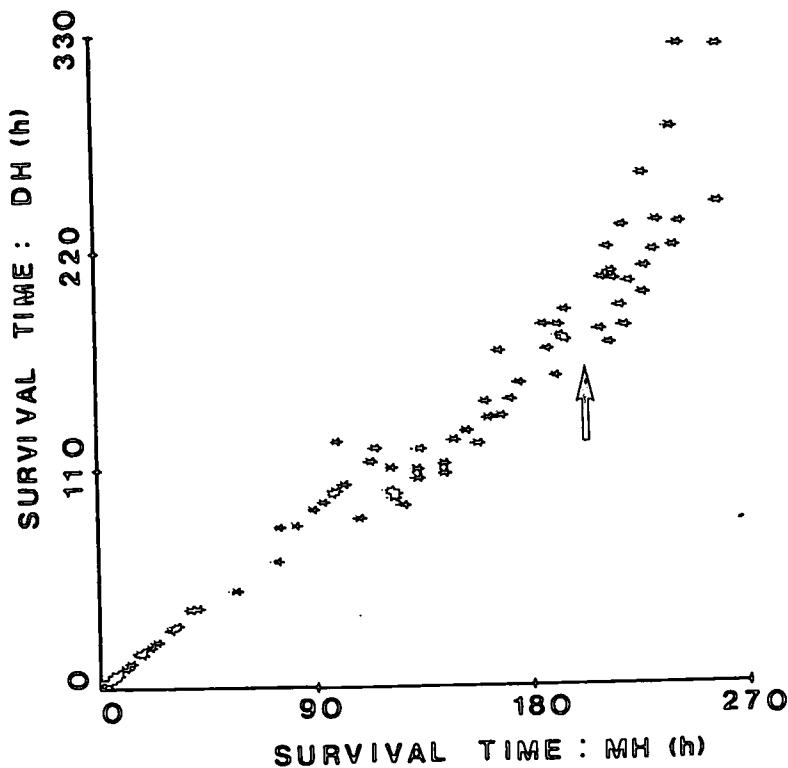


Figure 2: Comparison of the mean platelet survival time estimated by the Dornhorst (DH) and multiple hit (MH) models ($r = 0.962$). There was a deviation of some points from the regression line for survival times exceeding 200 hours.

platelet survival curves of the patients badly. The poor fit of the data by the regression curves resulted in an inaccurate estimate of the mean platelet survival time. This finding is in agreement with results reported by Paulus (20). Estimates of the mean platelet survival time were in many cases in error by 50 % to 300 %. These methods can therefore certainly not be recommended. The results obtained with the second group also indicate that the WM method applied as recommended by the ICSH, and the variations of the Dornhorst and Meuleman models where the potential age-dependent platelet life span is kept constant at 10 days (the normal platelet survival time) also fit the data relatively poorly (21,22). The third group which included our modification of the weighted mean model (MWM), MH, DH, ML, AO and PO models all performed well and there was no evidence that any one of these is definitely superior.

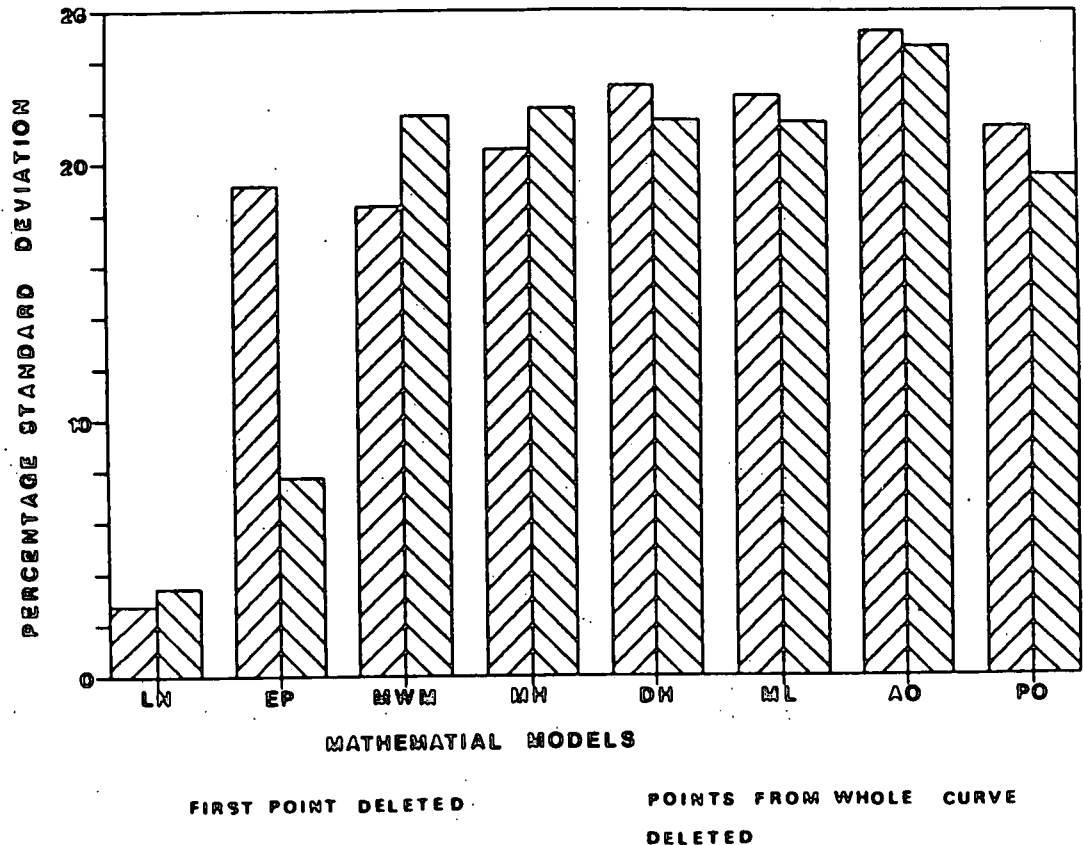


Figure 3: Comparison of differences in the mean platelet survival time estimated by the different mathematical models when the first survival curve point and points from the whole curve were deleted. There was not, with the exception of linear (LN) and exponential (EP) models, a statistical significant difference between the estimates by the models that fit the data well.

Although these mathematical models fitted the curves equally well, their estimates of the mean platelet survival times differed. There is no obvious physiological basis for these differences and it is obvious that the application of, or preference for, any specific model is arbitrary. The major implication of this finding is however, that it may be hazardous to directly compare results of studies where the platelet life spans were not estimated with the same mathematical model.

The usefulness of a mathematical model will also be determined by its

sensitivity to "noise". Sensitivity to noise will decrease the precision of the measurement of the mean platelet survival time. We have used the range of the mean platelet survival time observed in normal subjects (where the platelet survival curve is effectively linear) as an approximate indicator of sensitivity to noise. The multiple hit model is relatively insensitive to noise. This is reflected by the coefficient of variation: in normal subjects it was 7.9 % for the multiple hit, compared to 6.5 % obtained with the linear model. The other models that fitted the data well (MWM, DH, AO, ML and PO) are somewhat more sensitive to noise: in normal subjects their coefficients of variation varied from 9.9 % to 12.0 %. The differences in sensitivity to noise could be ascribed to the longer mean platelet survival time estimated in a few patients by the other models in relation to the MWM and MH models (Fig. 2). These differences occurred when concave shaped curves were fitted. The concave shaped regression curves could result from random error or from "physiological" noise. The sensitivity of the models to random error was evaluated by deleting selected points from the survival curve and compare the resultant mean platelet survival times. The results indicated that the mathematical models that fit the data well were all equally sensitive to noise (Fig. 3).

On the basis of the results of this study we would like to make the following recommendations for estimating the mean platelet survival time. The multiple hit model is recommended, but if limited computing facilities are available, our modified weighted mean method is satisfactory. These models are insensitive to noise, but do not indicate the presence of invalid physiological mechanisms. Therefore, it is always necessary to inspect the platelet survival curve visually. This will provide information on the influence of the collection injury, the presence of obvious outliers, and the shape of the platelet survival curve. This will alert the investigator to the possibility of an imbalance between platelet production and destruction, to problems with the labelling and harvesting of the platelets, and to the possibility of faulty techniques of blood sampling or counting of radioactivity. It is advisable to use at least one other mathematical model to facilitate the recognition of above mentioned pitfalls. The Dornhorst, Meuleman, alpha order or polynomial models are equally satisfactory for the detection of a concave shaped platelet survival curve, while the alpha order is recommended for the detection of higher order functions in patients with a very short mean platelet survival time.

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Quantification of the Distribution of ^{111}In -Labelled Platelets in Organs

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Abstract. A simple and practical approach to the in vivo quantification of ^{111}In -oxine labelled blood platelets with a scintillation camera and computer assisted imaging system was evaluated. Radioactivity of the 172 and 247 keV energies was measured in a phantom at various source distances from the collimator and the accuracy of anterior and posterior mode measurements compared with that of the geometrical mean (GM) method, with and without correction for Compton scatter (CS). Organ radioactivity, expressed as a percentage of whole body radioactivity, was determined in vivo in five baboons and the accuracy of the methods verified by post mortem quantification in the animals. Measurement in the anterior mode significantly overestimates hepatic and underestimates splenic radioactivity; posterior mode quantification reverses these results. Correction with the GM method made the accuracy and reproducibility very acceptable. Further correction for anterior-posterior attenuation and/or CS did not improve results materially. The GM method could readily be applied in five human subjects. This study shows that the GM method is an accurate and practical method for the in vivo quantification of organ and regional distribution of ^{111}In -labelled platelets.

stances. We investigated an approach that would be practical to perform. The methods evaluated were verified ex vivo in five baboons.

The geometrical mean method measuring the anterior and posterior counts corrected for attenuation was used. (Arimizu and Morris 1969; Graham and Neil 1974). The geometrical mean counts were expressed as a percentage of the total body geometrical mean counts to eliminate the effect of scintillation camera efficiency calibration, and the influence of organ geometry. The accuracy of organ uptakes without attenuation correction was also determined.

The dual photon energy emissions of 274 keV (0.9395 photons/disintegration) and 172 keV (0.8959 photons/disintegration) of ^{111}In results in an increase in the scattered radiation component in the lower energy window. To overcome this a Compton scatter correction was investigated.

Theory and Calculations

Phantom Studies. Phantom studies were performed to determine the Compton correction factor for each photon energy. Consider the anterior count rate P , observed with a scintillation camera fitted with a parallel-hole collimator, due to a radioactive source. Assuming that the radioactive source can be represented by an equivalent plane source, that the sensitivity of the camera/collimator system is depth independent, and that the attenuation is monoexponential, the unattenuated count rate, N , is given by

$$N = G \cdot T^{-1} \quad (1)$$

where

$G = (A \cdot P)^{1/2}$
= Geometrical mean (GM) of the anterior A and posterior P count rate

$T = \exp(-mB/2)$
= Attenuation correction constant for gamma photons with anterior-posterior distance B , and linear attenuation coefficient m .

The unattenuated count rate N can therefore be calculated without knowledge of the position of the source in the attenuating medium.

The anterior-posterior (A-P) distance B was kept constant for the phantom studies. The corrected count rate N obtained with the radioactive source at different A-P distances was expressed as a percentage of the unattenuated surface count

Introduction

^{111}In -oxine has recently been described as a suitable platelet label by Thakur et al. (1976). An advantage of ^{111}In in comparison with ^{51}Cr , the recommended radio-nuclide for platelet studies (ICSH 1977), is that the regional distribution of ^{111}In -platelets can be externally imaged and quantified with a scintillation camera and computer assisted imaging system (Lötter et al. 1980; Heyns et al. 1980a; Heyns et al. 1980b; Heyns et al. 1980(c); Van Reenen et al. 1980). The in vivo quantification of organ activity distribution is not easy to achieve, not often performed and in vivo verification seldom reported. Methods reported require anterior, posterior and lateral views as well as measurement of the gamma detection efficiency of the scintillation camera and gamma attenuation coefficients (Thomas et al. 1976; Ferrant and Cauw 1979; Fleming 1979). In the investigation of platelet kinetics whole body platelet distribution is a useful adjunct in assessing sites of sequestration or deposition of platelets in different diseases. The proposed methods would be difficult in these circum-

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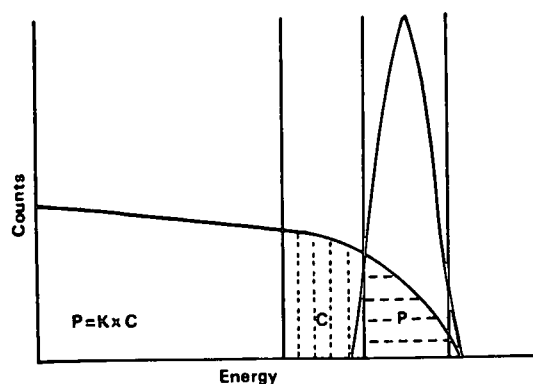


Fig. 1. Illustration of the method to correct for photopeak Compton contribution by subtracting a fraction of the Compton energy window counts from the photopeak counts

rate. This percentage Np is independent of the attenuation correction constant:

$$Np = G/G_o \times 100\%.$$

The derivation of the unattenuated count rate N (Eq. 1) is based on monoexponential attenuation. Inaccuracies will therefore occur when Compton scattering, resulting in multiexponential attenuation, is present. This is the case when 172 keV photons are detected in the presence of 247 keV photons emitted by ^{111}In . Correction for Compton scatter was investigated by obtaining scintillation camera images with an energy window selected over the Compton region in addition to the photopeak region (Fig. 1). Regional source or organ count rate was obtained using the same region of interest for both images. Compton corrected count rate was then obtained by subtracting a fraction K of the Compton region of interest (ROI) count rate from the photopeak count rate and expressed as a percentage of the unattenuated Compton corrected surface count rate.

$$N_c = \frac{(A - KC)(P - KO)}{(A_o - KC_o)(P_o - KO_o)} \times 100\% \quad (2)$$

where A and P anterior and posterior ROI count rate were respectively obtained from photopeak images, and C and O anterior and posterior ROI count rate respectively obtained from Compton scatter images. The Compton correction factor K was numerically calculated to ensure that

$$\sum_{i=1}^n (N_{ci} - \bar{N}_c)^2 = \min$$

where $\bar{N}_c$ is the average of all corrected counts N_{ci} at various depths in the phantom.

Baboon and Patient Studies

Anterior and Posterior Organ Distribution. In baboon and patient studies the percentage organ radioactivity distribution was calculated for the anterior and posterior images separately to investigate improvement in accuracy with the proposed methods. Organ radioactivity distribution was represented by expressing organ region of interest count rate as a percentage of total body count rate. Whole body count rates were obtained by acquiring images to include the whole body: three were necessary for baboons and five for humans. Counts obtained from organ and non-organ regions of interest to include the whole body were summed. The anterior (AUi) and

posterior (PUi) organ radioactivity distributions were calculated by

$$AUi = Ai / \sum Ai \times 100, \text{ and} \quad (3)$$

$$PUi = Pi / \sum Pi \times 100 \quad (4)$$

where Ai and Pi is the anterior and posterior count rate for the i th organ.

Geometrical Mean Distribution (GM). It can be seen from Eq. (1) that the geometrical mean of the anterior and posterior count rate is that count rate that would be measured when the activity is distributed on the anterior-posterior (A-P) midplane. GM count rate therefore allows comparison of organ activities independent of position, provided that attenuation for all organs remains constant, and organ size does not vary too much. Percentage geometrical mean distribution (GMi) was calculated by expressing organ GM count rate as a percentage of total body GM count rate.

$$GMi = (AiPi)^{1/2} / \sum (AiPi)^{1/2} \times 100. \quad (5)$$

Geometrical Mean with Attenuation Correction Distribution (GMA). To obtain the GMA distribution the attenuation correction constant Ti was measured using a ^{111}In transmission source to acquire an image with and without the subject. The anterior ^{111}In -platelet image was also acquired at the same time without moving the patient.

The regional attenuation was calculated using the counts obtained from the same region of interest as that used for obtaining organ counts in the anterior view. The attenuation was calculated by

$$Ti = (Cai - Ai) / Cfi$$

where Cai and Cfi are the count rates for the i th region of interest in the transmission images with and without the subject respectively.

The percentage geometrical mean with attenuation distribution ($GMAi$) was calculated by

$$GMAi = \left[\frac{AiPi}{Ti} \right]^{1/2} / \sum \left[\frac{AiPi}{Ti} \right]^{1/2} \times 100 \quad (6)$$

Geometrical Mean with Compton Scatter (GMA) and Attenuation Correction Distribution (GMAC). Compton scatter correction was applied to the baboon and human studies as for the phantom investigation. The organ geometrical mean with Compton distribution ($GMCi$) is represented by

$$GMCi = \frac{[(Ai - KCi)(Pi - KOi)]^{1/2}}{\sum [(Ai - KCi)(Pi - KOi)]^{1/2}} \times 100. \quad (7)$$

The percentage organ radioactivity distribution for geometrical mean and Compton scatter correction is given by

$$GMCAi = \frac{\left(\frac{(Ai - KCi)(Pi - KOi)}{Ti} \right)^{1/2}}{\sum \left(\frac{(Ai - KCi)(Pi - KOi)}{Ti} \right)^{1/2}} \times 100.$$

Materials and Methods

A Searle large field of view scintillation camera fitted with the medium-energy parallel-hole collimator was interfaced to a MDS MUGA CART imaging system. Two images of non-overlapping energy windows were acquired simultaneously

serial data acquisition mode, reconstructed and stored on disc for later processing.

Phantom Studies. The proposed Compton scatter correction factor was measured in a phantom. The phantom consisted of ten 25.6 × 450 × 450 mm perspex plates allowing the placement of the 250-ml plastic bag in any of ten positions. Approximately 20 MBq ^{111}In was placed in the plastic bag. Images were obtained with the source in phantom positions 1–10. The photopeak and their Compton region were acquired for the 247 keV and 172 keV energies respectively; analyser settings were such that maximum counts were obtained for the photopeak area and minimum count rate for Compton areas. A 30% count contour region of interest was selected with the second derivative edge detection computer program MUGA.

Animal Experiments. Autologous platelets of five baboons were separated by differential centrifugation and labelled with approximately 20 MBq ^{111}In -oxine (Radiochemical Centre, Amersham, U.K.), (Heyns et al. 1980a). The baboons were killed after 8 days. The liver and spleen were removed, drained free of blood, and replaced in their original anatomical positions. The whole body was anteriorly and posteriorly imaged in sections. The photopeak and Compton energy settings of the analyser were set as described for the phantom studies. Regions of interest were manually selected by two independent operators. Radioactivity of the organs was then separately measured in a whole body counter to minimize attenuation and organ to organ scatter. The two 10 × 13 cm scintillation detectors were arranged to obtain a geometry-independent counting configuration.

Patient Studies. Patient studies were performed after the reinjection of ^{111}In -labelled autologous platelets (Heyns et al. 1980b) and radioactivity distribution was measured in the anterior and posterior mode in five patients.

Results

Phantom Studies. The measured radioactivity at various source distances from the collimator and with GM correction expressed as a percentage of the unattenuated counts are presented in Table 1. The geometric mean corrected counts are

overestimated with depths up to the A–P midline with a mean of $105 \pm 3.2\%$ and $108.7 \pm 5.0\%$ for the 247 and 172 keV photons respectively. The GM counts of Table 1 were used to calculate the Compton correction constant K . The corrected phantom counts N_{ci} at different depths using K was virtually identical to the surface counts with a mean of $99.9 \pm 1.0\%$.

Animal Studies. The mean ex vivo organ radioactivity expressed as a percentage of whole body activity was measured in dissected baboons. Results are presented in Table 2. ^{111}In radioactivity was distributed between the spleen ($33.8 \pm 3.6\%$), the liver ($35.9 \pm 6.4\%$) and the remainder of the whole body ($29.7 \pm 2.8\%$).

In vivo and ex vivo quantification of ^{111}In activity were compared. The accuracy of the in vivo quantification of radioactivity was determined by calculating the difference be-

Table 2. ^{111}In platelet distribution measurements in baboons

Method	N	Spleen	Liver	Whole body remainder
Distribution as % of whole body activity (mean $\pm$ SD)				
Single organs of baboons post mortem	5	33.8 ± 3.6	35.9 ± 6.4	29.7 ± 2.8
In vivo error as % of organ uptake (mean $\pm$ SD)				
274 keV				
Anterior	5	-7.6 ± 14.2	4.6 ± 14.7	1.8 ± 8.8
Posterior	5	3.2 ± 23.6	-7.1 ± 18.2	8.6 ± 10.8
GM	5	-3.8 ± 6.3	-2.3 ± 4.3	5.4 ± 7.5
GMC	3	0.1 ± 8.5	3.8 ± 6.4	-4.0 ± 10.0
GMA	3	-11.6 ± 10.3	7.7 ± 6.7	4.2 ± 12.7
GMAC	3	-8.1 ± 14.4	12.5 ± 9.3	-4.9 ± 13.0
172 keV				
Anterior	3	-0.9 ± 7.1	13.1 ± 7.8	-5.0 ± 5.5
Posterior	3	-8.0 ± 19.0	-1.0 ± 18.1	10.3 ± 29.0
GM	3	-6.3 ± 6.4	0.4 ± 7.2	7.0 ± 10.7
GMC	3	2.7 ± 7.8	4.0 ± 8.2	-7.5 ± 13.9
GMA	3	-12.9 ± 9.1	8.8 ± 9.0	4.7 ± 10.1
GMAC	3	-3.2 ± 12.9	9.5 ± 11.5	-6.4 ± 10.3

Table 1. Geometrical mean correction applied to perspex phantom

Depth (cm)	247 keV		172 keV		
	Percentage counts	GM corrected	Percentage counts	GM corrected	GMC corrected
0.0	100.0	100.0 ± 0.0	100.0	100.0 ± 0	100.0
2.6	72.9 ± 5.6	102.6 ± 2.4	74.4 ± 1.4	106.3 ± 1.6	98.7
5.2	55.3 ± 1.0	105.6 ± 2.5	53.9 ± 1.3	110.4 ± 1.4	99.0
7.8	40.7 ± 0.6	107.5 ± 2.5	38.1 ± 1.2	111.8 ± 1.5	101.7
10.5	28.9 ± 0.6	107.8 ± 2.8	26.3 ± 1.0	113.1 ± 2.2	101.0
13.1	20.2 ± 0.5	108.2 ± 2.7	18.0 ± 0.8	113.6 ± 2.9	99.2
15.7	14.1 ± 0.6	107.8 ± 2.8	11.9 ± 0.6	113.1 ± 2.2	98.8
18.3	9.9 ± 0.5	107.5 ± 2.5	8.2 ± 0.4	111.8 ± 1.5	100.2
20.9	7.1 ± 0.4	105.6 ± 2.5	5.0 ± 1.9	110.4 ± 1.4	99.7
23.5	4.9 ± 0.2	102.6 ± 2.4	3.8 ± 0.2	106.3 ± 1.6	98.9
26.2	3.5 ± 0.1	100.0 ± 0.0	2.5 ± 0.2	100.0 ± 0.0	100.3
Mean $\pm$ SD		105.0 ± 3.2		108.7 ± 5.0	99.9 ± 1.0

N = 10

Table 3. Percentage difference of anterior and posterior in relation to GM organ radioactivity measurement in human subjects

Method	Time	Spleen		Liver	
	h	247 keV	172 keV	247 keV	172 keV
Anterior	2	-28 ± 11	-26 ± 10	14 ± 7	15 ± 12
	24	-26 ± 9	-26 ± 10	16 ± 9	15 ± 9
	48	-27 ± 17	-26 ± 10	17 ± 6	15 ± 8
	72	-28 ± 14	-25 ± 14	18 ± 10	18 ± 7
	144	-29 ± 11	-25 ± 14	15 ± 7	20 ± 14
	mean ^a	-27 ± 11	-26 ± 10	16 ± 7	16 ± 9
Posterior	2	41 ± 24	37 ± 20	-12 ± 5	-12 ± 9
	24	38 ± 20	37 ± 18	-13 ± 6	-13 ± 7
	48	45 ± 42	39 ± 28	-14 ± 4	-13 ± 6
	72	40 ± 28	37 ± 27	-15 ± 6	-15 ± 5
	144	45 ± 25	35 ± 16	-12 ± 5	-16 ± 9
	mean ^a	41 ± 26	37 ± 21	-13 ± 5	-13 ± 7

^a Mean ± SD calculated for all results

tween the in vivo and ex vivo measurements expressed as a percentage of the ex vivo activity. Results are given in Table 2. For the 247 keV photopeak the anterior method underestimated splenic and overestimated hepatic radioactivity. The posterior method overestimated splenic and underestimated hepatic radioactivity. Both methods showed a poor sensitivity. These inaccuracies were corrected by the GM method. Additional correction for Compton scatter, attenuation and Compton scatter with attenuation had no advantage over the GM method. For the 247 keV photopeak the organ radioactivity was therefore accurately calculated with the GM corrected method. For the 172 keV photopeak the accuracy was similar to results obtained with the 247 keV photopeak, but with a slightly poorer sensitivity.

Patient Studies. The accuracy and sensitivity of the anterior and posterior measurement of radioactivity was compared with the GM method in five patients. In Table 3 the percentage difference between anterior and GM, as well as posterior and GM, organ activity measurements are presented. The anterior method underestimated splenic and overestimated hepatic radioactivity at both the 247 keV and 172 keV photopeaks. Quantification by the posterior method overestimated splenic and underestimated hepatic radioactivity, while quantification of whole body radioactivity was similar to that of the GM method.

Discussion

This study focussed on the assessment of methods to measure the percentage organ platelet distribution. The emphasis of this investigation was to find a method that would be suitable for routine patient studies. Therefore, rather than using phantoms which never accurately simulates true radioactivity distribution, in vivo measurement was performed on baboons which were then killed to measure organ uptake. The accuracy and reproducibility obtained closely reflected the clinical situation.

Theoretical and phantom studies reported by Thomas et al. (1976), Ferrant and Cauw (1979) and Fleming (1979) using anterior and lateral images indicate that organ size could significantly affect quantitation. This technique is not suitable

when whole body distribution is required to measure platelet reticulo-endothelial bone marrow uptake or platelet utilization in the lower limbs in addition to uptake in the spleen and liver. Also, it is impossible to estimate organ thickness accurately. Lateral imaging to obtain organ size was therefore not done; but corrected organ uptake was expressed as a percentage of the whole body count rate as calculated by Eqs. (5) and (6). Since organ counts contribute towards whole body counts, corrected whole body counts were used as the standard. Current large field of view scanning cameras with appropriate digital imaging systems facilitate easy acquisition of whole body images and adequate matrix resolution of 256×256 pixels. We did not attempt to quantitate absolute organ activity, but it could be calculated indirectly, if injected activity is known. The error of $-3.8 \pm 6.3\%$ for the spleen, $2.3 \pm 4.3\%$ for the liver and $5.4 \pm 7.5\%$ for the whole body remainder indicates that acceptable accuracy is obtained by our method. The accuracy of radioactivity measurement is dependent on attenuation. By applying the geometrical mean and anterior-posterior attenuation correction, unattenuated organ counts are obtained. The measurement of the A-P attenuation is time consuming and attenuation regions of interest have to be accurately aligned with organ regions of interest. If A-P attenuation correction is not performed, results of organ uptake would be valid provided that the GM organ counts are expressed as a percentage of the whole body GM counts, and that attenuation of radioactivity is uniform. Our results indicate that in the in vivo baboon model, A-P attenuation correction did not improve the accuracy of measurement of organ activity.

Results in the phantom indicate that as a result of the increased contribution of Compton scatter by the 247 keV photons in the 172 keV photopeak, activity of the superficial organs will be underestimated in relation of organs centrally situated. In the phantom measurement laminar 'organs' that are situated at the surface and at the centre were compared. In patient studies these extremes do not occur. Compton correction introduced errors due to counting statistics and required acquisition of scatter images. It is therefore not recommended as a routine procedure or when a low-energy measuring mobile camera is used.

These techniques were also applied to five human subjects (Table 3). The splenic and hepatic radioactivity measured in the anterior mode were under- and overestimated respectively compared with the GM method (Table 3). The scintillation camera placed posteriorly reversed these results. These studies convey the magnitude of the errors if only anterior or posterior measurements are performed. It also confirms in vivo baboon results that the error due to the increased scatter contribution for the lower energy photons is reduced by expressing GM corrected regional counts as a percentage of whole body counts. The results of daily in vivo measurements in the patients show that the percentage error between the GM method and anterior or posterior measurement remains constant. The technique also has the major advantage that platelet accumulation can be retrospectively quantitated since whole body images are acquired. This facility may be of importance in the study of the role of platelets in thrombotic and other diseases.

This investigation shows that the application of the modern technology of the scintillation camera with computer-assisted regions of interest selection makes kinetic studies and in vivo quantification of ^{111}In -labelled platelets feasible and practical.

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Kinetics of In-111-Platelets in the Baboon:

II. In Vivo Distribution and Sites of Sequestration

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Key words

Platelets kinetics – Primates – Sites of platelet sequestration – Indium-111 – Indium-111-labelled platelets

Summary

The kinetics and sites of sequestration of a fully representative population of In-111-platelets were determined in 11 baboons. The *in vivo* method of quantification with computer assisted scintillation camera image analysis was validated by sacrificing 5 baboons and measuring and comparing the distribution of organ radioactivity. Recovery of platelets in the circulation was $87\% \pm 7$, and their mean survival time was $147 \text{ hr} \pm 15$. The mean splenic platelet pool was $16.0\% \pm 1.9$. At equilibrium $15.8\% \pm 2.9$ of the In-111-platelets were in the hepatic blood pool. Senescent platelets were destroyed in the reticulo-endothelial system. The major sites of sequestration were: liver ($37.6\% \pm 6.0$), and the spleen ($23.3\% \pm 4.6$). The bone marrow sequestered $14.4\% \pm 1.7$ of the labelled platelets, and $15.5\% \pm 4.0$ were present in various other tissues. We conclude that the *in vivo* method of In-111-quantification is accurate. Senescent platelets are mainly sequestered in the reticuloendothelial tissue, with the liver, spleen and the bone marrow important sites of sequestration.

Introduction

Exploitation of the technology of the scintillation camera interfaced with computer image analysis systems permits *in vivo* quantification of the distribution and sites of sequestration of platelets labelled with In-111 (1–3). It is generally accepted that senescent platelets are sequestered by the reticuloendothelial system (1–9). The spleen is recognized as a major site of platelet sequestration, but the role of the liver in this respect is controversial. Some regard the liver as important (1–5), but this is disputed by other investigators (6, 7, 9). The role of the bone marrow as a site of platelet sequestration has only recently been recognized (5). The diffuse nature of the bone marrow makes it impossible to accurately quantify the number of platelets sequestered in this organ. It has therefore been proposed that those platelets not sequestered by the spleen and the liver, should be considered as destroyed by the bone marrow (1, 3, 6, 7, 9).

The aim of this study was to investigate the kinetics of a representative population of In-111-platelets in normal baboons. This non-human primate is an important model for thrombosis and atherosclerosis, and closely reflects its human counterpart (10, 11). The geometrical mean method of *in vivo* quantification and the precise sites of sequestration of senescent labelled platelets was determined by sacrificing the animals.

Materials and Methods

Experimental Animals

Eleven baboons (*Papio ursinus*), 5 males, weighing $17.6 \text{ kg} \pm 5.4$, were studied in a project approved by the Ethical Committee of the University of the Orange Free State. The baboons were apparently free of disease, and in captivity for at least 6 months. The baboons were sedated with intramuscular ketamine hydrochloride, 10 mg/kg body mass, when necessary.

Platelet Labelling and Survival

Autologous blood, 42.5 ml in 7.5 ml acid-citrate dextrose NIH, formula A (Fenwall, Baxter Travenol Laboratories Inc., Illinois), was collected from the femoral vein by a two syringe technique. A fully representative platelet population was isolated from the blood, and labelled with In-111-oxine (25 to 29 MBq; Radiochemical Centre, Amersham, UK) as described in detail elsewhere (3, 12). The viability of the labelled platelets was assessed by the aggregation response to ADP (3, 13), and were not used unless aggregates consisting of at least 10 platelets were observed.

Platelet survival studies were done as recommended by the ICSH (14), and the mean platelet life span calculated by fitting the data points to a gamma function model (15). The recovery of In-111-platelets in the circulation at equilibrium was calculated from the blood volume, estimated at 63 ml/kg body mass (16), and back extrapolating the survival curve to zero time.

In Vivo Quantification of the Distribution of In-111-Platelets

Imaging was done with a large field of view scintillation camera with scanning facilities. Image analysis was with a MDS-A² data processing system. The whole body and organ In-111-radioactivity was quantitated with the geometrical mean method as is described in detail elsewhere (3).

Ex Vivo Determination of the Sites of Platelet Sequestration

Five baboons were sacrificed 8 days after reinjection of In-111-platelets. The baboons were deeply anaesthetized and exsanguinated by severing the femoral arteries. The baboons were dissected and the radioactivity of each organ determined in a whole body counter as described previously (2). Organ radioactivity was expressed as a percentage of total body radioactivity.

Table 1 Data of platelet isolation, labelling and survival

Parameter	Mean $\pm$ 1 SD
Blood platelet count ($\times 10^{-9}/\text{l}$)	372 ± 94
Labelling efficiency (%)	84 ± 4
Contamination of labelled platelets (%) with	
Red blood cells	1.3 ± 1.1
Plasma proteins	2.1 ± 0.9
Recovery in the circulation (%)	87 ± 7
Mean platelet life span (hr)	147 ± 15

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Statistical Analysis

Differences were tested with the t-test for paired data. All values are given as a mean $\pm$ 1 SD.

Results

Platelet Labelling and Survival

The results are summarized in Table 1. A mean of $8.8 \pm 3.7 \times 10^9$ platelets were reinjected. Mean recovery of In-111-platelets in the circulation at equilibrium was $87\% \pm 7$. The disappearance curve of platelets from the circulation fitted a linear function best, the mean platelet life span was $147 \text{ hr} \pm 15$.

In Vivo Distribution of In-111-Platelets

At equilibrium $16.0\% \pm 1.9$ and $15.8\% \pm 2.9$ of the labelled platelets were located in the spleen and liver respectively (Table 2).

During the platelet life span whole body radioactivity decreased from 100% at equilibrium to $93\% \pm 7$. The spleen and the liver were the main sites of platelet sequestration, and accounted for $61.0\% \pm 5.4$ of whole body radioactivity at the end of the platelet survival time (Table 2, Fig. 1).

Determination of the Sites of Platelet Sequestration

The results are summarized in Table 3. The results of the *in vivo* quantified and the *ex vivo* determined radioactivity in the liver and spleen did not differ significantly ($p > 0.10$). The spleen, liver, and bone marrow were the sites of sequestration of $84.4\% \pm 3.6$ of senescent In-111-platelets. The remaining $15.5\% \pm 4.0$ were sequestered in various other tissues.

Discussion

We have determined the sites of sequestration of a fully representative platelet population labelled with In-111-oxine. The viability of the labelled platelets was demonstrated by their *in vitro* aggregation response to ADP, the high recovery of labelled platelets in the circulation, and their normal mean life span in the circulation corresponding to that previously reported in this species with Cr-51-labelled platelets (16, 17).

The accuracy of the geometrical mean method of quantification of In-111-distribution *in vivo* was validated. The difference between results obtained with *in vivo* and *ex vivo* estimates of organ radioactivity was less than 3%. Computer-assisted image analysis may thus be accepted as an accurate, non-invasive *in vivo* method of determining the sites of platelet sequestration or deposition.

The high recovery of labelled platelets in the circulation and the relatively low In-111-radioactivity measured at equilibrium, shows that the splenic platelet pool is much smaller in baboons than in humans. Since the sum of the recovery of platelets in the circulation and the quantified splenic platelet pool totals $103\% \pm 9$, the labelled platelets were apparently distributed only in the circulation and the spleen. These results therefore do not support the view that there may be a hepatic platelet pool (6, 18) or the presence of a significant non-splenic platelet pool (19). Our results seem to indicate that the hepatic In-111-radioactivity at equilibrium is due only to platelets in the blood perfusing this organ.

The *in vivo* measurements demonstrate that the liver and spleen are major sites of sequestration of senescent platelets. The results in the baboon are similar to that reported in humans applying the same method of In-111-quantification (3). We have

Table 2 The distribution of In-111-platelets at equilibrium and at the end of the platelet life span

Baboon	Equilibrium			At end of platelet life span		
	Liver	Spleen ¹	Remainder	Liver	Spleen	Remainder
1	15.9	17.0	67.2	33.7	29.2	35.8
2	19.4	15.7	64.9	40.7	24.0	42.9
3	18.3	15.6	66.1	35.7	24.0	38.2
4	13.2	14.8	72.0	38.5	18.1	35.6
5	11.0	20.3	68.7	34.3	30.8	26.7
6	14.7	18.1	67.3	25.2	21.9	30.0
7	19.7	13.7	66.6	47.4	23.8	23.6
8	12.9	14.2	72.9	45.8	20.6	24.6
9	16.8	15.9	67.3	37.9	18.7	33.3
10	14.0	14.7	71.3	37.4	18.6	24.8
11	18.0	16.0	66.0	37.2	20.4	34.8
Mean	15.8	16.0	68.2	37.6	23.3	31.8
$\pm$ SD	2.9	1.9	2.7	6.0	4.6	6.3

¹ Spleen and liver radioactivity were subtracted from that of whole body regarded as 100%, to obtain radioactivity in the "remainder". Organ In-111-radioactivity is expressed as a percentage of whole body radioactivity.

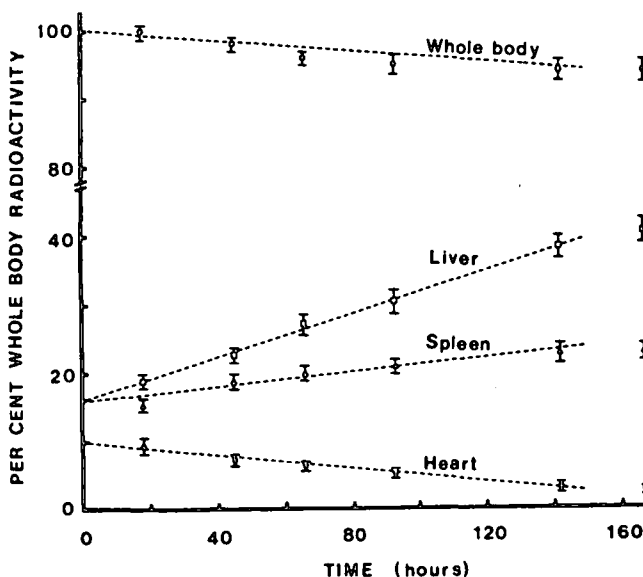


Fig. 1 Time-radioactivity regression curves illustrating the redistribution of In-111-platelets during their life span. Values are expressed as a percentage of whole body radioactivity (± 1 SEM), assumed to be 100%.

Table 3 The distribution of In-111-platelets in 5 baboons sacrificed days after reinjection of In-111-labelled platelets

Organ	Quantification in excised organs	In vivo quantification
Whole body	100.0	100.0
Spleen	33.6 ± 4.1	32.8 ± 3.6
Liver	36.7 ± 7.1	35.9 ± 3.5
Skeleton	14.4 ± 1.7	—
Muscle and skin	6.7 ± 1.4	—
Intestines	4.9 ± 1.6	—
Kidneys	2.5 ± 0.6	—
Lungs	0.9 ± 0.6	—
Heart	0.5 ± 0.2	—
Blood	0.5 ± 0.3	—

Organ radioactivity is expressed as a percentage of that of whole body radioactivity, regarded as 100%.

no ready explanation for the reports that liver is not an important site of sequestration of platelets (6, 7, 9), but the discrepancy may be due to methodological differences. The post mortem quantification of In-111-radioactivity confirms that the bone marrow is an important site of platelet sequestration, accounting for almost 15% of whole body radioactivity. Since there is only slight elution of In-111 from the phagocytes of the spleen (20), our results may be regarded to reasonably accurately reflect the true sites of platelet sequestration.

A small component, about 15%, of the platelet population were sequestered or deposited in various other tissues. These platelets may have been phagocytosed by macrophages located in these organs, or platelets may have been utilized for maintenance of the vascular endothelium (20). Our study was however, not designed to distinguish between these two possibilities.

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Quantification of In Vivo Distribution of Platelets Labeled with Indium-111 Oxine

The recent paper by Scheffel et al. (1) in the *Journal of Nuclear Medicine* raises some important questions in regard to the sites of platelet sequestration and the accuracy of the different methods used to quantitate the in vivo distribution of platelets labeled with In-111 oxine. The observation that there is no appreciable increase in splenic and hepatic radioactivity at the end of platelet life span raises the question of where the nearly 50% of the injected dose that remained unaccounted for was deposited, and whether this occurs in the bone marrow as has been suggested by Klonizakis et al (2).

We have improved the accuracy of our original method (3) of in vivo quantification of In-111-labeled platelets in organs (4) and present some of the relevant results with this method. This may throw some light on the sites of final disposal of platelets.

Briefly, autologous platelets were labeled with In-111 oxine as previously described (5). Anterior and posterior images of the whole body were acquired every second day with a scintillation camera and a computer-assisted imaging system. The geometrical mean method of correcting for attenuation was used for in vivo measurement of whole-body and organ radioactivity. Distribution of In-111 activity at equilibrium and at the end of platelet life span was determined by fitting the data with the method of linear least squares regression analysis. The geometrical mean counts were expressed as a percentage of the total body's geometrical counts to eliminate the effect of scintillation camera efficiency calibration and the influence of organ radiation attenuation. The corrected counts calculated by this method represent the radioactivity of the organ as if it were situated in the mid anteroposterior plane of the body (4).

The results of In-111 distribution, at equilibrium and at the end of platelet survival, as measured in normal volunteers are given in Table 1. There was a slight but significant increase in splenic radioactivity with time. Hepatic activity increased threefold. Total-body In-111 activity decreased minimally.

The accuracy of the method of quantification was verified, and the sites of deposition of In-111 determined in 10 baboons (*Papio ursinus*). The equilibrium and final in vivo In-111 distributions, determined with the scintillation camera, are given in Table 2. Radioactivity in both the liver and spleen increased. In the baboon the spleen is a more active site of platelet sequestration than in man, but hepatic activity increased twofold with time. The baboons were killed at the end of the study and the excised organs measured. As is evident from Table 2, there is a close agreement between in vivo and ex vivo measurements. Dissection of the skeleton revealed that (14.4 ± 1.7)% of radioactivity was located in the bone marrow

TABLE 1. IN VIVO QUANTIFICATION OF IN-111 ORGAN RADIOACTIVITY IN NORMAL HUMAN SUBJECTS (n = 6)

Organ	Equilibrium % activity	Final % activity
Whole body	100 ± 0	96.6 ± 5.2*
Spleen	31.1 ± 6.1	35.6 ± 9.7*
Liver	9.6 ± 1.2	28.7 ± 8.3*

* Whole-body In-111 activity did not decrease significantly ($p > 0.1$), but that of the spleen ($p < 0.01$) and liver ($p < 0.005$) did increase significantly with time (student's t-test for paired data).

at the end of platelet life span. Most of the In-111 activity lumped under "other organs" was accounted for by muscle and skin (6.7 ± 1.4)%, gastrointestinal system (4.9 ± 1.6)%, and kidneys (2.5 ± 0.6)%.

These results, and studies performed in a phantom (4), clearly validate the accuracy of the geometrical mean method for the in vivo quantification of In-111 activity.

The discrepancies between our results and those of Scheffel et al. (1) are difficult to explain if they are not due to differences in the techniques of In-111 quantification. We find it difficult to accept the suggestion that the bone marrow is responsible for 35-40% of platelet sequestration (2). The bone-marrow activity of 14.4% that we found in the baboon corresponds closely to the 14% measured in rabbits (1). Our results favor the liver as a major site of platelet sequestration. Analysis of the data given by Scheffel et al. (1) in their Table 4 with Student's t-test for paired data also shows that hepatic activity in subjects 1, 4, 6, 7, 8, and 9 increased significantly ($P < 0.01$) during the time from 90 min after injection of labeled platelets to the final measurement of In-111 activity. Although the spleen, liver, and bone marrow are the major components of the reticuloendothelial system, the wide distribution of this tissue throughout the body may account for the final distribution of the remainder of In-111 activity. Some platelets may also be utilized in the vascular system, but we have not been able to measure this in vivo in normal subjects (3).

Our results confirm the validity of the geometrical mean method for the in vivo quantification of labeled platelets. The results also confirm the findings of Aster (6), who in his classical studies

TABLE 2. IN VIVO AND EX VIVO QUANTIFICATION OF IN-111 RADIOACTIVITY IN BABOONS (n = 10)

Organ	In Vivo Quantification		Ex Vivo Quantification
	Equilibrium % activity	Final % activity	
Whole body	100 ± 0	95.2 ± 3.7*	100 ± 0
Spleen	17.3 ± 3.2	33.5 ± 3.1*	33.7 ± 5†
Liver	15.6 ± 2.0	35.2 ± 6.5*	36.7 ± 7.1†
Bone marrow	—	—	14.4 ± 1.7
Other organs	68.1 ± 4.5	31.4 ± 4	15.5 ± 4.0

* Whole-body In-111 activity did not decrease significantly ($p > 0.05$), but that in the spleen ($p < 0.001$) and liver ($p < 0.0005$) did increase highly significantly with time. (Student's t-test for paired data).

† In vivo and ex vivo quantification of splenic and hepatic In-111 activity did not differ statistically ($p > 0.1$).

showed that the liver and spleen are the major sites of platelet sequestration. The role of the bone marrow has certainly been underestimated in this regard, but it seems to play a relatively subsidiary role to that of the liver and spleen.

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Dual-isotope Scintigraphy and the Quantitation of Platelet Deposition

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ABSTRACT

In the last decade since the first introduction of In-111 as platelet label, intensive research has been done on basic platelet research and on the clinical applications of this radionuclide. The varied aspects of research include platelet kinetics in humans and animals, atherosclerotic disease and the detection of acute organ transplant rejection. More recently, innovative developments in scintigraphic techniques have included dual isotope scintigraphy and quantitative digital blood pool subtraction.

Dual isotope subtraction methods mostly use Tc-99m labelled red cells to delineate and subtract the blood pool in the region of suspected In-111 platelet deposition. By eliminating the blood pool signal, subtle foci of platelet deposition, otherwise not visible, can be easily visualised and quantitated.

The dual radionuclide subtraction method has been thoroughly evaluated in 5 animal models and has been shown to increase the sensitivity of detection of thrombi in man. In the animal models, a high correlation exists between the in vivo measurement (% Indium-excess) and the ex vivo measurement of platelet deposition (% injected dose) (r is more than 0,8). The in vivo measurement of %Indium-excess is thus a good indicator of platelet deposition and, although not an absolute quantitative measurement, can be used for non-biased inter-individual comparisons. Clinical applications include the detection of atrial and ventricular thrombi, coronary artery disease and acute myocardial infarction. An indication of the sensitivity of the dual isotope method may be seen from the results of a study of patients with acute myocardial infarction, in which more than 80% of patients with myocardial infarction had positive scintigrams against only 7% of controls.

In summary, quantitative dual radiotracer scintigraphy promises to be a useful less time-consuming method for the detection of platelet deposition in small vascular lesions and aneurysms, when absolute quantitation is not deemed necessary. The technique is especially recommended as an adjunctive diagnostic aid in the detection of cardiac thrombi, where the blood pool can easily obscure platelet deposition.

Introduction.

¹¹¹In is an effective platelet radiolabel, primarily because of its high labelling efficiency and high gamma photon emission. These characteristics of ¹¹¹In allow accurate quantitative in vivo studies of platelet survival, turnover and thrombosis.

Platelet kinetics in normals and in many different platelet-associated diseases have already been investigated. Some of the diseases that have been studied include immune thrombocytopaenic purpura, hypercholesterolaemia, splenomegaly and thrombotic thrombo-cytopaenic purpura. A further example of the application of ¹¹¹In-labelled platelets (¹¹¹In-platelets) includes the diagnosis of kidney transplant rejection ^{7,16}.

Many clinical applications of ¹¹¹In have focussed on the study of lesions of the circulatory system. For example, ¹¹¹In-platelets have been used to detect

left ventricular thrombi, atherosclerosis, coronary artery thrombi, platelet deposition in aortic aneurysms and venous thromboembolism. The detection of thrombi with ^{111}In -platelets has been reviewed in more detail by Powers and Siegel ¹².

In most of the platelet imaging techniques, the limits of scintigraphic technology have been reached, especially regarding the amount or size of platelet deposition that can be quantitated. There is, however, an answer to one problem in ^{111}In -platelet studies i.e., the discrimination between circulating and deposited platelets. This has quite recently been addressed by using dual radionuclide techniques with $^{99\text{m}}\text{Tc}$ -red cells and ^{111}In -platelets.

Methods

Basically, the $^{99\text{m}}\text{Tc}$ -red cells are used to identify the red cell pool (4), and thus indirectly the circulating platelets. The radioactivity from $^{99\text{m}}\text{Tc}$ can be readily distinguished from the ^{111}In radioactivity, since $^{99\text{m}}\text{Tc}$ emits gamma photons at 140 keV and ^{111}In at 173 and 247 keV. This method assumes that both platelets and red cells have similar distributions in the area of interest. Separate images of $^{99\text{m}}\text{Tc}$ -red cells and ^{111}In -platelets can thus be obtained. The actual acquisition of images can be done either sequentially or simultaneously. A computerised imaging processor would be most suitable for dual isotope scintigraphy.

With the aid of computer region of interest analysis and digital subtraction of the $^{99\text{m}}\text{Tc}$ image from the ^{111}In image, the amount of platelet deposition in a specific area can be visualised, especially in subtler foci with a large blood pool background (13).

There are some assumptions that have to be made concerning the use of the dual radiotracer technique⁹. These include:

- 1) The radiolabelled cells should be physiologically similar to their native unlabelled counterparts. It is already well known that ^{111}In -platelets are functionally viable and do not lose ^{111}In into the circulation. $^{99\text{m}}\text{Tc}$ red cells have also been shown to retain the $^{99\text{m}}\text{Tc}$ effectively.
- 2) The amount of $^{99\text{m}}\text{Tc}$ -red cells incorporated into a thrombus should be negligible. There is always some uptake of red cells into a thrombus, and this may cause an underestimation of platelet uptake.
- 3) The physical considerations of gamma camera scintigraphy are similar for both radionuclides within the region of interest. The main problem here would be differences in attenuation and Compton scatter.
- 4) The reference region of interest is representative of the true blood pool of both red cells and circulating platelets. This therefore means that platelets and red cells are distributed similarly in the region of interest. Correction cannot be made for the axial streaming of red cells in the arteries.
- 5) The images of both radionuclides are properly superimposed. Incorrect superimposition of images will lead to erroneous results.

Two types of dual radionuclide imaging can be described: firstly, the qualitative method and secondly, the quantitative method. The qualitative method is based on the simple comparison of $^{99\text{m}}\text{Tc}$ and ^{111}In images (8,11). In this way, a "hot spot" in an $\text{In-}^{111}\text{In}$ -platelet image can be identified as either true platelet deposition or the result of a local pooling of blood. This method is still subject to observer bias and is therefore not completely free of error.

In our laboratory, we have refined the qualitative method by computer subtraction of the ^{99m}Tc image from the ^{111}In image. The reference blood pool is delineated by computer region of interest analysis. The counts in both the ^{99m}Tc and ^{111}In images are then normalised by multiplying the ^{111}In image by a correction factor obtained from the ratio of ^{99m}Tc to ^{111}In counts in the reference region. The normalised ^{99m}Tc image is then digitally subtracted from the ^{111}In image to provide the ^{111}In -excess image.

The group at the Mallinckrodt Institute of Radiology have further refined the method so that platelet deposition can be quantified relative to the circulating platelets (9).

The actual deposition of platelets in an area of interest is calculated as follows (9):

$$\text{Platelet deposition} = \frac{(\text{Total } ^{111}\text{In activity}) - (^{111}\text{In BP})}{^{111}\text{In BP}} \dots\dots\dots(1)$$

where:

Total ^{111}In activity = the radioactivity due to circulating and deposited platelets.

$^{111}\text{In BP}$ = radioactivity due to circulating platelets

The $^{111}\text{In-BP}$ is derived from the ratio of ^{99m}Tc to ^{111}In counts obtained in the reference blood pool region as follows:

$$^{111}\text{In-BP} = \frac{(\text{In-ref})}{(\text{Tc-ref})} \times \text{Tc-roi} \dots\dots\dots(2)$$

where :

In-ref = the activity in the blood pool reference region of the ^{111}In image

Tc-ref = the activity in the blood pool reference region of the ^{99m}Tc image

Tc-roi = the ^{99m}Tc activity in the area of interest.

The amount of platelets deposited in an area can also be expressed as a percentage:

$$\begin{aligned} \% \text{In-excess} &= \frac{(\text{Total } ^{111}\text{In activity}) - (^{111}\text{In-BP})}{^{111}\text{In BP}} \times 100 \dots\dots\dots(3) \\ &= \text{equation}(1) \times 100 \end{aligned}$$

The %In-excess (%IE) is essentially equivalent to the ratio of deposited platelet activity to blood activity (platelet deposition) in equation (1). The quantitative dual radionuclide method is suitable for inter-individual comparisons, thus allowing more accurate investigation of certain platelet-associated diseases in experimental models and clinical studies. More objective

analysis of results is possible, since quantitative studies eliminate the observer bias present in the qualitative comparison method.

Results

Experimental studies:

The use of dual radiotracer techniques has been validated in various animal models mainly by the group at the Mallinckrodt Institute for Radiology (1,2,3,9,10,14,15,19,20). The models used were:

1) An electrocoagulation model in rabbits (15).

In this model, thrombi were generated in the abdominal aortas of rabbits by electric coagulation. The in vivo measurement of % Indium-excess, as calculated according to the quantitative dual radiotracer technique, was linearly related to the % of injected dose, measured in vitro ($r = 0,88$).

2) Vascular grafts in dogs (1,2,10,9).

The % Indium-excess and the calculated % injected dose incorporated in different types of carotid and femoral grafts in dogs also show a linear relationship ($r = 0,94$ ⁹).

3) Atherosclerosis in non-human primates (9,14,19).

Dual radiotracer techniques have also been used to study atherosclerosis in some primates on a high cholesterol diet. Powers et al (1982)¹⁴ first applied the technique when they could not detect atherosclerosis by conventional ¹¹¹In-platelet imaging. However, with quantitative blood pool correction, platelet deposition was demonstrated in the abdominal aortas of hypercholesterolaemic monkeys. The % Indium-excess (%IE) was also confirmed histologically. Aspirin was also found to prevent platelet deposition. The effect was not dose-related (Table 1).

Table 1

MEAN %IE IN THE ABDOMINAL AORTA
OF MACAQUES 24 HR AFTER ADMINISTRATION
OF IN-¹¹¹I-LABELLED PLATELETS ⁹

	Control diet	High cholesterol diet
No treatment	15,8 (9,9-18,5)	41,5 (15,4-92,5)
Low dose ASA	18,7 (14,5-22,9)	16,2 (10,4-21,7)
High dose ASA	11,7 (8,4-14,9)	16,9 (9,7-23,3)

Low dose ASA = 3 mg aspirin/kg/day

High dose ASA = 30 mg aspirin/kg/day

4) Acute coronary artery thrombosis in dogs (3).

The dual radionuclide method is also useful in visualising and quantitating the deposition of platelets in electrically-induced coronary artery thrombi in dogs. Accumulation of platelets at the point of injury was reportedly apparent only after subtraction of the blood pool images. The %Indium-excess (%IE) again correlated linearly with the in vitro measurement of % injected dose ⁹.

5) Acute endothelial injury in non-human primates (9,20).

With the dual radionuclide method, detection of small deposits of platelets in a large blood pool is possible. The use of this method was assessed in an acute endothelial injury model in certain species of monkey. Endothelial damage to the abdominal aorta could be visualised only after subtraction of the blood pool image. PGI₂ was found to reverse platelet deposition on the damaged endothelium, but this reversal was dependent on the time after injury. PGI₂ did not cause reversal of platelet deposition if administered 46 minutes after injury.

Clinical studies:

The ^{99m}Tc red cell subtraction technique has also been applied successfully in clinical diagnosis. Some examples are presented below:

1) The detection of left atrial thrombi (21). The use of blood pool subtraction was shown to decrease the incidence of false-positive results due to overlap of atria or other blood-containing structures.

2) The detection of platelet deposition in giant intracranial aneurysms (18). Quantitative subtraction of the blood pool showed that platelet aggregation in the aneurysms is more frequent than realised before.

3) Acute myocardial infarction can also be visualised and quantitated with the dual radionuclide method (6,9). According to the group at the Mallinckrodt Institute for Radiology, more than 80% of patients with suspected myocardial infarction had positive scintigrams against only 7% of controls.

4) Atherosclerosis of the carotid arteries. Pilot studies on patients with high incidence of atherosclerosis (hypercholesterolaemia) in this laboratory have shown that platelet deposition can be visualised in some patients.

5) Arterial bypass grafts. Mathias & Welch (1985)⁹ recently reported on the use of quantitative dual isotope subtraction to study platelet deposition on ileo-femoral and femoral-popliteal grafts in patients. They found among other things, that the absence of platelet inhibitors markedly increased deposition of platelets on synthetic grafts.

6) Coronary artery disease in hypercholesterolaemics was investigated in our laboratory. Positive scintigrams were found in only two out of five studies performed. One of the patients with a positive scintigram had extensive coronary bypass grafts.

7) Left ventricular thrombi are usually more difficult to distinguish from the blood pool background. One way to circumvent this problem is by determining whether the "hot spot" increases in activity relative to the blood pool with time (5). The ¹¹¹In-platelet images can also be compared with echocardiography (17). The thrombi can however only be visualised about 48 hours after infusion of the ¹¹¹In-platelets, due to interference from the blood pool (17).

With the dual radionuclide subtraction technique, thrombi can be readily distinguished from the local blood pool within 12 hours. This was demonstrated in a patient with a left ventricular aneurysm. The excised aneurysm contained a 2cm radioactive thrombus which could also be imaged with a gamma camera.

Limitations

Some limitations with the use of dual radionuclide scintigraphy remain. These include:

- 1) The interference of reticuloendothelial deposition of platelets in overlying or underlying tissues. In some studies, such as the study of platelet deposition in the abdominal aorta, platelet uptake in the bone marrow may cause overestimation of deposition of platelets, unless proper controls are used.
- 2) The region of analysis and region of reference (for the blood pool) must be at the same geometrical location in the body. The overlying tissues for both regions must also be of similar structure so as not to affect the count ratio of the two radionuclides. The importance of tissue depth can be seen by the simple expedient of determining the ratio of activity of two radionuclides, In-113 and ^{99m}Tc , in increasing thicknesses of tissue-equivalent material. A graph of the ratio of the activities of the two radionuclides, shows that the ratio is exponentially dependent on tissue thickness (Fig 1).
- 3) As mentioned earlier, red cell incorporation into an active thrombus can result in underestimation of platelet deposition.
- 4) The quantitative dual radionuclide technique cannot determine the absolute amount of radioactive platelets deposited in an area. However, inter-individual statistical comparisons are valid with this method.
- 5) Compton scatter from adjacent organs with high radioactivity can significantly affect the counts in an adjacent area.

Conclusions.

The dual radiotracer technique is a valid means of visualising and quantitating deposition of ^{111}In -platelets in thrombi. It is especially useful in situations where the blood pool is proportionally large. The technique is suitable for various experimental studies and as a diagnostic aid. It is easier to apply and less time-consuming than other quantitative methods. Mathematical manipulation is kept to a minimum, allowing for easier analysis of experimental results.

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FIGURE

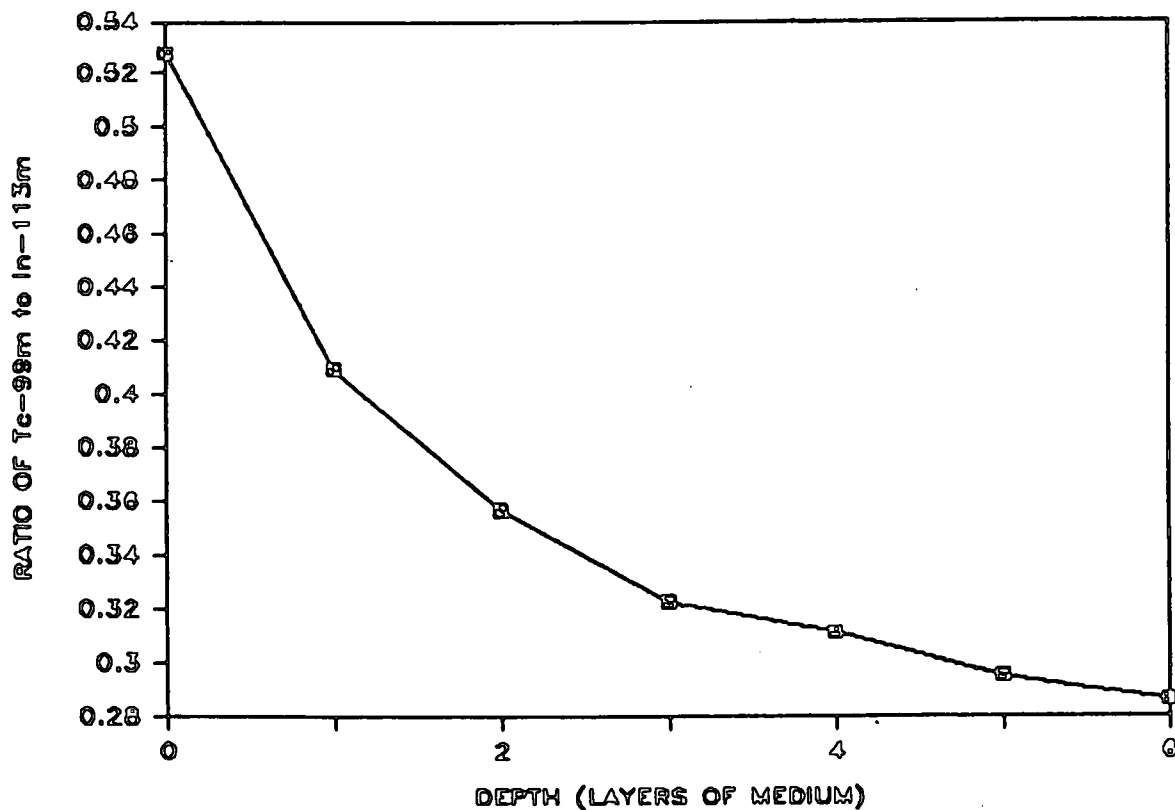


FIGURE 1: The effect of attenuation on the ratio of counts of Tc-99m and In-113.

¹¹¹In-Labelled Baboon Platelets: The Influence of *in vivo* Redistribution and Contaminating ^{114m}In on the Radiation Dose

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In five normal baboons, whole body ¹¹¹In decreased slowly ($t_{1/2} = 60$ days) after the end of platelet lifespan. There was slight elution of radioactivity from the liver and spleen, the important sites of platelet sequestration. A fraction of this eluted radioactivity accumulated in the bone marrow, but also in other tissues. This redistribution did not have a significant effect on the measurement of *in vivo* platelet kinetics. It did however, diminish the total weighted radiation dose, especially that due to contaminating ^{114m}In.

Introduction

Indium-111 is the platelet label of choice for *in vivo* kinetic studies. Its physical characteristics and high platelet labelling efficiency permit *in vivo* estimation of the number of labelled platelets in an organ.⁽¹⁻⁴⁾ The patients are however, exposed to radiation and 86 MBq ¹¹¹In is the maximum dose recommended for platelet kinetic studies.⁽⁴⁾ ^{114m}In is a byproduct of ¹¹¹In produced from cadmium. ^{114m}In, with a half-life of 49.5 days, may contribute significantly to the radiation dose. Thus, in studies of platelet kinetics, contamination of the ¹¹¹In-product with as little as 0.04% ^{114m}In may increase the effective radiation dose equivalent by as much as 6%,⁽⁵⁾ while 0.1% contamination may increase the radiation dose to the spleen by up to 4.6%.⁽⁶⁾

In studies with ¹¹¹In-labelled platelets, the radiation dose to the whole body and organs has been established.^(4,7-9) The calculations were based on the assumption that ¹¹¹In is not excreted from the body and that the radionuclide is retained in the macrophages of the spleen, liver and bone marrow after the sequestration of ¹¹¹In-labelled platelets. Studies with heat-damaged ¹¹¹In-labelled red blood cells sequestered in the spleen have however demonstrated that the radionuclide elutes slowly from the spleen and accumulates in the liver.⁽¹⁰⁾ It is not known whether

a similar redistribution of ¹¹¹In occurs during studies of platelet kinetics.

The aims of this study were to assess the influence of the *in vivo* redistribution of ¹¹¹In on the organ radiation dose, and to determine the effect of contamination with ^{114m}In on radiation. Although there was indeed *in vivo* redistribution of the radionuclide between organs, this was slight. This redistribution did nevertheless affect the radiation dose contributed by ^{114m}In.

Methods

Experimental animals

In a project approved by the Ethical Committee of the Provincial Administration and the University of the Orange Free State, five healthy male baboons, weighing 21.5 ± 5.2 kg, were studied. Anaesthesia with ketamine hydrochloride (± 10 mg/kg body mass) facilitated handling for blood sampling and scintillation camera imaging. The mean peripheral blood platelet count of the five baboons was normal, $430 \pm 84 \times 10^9/L$.

Platelet labelling and determination of platelet survival

Autologous platelets were isolated and labelled in saline with ¹¹¹In-oxine (Radiochemical Centre, Amersham, U.K.) as described in detail.^(3,11) Briefly, platelets were isolated from the whole blood by differential centrifugation and repeated washings of the red cell layer. The platelets were concentrated by centrifugation, resuspended in saline, and ¹¹¹In-oxine

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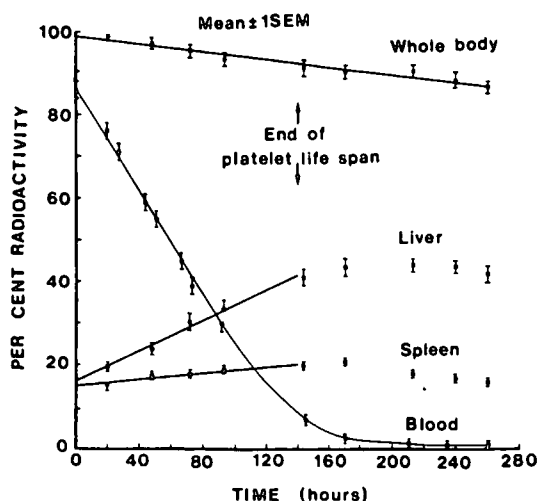


Fig. 1. The redistribution of ^{111}In after injection of ^{111}In -labelled platelets. Whole body and organ radioactivity was expressed as a per cent of whole body radioactivity (assumed to be 100%) on the day of injection. Blood radioactivity was expressed as a per cent of total radioactivity/mL blood injected. Values are given as a mean $\pm$ 1 SEM.

added. After incubation, the platelets were concentrated and resuspended in platelet poor plasma for reinjection. Labelling efficiency was $82 \pm 2\%$. Contamination of the labelled platelet injectate with red cells, plasma proteins and white cells was less than 3%. The recovery of labelled platelets in the circulation at equilibrium was calculated. Their mean lifespan (MPLS) was estimated by fitting the blood disappearance curve of the labelled platelets to a gamma function⁽¹²⁾ The recovery at equilibrium was $86 \pm 4\%$, and MPLS was 140 ± 13 h.

The labelled platelets were viable, as assessed *in vitro*.⁽²⁾ *In vivo* function was normal. This was reflected by a linear disappearance of labelled platelets from the circulation (Fig. 1); normal recovery of labelled platelets in the circulation; and a normal MPLS compared to those of other studies.⁽¹²⁻¹⁵⁾

Quantification of the *in vivo* distribution of labelled platelets

The procedures are described in detail elsewhere.⁽¹⁶⁾ Briefly, anterior and posterior images of the whole body were acquired daily for 11 days with a large field of view scintillation camera, starting on the day of injection of the labelled platelets. Whole body and organ radioactivity was determined by region of interest analysis. The percent of whole body radioactivity in an organ was quantified with the geometric mean method, and time/radioactivity curves were generated. Whole body radioactivity at equilibrium was assumed to be 100%, and all subsequent whole body and organ radioactivities were expressed relative to this. The radioactivity in the pelvis was also determined by region of interest analysis. It was assumed that this radioactivity represents seques-

tration of senescent labelled platelets in the bone marrow.

In two of the baboons, whole body radioactivity was determined with anterior and posterior scintillation detectors in a whole body counter on days 13, 20, and 33 after the labelled platelets were injected.^(3,4) The geometric mean hepatosplenic count rate was determined and expressed as a per cent of the geometric mean whole body radioactivity at equilibrium. The whole body ^{111}In -radioactivity measured with the whole body counter on day 13 was normalized to that measured with the scintillation camera on day 11.

Radiation dose

The radiation dose was calculated from the data according to the recommendations of the Medical Internal Radiation Dose Committee of the Society of Nuclear Medicine (MIRD).⁽¹⁷⁾ To calculate the cumulative organ radioactivity, the radioactivity in the spleen, liver and blood during the platelet survival time was fitted to a linear function. In the time period after the end of platelet survival, an exponential function was fitted to the data. The weighted dose equivalent was calculated according to the International Commission on Radiological Protection.⁽¹⁸⁾

Contamination with ^{114m}In of commercial ^{111}In -oxine (Radiochemical Centre, Amersham, U.K.) was measured in a well-type scintillation counter after complete decay of a known amount of ^{111}In . The measured contamination was 0.05%. The effect of contamination with 0.08% ^{114m}In , the value specified by the manufacturer, on the radiation dose was also calculated.

For purposes of comparison, we have extrapolated the results on redistribution of the ^{111}In in this study to humans, and calculated the maximum annual dose that may be administered. The organ radioactivities reported by Wessels *et al.*⁽¹⁹⁾ were used in the calculations.

Results

In vivo distribution of radioactivity

The distribution of radioactivity at equilibrium, at MPLS, and after 261 h is given in Table 1. The changes in blood-, whole body-, spleen-, and liver radioactivity during the 261 h of the study, are presented in Fig. 1. This graph can conveniently be divided into a survival phase (from the time of reinjection of labelled platelets to the end of MPLS) and a post-survival phase (from MPLS to 261 h). Whole body radioactivity decreased significantly from equilibrium, (assumed to be 100%) to $89 \pm 5\%$ at 261 h ($P = 0.008$; *t*-test for paired data). The biological half-life of ^{111}In in the body was 60 days. Splenic radioactivity decreased with 3.4% in the post-survival phase. This decrease was significant; $P = 0.02$ (*t*-test for paired data). The biological half-life of the radionuclide in the spleen was 18 days. Although hepatic radioactivity tended to increase

Table 1. The redistribution of ^{111}In following injection of ^{111}In -labelled platelets. Values are given as a mean per cent of equilibrium whole body radioactivity, assumed to be 100%

Baboon	At equilibrium			At platelet life span			After 261 h		
	Spleen	Liver	Pelvis*	Spleen	Liver	Pelvis*	Spleen	Liver	Pelvis*
1	13.7	19.7	10.6	23.8	47.4	4.2	18.9	42.9	5.7
2	14.2	13.7	11.3	20.4	45.3	5.4	17.8	52.4	5.2
3	15.9	16.8	11.3	18.7	37.9	6.9	14.6	43.4	6.4
4	14.7	14.0	15.2	18.6	37.4	6.1	19.1	37.5	5.6
5	16.0	18.0	10.8	20.4	37.2	6.9	14.8	35.9	5.4
Mean	14.9	16.4	11.8	20.4	41.0	5.9	17.0	42.4	5.7
SD	1.0	2.6	1.9	2.1	4.9	1.1	2.2	6.5	0.5

* The radioactivity in the pelvis was used as an indication of bone marrow ^{111}In , either due to blood flow (equilibrium) or sequestered platelets.

slightly at first, and then decreased (Fig. 1), hepatic and bone marrow radioactivity remained relatively constant in the post-survival phase (Table 1).

The results obtained with the whole body counter are given in Table 2. Whole body and hepatosplenic radioactivity decreased, and there was an increase of the radioactivity in the remainder of the tissues. The biological half-life of ^{111}In , calculated from both the scintillation camera and whole body counter data, in baboons 3 and 5 was 67 days.

Radiation dose

The biological data in Tables 1 and 2 were used to calculate the radiation dose (Table 3A). A biological

Table 2. The redistribution of ^{111}In measured in the whole body counter. Results are expressed as a per cent of whole body radioactivity at equilibrium, assumed to be 100%

	Baboon	Time (h)		
		291	459	796
Whole body	3	95	92	89
	5	87	84	83
	Mean	91	88	86
Hepatosplenic	3	81	76	69
	5	71	66	61
	Mean	76	71	65
Remainder*	3	14	17	19
	5	17	18	23
	Mean	16	18	21

* Remainder is radioactivity neither in the spleen nor in the liver.

half-life of 60 days, as estimated from the decrease in whole body radioactivity from 0 to 11 days, was used for ^{111}In . It was assumed that non-hepatosplenic radioactivity was evenly distributed in the remainder of the body.⁽¹³⁾ The spleen received the highest radiation dose, 4.66 mGy/MBq. ^{114m}In contributed approximately 8% to the total weighted radiation dose; the liver was the major recipient, i.e. 15%.

For safety purposes, the absorbed dose was also calculated assuming that: (1) the radionuclide was not excreted but was indefinitely retained in the macrophages and that non-hepatosplenic radioactivity was evenly distributed in the body (Table 3B), and (2) excretion of ^{111}In and redistribution of radionuclide between organs took place, but non-hepatosplenic radioactivity accumulated only in the bone marrow (Table 3C). Loss of radionuclide did not affect the calculated radiation dose received from ^{111}In . The radiation dose from the ^{114m}In was influenced (Table 3B vs Table 3A). As a result of the loss of radioactivity, ^{114m}In contributed 8% to the total weighted radiation dose equivalent, in contrast to 15–17% if radioactivity was not lost. This is a reduction of about 50%. Elution of radioactivity from the spleen reduced the radiation dose by 15%. Again, this was the result of a decrease in the absorbed dose from ^{114m}In , i.e. 0.78 mGy/0.8 kBq if radioactivity was indefinitely retained in the spleen vs 0.24 mGy/0.8 kBq if elution occurs. If it was assumed that non-hepatosplenic radioactivity accumu-

Table 3. The absorbed radiation dose, in mGy, received from 1 MBq ^{111}In and 0.8 kBq ^{114m}In . The absorbed dose (A) is calculated from the results in Table 1. In (B) it was assumed that no excretion of ^{111}In took place and that the radionuclide was indefinitely retained in the macrophages. In (C) it was assumed that excretion and redistribution of radionuclide took place, but that non-hepatosplenic radioactivity accumulated only in the bone marrow

Radionuclide	Whole body	Spleen	Liver	Bone marrow	Total weighted dose
(A)					
^{111}In	0.16	4.41	1.10	0.16	0.45
^{114m}In	0.01	0.25	0.16	0.00	0.04
Total	0.17	4.66	1.26	0.16	0.49
(B)					
^{111}In	0.16	4.56	1.10	0.16	0.45
^{114m}In	0.01	0.78	0.17	0.00	0.08
Total	0.17	5.34	1.27	0.16	0.53
(C)					
^{111}In	0.16	4.19	1.08	0.52	0.49
^{114m}In	0.01	0.25	0.16	0.02	0.03
Total	0.17	4.44	1.24	0.54	0.52

lated in the bone marrow alone, the absorbed radiation dose to this organ increased 3.4 fold (Table 3C vs Table 3A).

Discussion

The radiation dose to the whole body and different organs due to ^{111}In -labelled platelets has been established.^(4,7-9) Apart from the half-life of the radionuclide, *in vivo* redistribution of radionuclide between organs, and loss of radionuclide from the body may influence the calculation of the radiation dose. The influence of these factors, and of contaminating ^{114m}In , were determined in this study. However, to calculate the radiation dose accurately, it is necessary to have an accurate estimate of the radioactivity in the different organs. The geometric mean method of quantification has been showed to be suitable.⁽²⁰⁾

Whole body radioactivity decreased (Fig. 1, Table 2) with a biological half-life of 60 days. This is similar to results reported in some studies on human patients^(21,22) and in our previous study in baboons.⁽¹³⁾ The estimate of the biological half-life of 67 days, based on the decrease in whole body radioactivity from day 0 to 33, was slightly longer. Since only two baboons, each with only three observations were studied by this means, we consider the estimate of the biological half-life as 60 days more reliable.

In studies of normal humans, however, ^{111}In -radioactivity was not lost.^(3,19) The reason for the decrease in whole body radioactivity is not entirely clear. It is apparently not due to excretion of radionuclide, because radioactivity has not been detected in urine or feces.^(3,7,8) It may not be a true loss of radionuclide, but due to the influence of attenuation of the gamma photons. It seems likely that the redistribution of labelled platelets from the circulation to the organs of the reticulo-endothelial system may affect the estimate of organ and whole body radioactivity.

In the post-survival phase, ^{111}In eluted from the spleen (Fig. 1, Table 1) at a rate of approximately 0.7%/day. If it is assumed that elution started on the day of injection of the labelled platelets, the spleen would have lost only 4% (0.7%/day $\times$ MPLS) of its equilibrium radioactivity in the survival phase. This will not influence the estimate of splenic radioactivity significantly, because it is less than the accuracy of 4–6% inherent in the methodology.⁽²⁰⁾ Pelvic radioactivity remained relatively constant in the post-survival phase (Table 1). Hepatic radioactivity did seem to increase slightly, although not significantly. This was followed by a decrease (Fig. 1). After 260 h, there was a loss of ^{111}In from the hepatosplenic area, and an accumulation of radioactivity in the remainder (Table 2). It seems reasonable to assume that this radioactivity will be located equally in the bone marrow and other tissues. This assumption is based on our previous findings in baboons.⁽¹³⁾

The results demonstrate that the spleen is the major target for radiation (Table 3A). Contamination with 0.08% ^{114m}In contributed about 5% of this absorbed dose. The liver received less radiation, but was more susceptible to the effect of contaminating ^{114m}In (Table 3A). The ^{114m}In contributed about 8% to the total weighted radiation dose.

The influence of redistribution of ^{111}In on the radiation dose was also determined. The effect on the spleen was marked, i.e. a reduction of 15% in the absorbed dose (Table 3B vs Table 3A). The effect on the bone marrow was slight if it was assumed that all radioactivity lost from the spleen and liver accumulated in the bone marrow and other tissues (Table 3B vs Table 3A). However, if this redistribution was solely to the bone marrow, the radiation dose of this organ increased 3.4 fold (Table 3C vs Table 3A). It should be noted that only about half the non-hepatosplenic radioactivity accumulates in the bone marrow.⁽¹³⁾

These results on the effect of redistribution of radionuclide on radiation pertain to baboons, but can readily be extrapolated to humans. One should however, note that the splenic platelet pool of baboons is about half that of humans.^(3,4,13,19-22) The much higher radiation dose to the human spleen will therefore increase the importance of ^{114m}In contamination as a source of radiation.

We conclude that the radiation dose due to ^{111}In is acceptable for platelet kinetic studies in humans. The maximum permissible annual dose should however, not exceed 61 MBq. This will limit the absorbed dose to be within the specified permissible annual radiation dose.^(17,18) Far less ^{111}In , 15–23 MBq, is adequate for platelet kinetic studies in humans.^(3-9,19-22) It is also evident that contamination with as much as 0.08% ^{114m}In will not appreciably increase the radiation dose. Investigators should however have information available, or, measure the extent of this contamination.

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Evidence that ^{111}In -labelled platelets pool in the spleen, but not in the liver of normal humans and baboons

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The kinetics of pooling of platelets in the spleen may be used to measure spleen function. However, pooling of platelets in other organs, especially the liver, may affect these kinetic measurements. We have therefore compared, in man and baboon, the kinetics of ^{111}In -labelled platelets and ^{111}In -labelled red cells in the liver and spleen during the first 60 min after reinjection. This was determined in vivo with a scintillation camera and computer-assisted image analysis. Organ radioactivity was expressed as a percentage of that of the whole body. In both humans and baboons, the spleen accumulated many more platelets than red cells. Also, the red cells equilibrated more rapidly. The presence of a splenic platelet pool was thus confirmed. In contrast, the percentages of labelled platelets and labelled red cells in the liver were similar in both species. In both species, the sum total of labelled platelets in the circulation, i.e. recovery, and that quantified in the spleen at equilibrium, were equal to approximately 100%. These results confirm the presence of the exchangeable splenic platelet pool, and indicate that there are no significant exchangeable platelet pools in either the liver or any other organs.

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Approximately one-third of reinjected radio-labelled platelets are not recovered in the circulation, but accumulate in an exchangeable splenic platelet pool (1-7). The mechanism of pooling is not known, but may be related to the circulation of the spleen. Approximately 90% of blood delivered to the spleen enters the splenic cords and flows through the sinuses. This is the 'open' circulation. The remainder flows through a 'closed' system, where the

splenic arteries and veins are in continuity (8, 9). Many platelets apparently enter the 'open' circulation and their transit through the spleen is therefore delayed (10, 11). In contrast, less than 10% of the red cells entering the spleen are subject to intermediate or slow flow through the spleen (12), but they enter the 'open' circulation nonetheless.

The rate of accumulation of labelled platelets in the spleen is monoexponential. It has been

suggested that platelet kinetics and in vivo distribution conform to a simple two-compartmental, closed system describing movement of platelets between the circulation and the spleen (4-7). Based on this assumption, compartmental analysis has been used to noninvasively measure spleen function, splenic blood flow, and the transit time of platelets through the spleen (7, 11, 13-15). However, if platelets were to accumulate in other organs, or damaged platelets are sequestered in a non-exchangeable pool in the spleen, the measurement of the kinetics of platelets by compartmental analysis will be affected (13-16). The suggestion that platelets may be pooled in the liver (7, 13-16) is therefore important.

We have now tried to resolve the question of the presence or absence of an extrasplenic platelet pool by comparing the kinetics and in vivo redistribution of ^{111}In -labelled platelets and ^{111}In -labelled red cells in the spleen and liver of normal humans and baboons. The baboons were studied because the size of their splenic platelet pool is about half that of humans (19). It was thought that species differences in the kinetics of pooling of platelets in the spleen may point to a factor, or factors, that may govern the mechanism of pooling.

Methods

Subjects and experimental animals: 11 healthy human volunteers, who gave informed and signed consent, and 6 healthy baboons (*Papio ursinus*) were studied in a project approved by the Ethical Committee of the University of the Orange Free State. Platelet kinetics were studied in 6 humans and 6 baboons; and red blood cell kinetics in 5 other humans and 3 of the baboons. The baboons were anaesthetized with ketamine hydrochloride (10 mg/kg body mass).

Blood cell labelling: Autologous platelets were isolated from the whole blood and labelled with ^{111}In -oxine (Radiochemical Centre, Amersham, UK) as described in detail elsewhere (17-19). Briefly, the platelets were isolated by repeated 'washing' from the red cell layer. The platelets were concentrated by centrifugation, resuspended in saline, and labelled with ^{111}In -oxine. Labelled platelets, resuspended in platelet poor plasma,

were reinjected 1 h later. Viability of labelled platelets was assessed in vitro by their aggregation response to ADP (20), and they were not reinjected unless aggregates consisting of 10 or more platelets were observed. Recovery of labelled platelets in the circulation at equilibrium was estimated (17).

Autologous red blood cells were labelled as described elsewhere (21). Blood, 17.5 ml, was collected in 2.5 ml acid-citrate-dextrose and the red cells sedimented with hydroxyethyl starch. Platelets and leucocytes retained in the red cell layer were removed by washing 3 times, and the red blood cells labelled with ^{111}In -oxine. The red cells were resuspended in 5 ml platelet poor plasma and reinjected. The labelling efficiency with ^{111}In -oxine was consistently greater than 90%. Contamination with labelled platelets and leucocytes was less than 4%.

Scintillation camera imaging: The redistribution of labelled cells in the spleen and the liver during the equilibrium phase was dynamically imaged with a large field of view scintillation camera interfaced with an A²-MDS data processing system (17-19). The subject was positioned so that the heart, liver and spleen were included in the field of view of the camera. Labelled cells were injected as a bolus and 64×64 byte mode images acquired: 30 images, each lasting 30 s, followed by 15 images of 180 s duration. Radioactivity in the liver, spleen and heart was expressed as counts/pixel/min (17). At equilibrium, reached 90 min after the reinjection of the labelled cells, the in vivo distribution of the labelled cells in organs and the whole body was quantified with the geometric mean method (17, 18). The mean count rate of the dynamic curves was normalized to the radioactivity present in the organ at equilibrium. Organ radioactivity was expressed as a percentage of whole body radioactivity.

Calculations: The accumulation of ^{111}In -labelled platelets in the spleen was fitted to the equation:

$$\text{Accumulation} = A_1 + A_2 e^{-kt} \quad (1)$$

where A_1 is the asymptotic value, $A_1 + A_2$ is the Y-intercept, and k is the rate constant of accumulation of platelets in the spleen.

Splenic blood flow and the transit time of platelets through the spleen were calculated by compartmental analysis (7, 11, 15). The rate constant of accumulation of cells in an organ (K_0) is equal to the sum of the input rate constant (k_1) and the output rate constant (k_2). Therefore, at equilibrium:

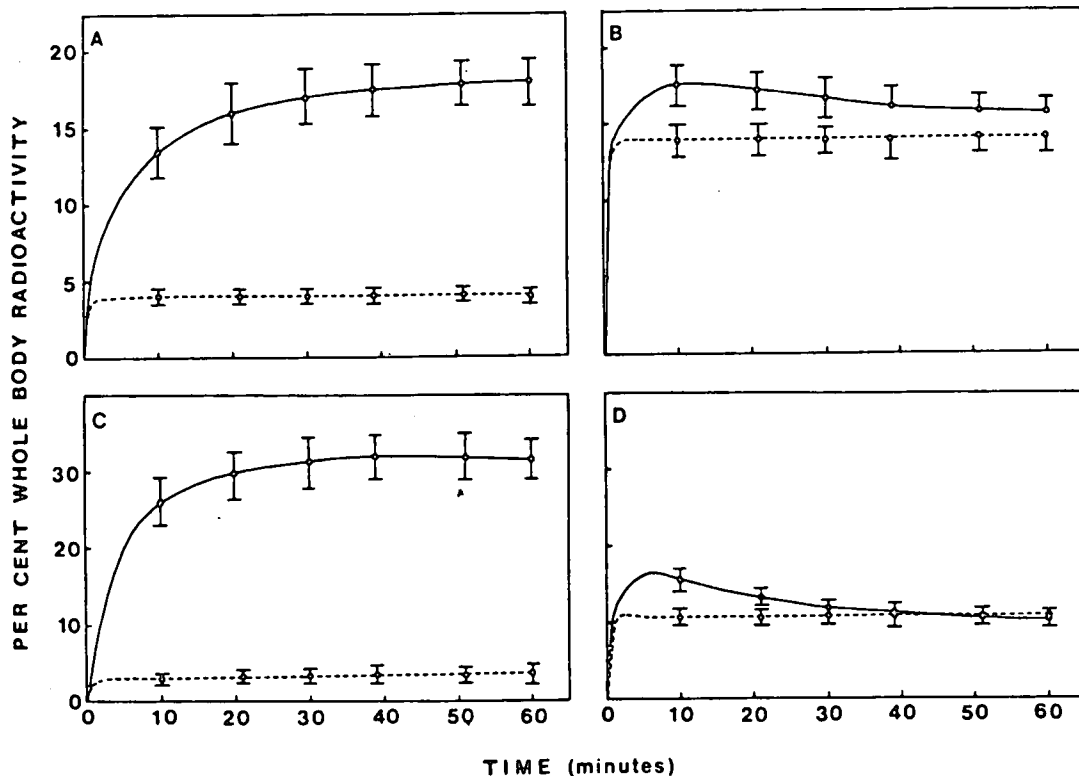


Figure 1. The attainment of equilibrium distribution of ¹¹¹In-labelled platelets (solid lines) and ¹¹¹In-labelled red blood cells (broken lines) in the spleen (A and C) and liver (B and D). Panels A and B are for baboons and panels C and D for humans. Values are given as a % of whole body radioactivity, mean $\pm$ 1 SEM.

$$k_1/k_2 = Q_0 \quad (2)$$

where Q_0 is the fraction of ¹¹¹In-labelled platelets quantified in the organ.

The ratio of the transit time of labelled platelets to labelled red cells in the different organs was calculated (22). The equation used was:

$$t_P/t_R = O_P \times B_R/O_R \times B_P \quad (3)$$

where t is the transit time of platelets (P) and red cells (R), O the quantified organ radioactivity for the different cells and B the recovery of platelets and red cells in the circulation. Since red cells are not pooled in an organ (12, 13), it was assumed that the recovery of these cells was 100%.

Statistical analysis: Paired or unpaired data were evaluated with the t -test for different means.

Results

Cell kinetics in the spleen: In humans, labelled platelets accumulated at a rate constant of $0.177 \text{ min}^{-1} \pm 0.057$, reaching 90% of the maximum concentration after about 12 min (Figure 1C). Labelled red cells accumulated at a much faster rate, and equilibrated within 4 min (Figure 1C). Mean splenic blood flow was calculated to be $5.7\% \pm 2.4$ of the cardiac output. The transit time of platelets through the spleen was $9.01 \text{ min} \pm 2.72$. At equilibrium, a significantly greater percentage of labelled platelets than labelled red cells was present in the spleen (Table 1). The sum total of the recovery of labelled platelets in the circulation ($66\% \pm 13$) and that quantified in the spleen at equilibrium ($30.7\% \pm 6.0$) was $97\% \pm 10$.

In baboons, the labelled platelet accumulation

was exponential at a rate constant of $0.158 \text{ min}^{-1} \pm 0.077$. The rate constant was not significantly different from that observed in humans ($p > 0.05$). The red cells accumulated much faster than platelets, and equilibrated within 4 min after reinjection (Figure 1A). Splenic blood flow was $2.7\% \pm 1.4$ of cardiac output, significantly less than that in humans ($p < 0.05$). The transit time of platelets through the spleen of baboons, $9.02 \text{ min} \pm 3.65$, was similar to that of humans. The splenic platelet pool of baboons, $16.8\% \pm 2.6$, was significantly smaller ($p < 0.01$) than that of humans (Table 1). At equilibrium, approximately 3.5% of the labelled red cells were present in the spleen (Table 1). The sum total of the fraction of labelled platelets quantified in the spleen ($16.8\% \pm 2.6$) and that recovered in the circulation ($87\% \pm 6$) was $104\% \pm 7$. Although it is possible that the anaesthesia may have influenced the rate of accumulation of the labelled platelets in the spleen, similar to that seen in dogs (13, 14), it is considered to be unlikely since, in these baboons, the recovery of labelled platelets in the circulation was similar to that described in unanaesthetized baboons (23).

Cell kinetics in the liver: In both species there was a transient accumulation of labelled platelets in the liver (Figures 1B and 1D). After an initial rapid accumulation, the hepatic concentration of labelled platelets increased more slowly and reached a maximum at about 6–10 min. Radioactivity then decreased gradually and equilibrated after 30–40 min.

Labelled red cells, on the other hand, equilibrated within 4 min in both species. The distribu-

tion of labelled platelets and labelled red cells at equilibrium did not differ significantly ($p > 0.10$ in both species, Table 1).

Ratio of platelet/red cell transit times: The mean ratio in the heart, where no pooling of blood cells can take place, was 1.5 in the humans and 1.4 ± 0.2 in the 3 baboons that were studied with both labelled red cells and labelled platelets. The ratio in the liver was 1.4 and 1.3 ± 0.2 , respectively. The ratios between the heart and liver of the 3 baboons were not significantly different ($p > 0.20$). The ratio in the spleen was 13.6 in the humans and 4.9 ± 2.1 in the baboons.

Discussion

A 'pool' for platelets may be defined as an organ, or region, with an increased blood cell to plasma ratio compared to the same ratio measured in the peripheral blood (14). We have defined a 'pool' for platelets as an organ where the concentration of platelets exceeded that of red cells. This approach was based on the assumption that in normal subjects red cells are not pooled in an organ (12, 13), but that their concentration reflects the perfusion of blood in the organ.

It is well established that approximately one-third of the total platelet population is pooled in the spleen: in normal humans, only about two-thirds of injected ^{51}Cr -labelled platelets are recovered in the circulation (1–3). Quantitative imaging of ^{111}In -labelled platelets has confirmed this (4, 6, 16–19). In the present study, the concentration of labelled platelets in the spleen of both species was much higher than that of labelled red cells (Table 1). The ratio of the transit time of labelled platelets to red cells was 13.6 in humans and 4.9 ± 2.1 in baboons. The presence of a splenic platelet pool, but not a red cell pool, was confirmed by the observation that the red cells equilibrated at a much faster rate than platelets. The transit time of red cells through the spleen was therefore also much faster than that of platelets. These results all confirm that platelets are pooled in the spleens of humans

TABLE 1
The in vivo distribution of ^{111}In -labelled platelets and ^{111}In -labelled red cells at equilibrium. Values are given as a mean % of whole body radioactivity $\pm 1 \text{ SD}$

Species	Organ	Platelets	Red cells
Humans	Spleen	30.7 ± 6.0	3.5 ± 0.6
	Liver	9.6 ± 1.2	10.6 ± 2.0
	Heart	7.3 ± 1.4	7.4 ± 1.8
Baboons	Spleen	16.8 ± 2.6	4.0 ± 0.7
	Liver	15.6 ± 1.9	14.1 ± 1.6
	Heart	7.7 ± 2.3	6.1 ± 2.1

and baboons and established the validity of the method we have used to demonstrate the presence of a pool.

Since only about 90% of all injected labelled platelets are recovered in the circulation of asplenic subjects (24, 25), it has been suggested that there is a nonsplenic pool for platelets (26), possibly situated in the liver (7, 14, 15). Our results do not support this view. In the livers of both species the concentration of labelled platelets and labelled red cells, expressed as a percentage of whole body radioactivity, were similar. The ratio of the transit time of labelled platelets to the transit time of labelled red cells corresponded to the ratio of these cells measured in the heart. It may be assumed that pooling does not occur in the heart. We could also account for all the injected ¹¹¹In-labelled platelet radioactivity by adding that recovered in the circulation to the splenic platelet pool, estimated by quantitative imaging: $97\% \pm 10$ in humans and $104\% \pm 7$ in the baboons. This is in accord with the results published by several other investigators (4, 18, 27), and supports the view that platelets are distributed only in two compartments, namely the spleen and the circulation. We therefore consider that the ¹¹¹In-labelled platelets in the liver at equilibrium are present in the blood perfusate and not in a pool.

Before equilibrium was established, a slight excess accumulation of ¹¹¹In-labelled platelets in the liver, maximal within 10 min after injection, was evident. This phenomenon was transient, and may be ascribed to the 'collection injury' sustained by the platelets during the labelling process (28). It is important to note that this excess accumulation should not be considered as a hepatic platelet pool. The 'collection injury' is reversible (18), and has a very small effect on the measurement of the mean platelet life span, in vivo distribution and sites of sequestration of labelled platelets (28).

The human spleen pooled approximately twice as many platelets as that of baboons. We have no explanation for this difference, but some factors may be considered. The size of the spleen may be important, since significantly more plate-

lets are pooled in patients with splenomegaly (24). However, since the human spleen contributes approximately 0.16% to the total body mass (29), and that of the baboon approximately 0.23% (our own unpublished observations), it seems unlikely that the size of the spleen explains our observations. The size of the splenic platelet pool is related to splenic blood flow and the transit time of platelets through the spleen (11, 15). Our results support this view that splenic blood flow does determine the size of the platelet pool. Splenic blood flow in baboons was approximately half that measured in humans. The transit time of platelets through the spleen, however, did not differ in the two species. Transit time therefore does not explain the difference in the size of the splenic platelet pool in the two species.

Hence, our results in both humans and baboons are consistent with the view that normal subjects do not have a nonsplenic platelet pool. If such a pool does exist, it must be very small, and in rapid equilibrium with the platelets in the circulation. It is therefore not necessary to correct for a nonsplenic platelet pool when measuring the kinetics of the splenic platelet pool. The transient, excess accumulation of labelled platelets in the liver due to the 'collection injury' will, however, have an influence on the time taken to attain equilibrium. This should be taken into account when measuring in vivo platelet redistribution and mean platelet life-span.

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The influence of the 'collection injury' on the survival and distribution of Indium-111-labelled canine platelets

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SUMMARY. The extent of the 'collection injury' sustained by platelets during labelling with In-111-oxine was investigated in three matched pairs of beagle dogs. The influence of the procedure on the survival, kinetics, *in vivo* distribution and fate of the labelled platelets was determined by serial blood sampling and quantitative computerized scintillation camera studies. Injured labelled platelets were removed in the matched dog acting as a biological filter. The survival, distribution and fate of the 'filtered' and 'unfiltered' platelets were compared. The mean platelet lifespan of the 'filtered' and 'unfiltered' platelets did not differ significantly, but the shape of the survival curve of the filtered platelets fitted a linear function more closely than that of the unfiltered platelets. Radioactivity in the different organs and regions was serially quantitated and expressed as a percentage of whole body radioactivity. Splenic and hepatic radioactivity of filtered and unfiltered platelets did not differ significantly at equilibrium or at the end of platelet lifespan. It is concluded that the currently employed isolation and labelling techniques for platelets are suitable for quantitative *in vivo* studies with a computerized scintillation camera system. The shape of platelet survival curves should, however, be interpreted with some caution as it may be influenced by these procedures.

The introduction of ¹¹¹Indium-oxine as a platelet label (Thakur *et al.*, 1976) added a new dimension to the study of platelet kinetics. The physical properties of ¹¹¹In permit quantitative studies of *in vivo* platelet distribution in health (Lötter *et al.*, 1980; Heyns *et al.*, 1980a) and disease (Heyns *et al.*, 1979). However, the currently available techniques for the labelling of

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platelets with radioactive tracers are associated with some degree of platelet damage—the so-called 'collection injury' (Harker, 1978). After reinjection, damaged labelled platelets are rapidly cleared from the circulation, whilst the undamaged portion persists for a much longer time (Kaplan & Saba, 1978). Platelet survival studies deal almost exclusively with the latter group. *In vivo* scintillation camera studies, however, will measure distribution of both populations. The validity of the results of quantitative studies of platelet kinetics may therefore be questioned.

An experimental study was devised to determine the influence of the 'collection injury' on the survival, kinetics, distribution and fate of ^{111}In -labelled canine platelets. Platelets damaged during the labelling process were removed by 'filtering' through another dog and the survival and kinetics of the filtered and unfiltered platelets were then compared.

MATERIALS AND METHODS

Platelet isolation and labelling. Platelets were isolated by differential centrifugation, labelled in physiological saline with ^{111}In -oxine, washed and resuspended in autologous plasma as previously described (Heyns et al, 1980b; Lötter et al, 1980). Platelet viability was assessed and only injected if the *in vitro* aggregation response to ADP and collagen was normal (Heyns et al, 1980b). An aliquot was fixed for electron microscopy.

Dogs. Six beagle dogs with a mean mass (± 1 SD) of 12 ± 2 kg were matched in pairs of a donor and a filter dog.

Donor dog. Two weeks prior to the exchange experiment, 127.5 ml venous blood was collected aseptically in a plastic bag containing 22.5 ml acid citrate dextrose NIH formula A (ACD-A) as anticoagulant. One week later, this blood was reinfused after 255 ml blood was collected in 45 ml ACD-A. Another week later, 425 ml blood was collected in 75 ml ACDA and the blood collected the previous week was reinfused. Platelets were isolated from this 425 ml blood and labelled with ^{111}In -oxine. The red cells were preserved.

Filter dog. The filter dog was positioned under a scintillation camera to visualize the heart, liver and spleen. The ^{111}In -labelled platelets of the paired donor dog were rapidly injected intravenously.

The exchange experiment. Twenty hours after the injection of the ^{111}In -labelled platelets the filter dogs were anaesthetized with 0.25 g Thiopentone sodium and positioned under the scintillation camera. A no. 16 gauge needle was inserted into the external jugular vein and 425 ml blood was drawn consecutively into 10 $\times$ 50 ml plastic syringes, each containing 7.5 ml ACD-A. One ml aliquots were removed from each syringe for electron microscopy of platelets and to serve as a ^{111}In -standard. The radioactivity of the remaining blood was measured in a Picker radionuclide dose calibrator.

Packed red cells obtained from the 425 ml blood collected from the donor dog the previous day were then infused into the filter dog.

The donor dogs were anaesthetized and positioned under the scintillation camera collimator. 200 ml blood was withdrawn and replaced with the 490 ml ACD-blood containing ^{111}In -labelled filtered platelets.

Scintillation camera imaging and quantitation. Image acquisition and quantitation of

platelets distribution were performed with a Searle Pho Gamma scintillation camera interfaced with a computer-assisted imaging system as previously described (Lötter *et al.*, 1980). Geometry was maintained by positioning the dogs in a fixed frame on a rigid table. A high-energy divergent collimator was positioned anteriorly to visualize the heart, liver and spleen. Images were obtained by integrating for 30 s intervals for 15 min, 9 min images were acquisitioned for the following 90 min, and 9 min images daily for 7 d. Whole body profile scanning was done daily with a slit collimator in a low background steel room. Areas of interest were selected over the heart, liver and spleen and the radioactivity in each organ measured by computer-assisted scintillation camera imaging. Radioactivity of a selected thoracic area was integrated in both the scintillation camera image and whole body counter profile. These activities were related and a correction factor derived to normalize the radioactivity measured by the scintillation camera to that measured by the whole body counter. Time-radioactivity distributions for the whole body and the specific organ on days 1-7 were subjected to linear least squares analysis. Zero-time equilibrium was derived by back-extrapolation of regression activity curves. Final organ and region radioactivity was determined at the end of the platelet lifespan in the circulation.

Electron microscopy. The filtered and unfiltered platelets were examined by a technique modified after White (1968) (Heyns *et al.*, 1980b). One volume of platelet rich plasma was fixed in 5 vol 2.5% glutaraldehyde, pH 7.3; post fixed in 1% osmium tetroxide; after dehydration samples were embedded in epoxy resin, sectioned and stained with uranyl acetate and lead citrate.

Platelet-survival studies. Platelet-survival studies were largely performed as recommended by the ICSH (1977) as previously described (Lötter *et al.*, 1980; Heyns *et al.*, 1980a). Mean autologous platelet survival was calculated by least squares computer fitting to a gamma function (Murphy & Francis, 1971) and by least squares analyses of the linear and logarithmic estimates (ICSH Panel, 1977). Platelet recovery in the circulation was determined from a blood sample taken 5 min after infusion of labelled platelets.

RESULTS

Ultrastructure of the platelets

Electron microscopy of the unfiltered platelets, done immediately prior to injection, showed some changes characteristic of activation (Fig 1). The platelets retained their discoid shape and granules were distributed normally. Surface projections were present and the canalicular system was dilated.

Electron microscopy of the filtered platelets (Fig 1B) revealed only a few small surface projections but the canalicular system was dilated.

Platelet survival

The relevant subject data, radioactivity and platelets administered, platelet recovery in the circulation and survival data are given in Table I. The mean platelet lifespan of the filtered

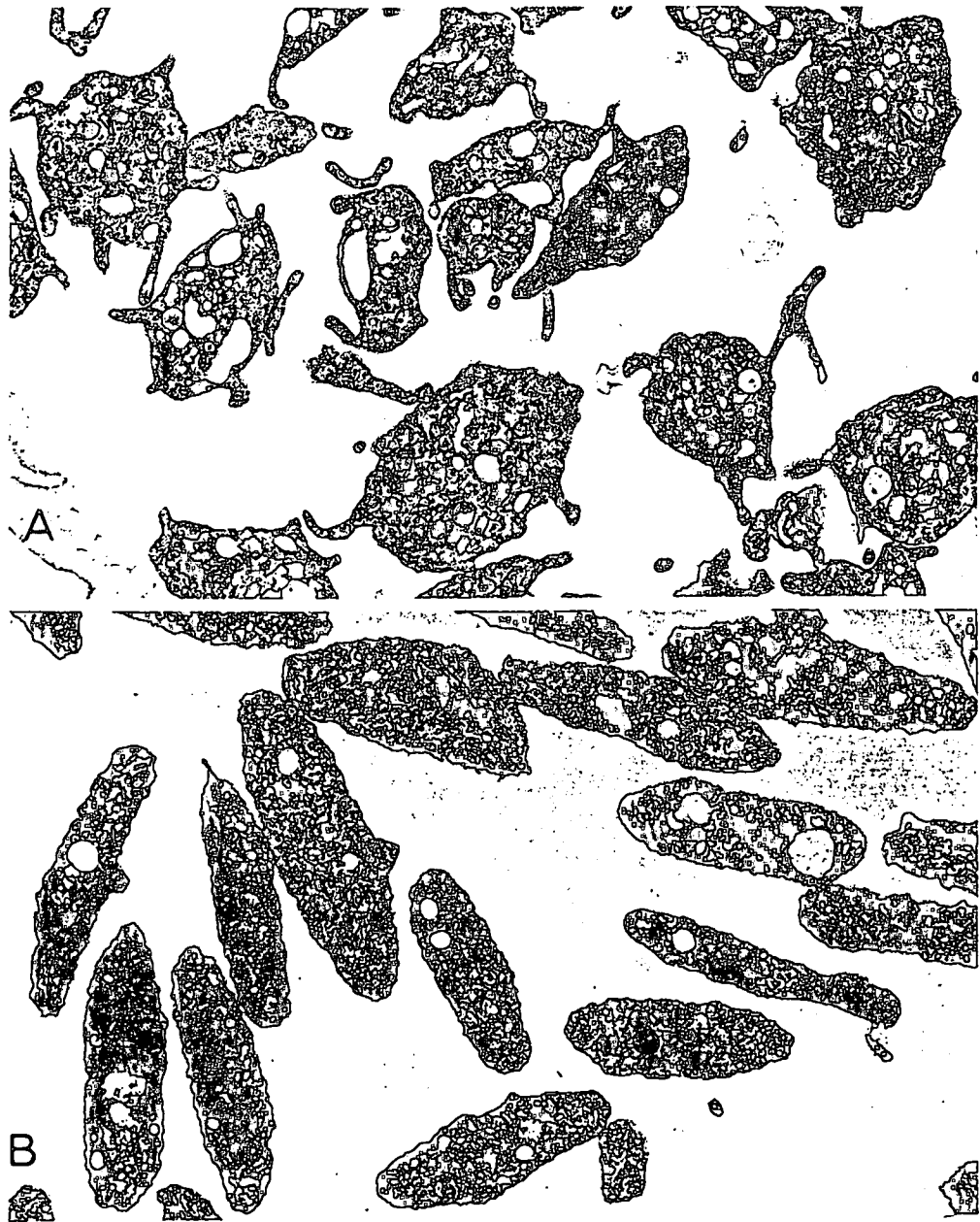


Fig 1. (A) Indium-111 labelled platelets immediately before injection. Platelets are discoid with normal distribution of granules. Surface projections are present and the canalicular system dilated. ($\times 11\,500$, enlarged). (B) Platelets after circulating for 20 h through a biological model. Surface projections are inconspicuous with some dilatation of the canalicular system. ($\times 11\,500$, enlarged).

Table I. Subject and platelet survival data

Dog	Indium-111 injected MBq (μ Ci)	Indium-111-labelled platelets injected ($\times 10^9$)	5 min recovery of In-111 in blood (%)	Platelet survival (h)		
				Weighted mean	Gamma function	'Hits'
1. Filter A	29.23 (792.4)	18.2	55	183	189	87
2. Filter B	26.58 (718.5)	22.3	49	157	162	100
3. Filter C	29.49 (795.0)	19.8	50	137	146	80
Mean	28.49 (768.6)	20.1	51.3	159	166	89
± 1 SD	1.63 (43.4)	2.07	3.2	23	22	10
1. Donor A	4.99 (134.6)	Unknown	50	171	167	17
2. Donor B	3.93 (106.2)	Unknown	45	133	139	34
3. Donor C	4.98 (134.5)	Unknown	43	134	134	28
Mean	4.63 (125.2)	Unknown	46.0	146	147	26
± 1 SD	0.6 (16.4)	Unknown	3.61	22	18	19

platelets (measured in donor dogs) was longer, but the difference was not statistically significant when compared with Student's *t*-test for paired data ($0.05 < P < 0.1$).

Platelet survival curves fitted to a gamma function model are depicted graphically in Fig 2. The mean curve for the filtered platelets fits a linear function better than that of the unfiltered platelets.

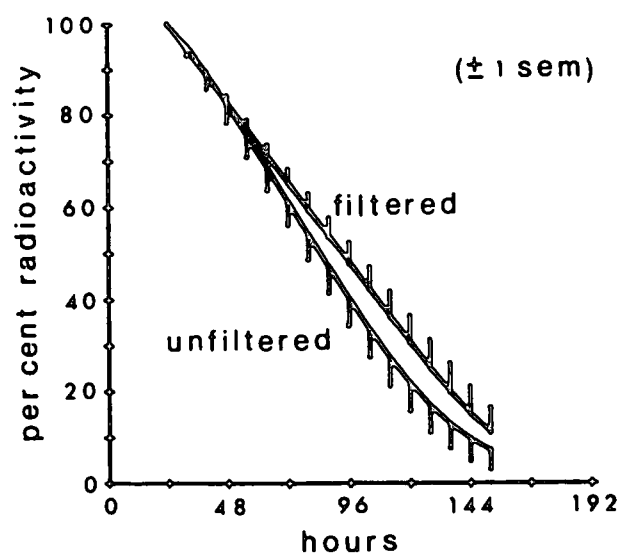


Fig 2. Platelet survival curves generated by computer fitting of the data to a gamma function. The curve for the filtered platelets is linear whereas that for the unfiltered platelets is curvilinear.

Table II. Equilibrium in late *in vivo* organ In-111 distribution

Dog	Heart		Liver		Spleen		Whole body	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Filter A	3.3†	0.7	29.9	37.3	38.3	52.2	100	92.5
Donor A	2.4	0.5	27.8	37.4	45.1	60.1	100	83.2
Filter B	5.3	1.4	19.4	22.6	36.0	43.2	100	84.4
Donor B	4.3	1.4	20.8	33.3	37.0	38.7	100	87.9
Filter C	4.1	1.7	22.3	27.5	42.7	47.1	100	91.6
Donor C	7.1	1.3	28.4	38.4	28.3	31.0	100	84.0
Mean filter	4.2	1.3	23.9	29.3	39.0	47.5	100	86.2
± 1 SD	1.0	0.5	5.4	7.7	3.4	4.5	—	4.8
Mean donor	4.6	1.0	25.7	36.0	36.8	43.3	100	85.1
± 1 SD	2.4	0.5	4.2	2.6	8.4	15.1	—	2.7
P-value*	0.45 > P > 0.40	0.15 > P > 0.10	0.37 > P > 0.25	0.2 > P > 0.15	0.4 > P > 0.35	0.35 > P > 0.30	—	0.20 > P > 0.15

* Student's t-test: the means of initial (equilibrium) and final (post sequestration) values of the filter and donor pairs were compared.

† Organ radioactivity is expressed as a percentage of initial whole body In-111-activity.

This difference in the shape of the curves is also reflected by the statistically significant ($P < 0.05$) greater number of 'hits' in the gamma function model calculated for the filtered (89 ± 10) compared to that of the unfiltered platelets (26 ± 9).

In vivo distribution of ^{111}In -labelled platelets in the donor and filter dogs

Radioactivity in the heart, liver and spleen expressed as a percentage of whole body radioactivity quantitated at equilibrium and at the end of platelet lifespan is given in Table II. Both the early and final splenic radioactivity was slightly higher in the filter dogs and the liver activity higher in the donor dogs, but these differences were not statistically significant ($P < 0.10$, $P < 0.30$ respectively).

DISCUSSION

The use of In-111 oxine as a platelet label, in conjunction with a scintillation camera interfaced with a computer-assisted imaging system, allows an exciting new approach to the study of platelet kinetics. The *in vivo* distribution of platelets can be quantitated accurately and the technique can also be applied to the study of diseases associated with abnormal platelet utilization and destruction. However, the viability of the labelled platelets is critical

for the success and interpretation of quantitative studies. The technique of labelling platelets is known to damage platelets. It is important to know the nature and extent of the so-called 'collection injury' and how it influences the results of quantitative studies. *In vitro* platelet function studies are of limited value because normal platelet aggregation curves have been reported when only 10% of the test platelet population is functionally normal (Cerskus *et al.* 1980). These considerations led to the exchange experiment in which the platelets damaged by the labelling technique were removed by filtering the labelled platelets through a biological model.

The labelled platelets showed ultrastructural signs of activation which was less evident in the filtered platelets (Fig 1). This finding is in accord with the view that manipulated platelets may recover after they are returned to the circulation (Ebbe, 1971), but may of course also be ascribed to removal of the severely damaged subpopulation from the circulation.

The mean platelet survival of the filtered platelets was slightly longer than that of unfiltered platelets, but the difference was not statistically significant.

This indicates that the labelling procedure does not affect platelet lifespan significantly. The labelling procedure may, however, influence the shape of the survival curve (Fig 2): that of the filtered platelets is linear and that of the unfiltered platelets curvilinear. This is also reflected by the difference in the calculated number of 'hits' sustained by the platelets before removal from the circulation. This finding should, however, be interpreted with caution. The multiple-hit model of analysis of platelet survival may not be entirely physiological and analysis of the shape of the survival curve as described by the gamma function model may thus not be accurate. Heparin disappearance from the blood has been studied by a general compartment, non-linear differential model (Simon *et al.* 1978). This model also allows description of the curve shape and may be more suitable than the multiple hit model for this purpose. However, data on platelet kinetics examined with this technique to our knowledge has not been published.

It is to be expected that irreversible damage to some of the labelled platelets may cause a different equilibrium *in vivo* distribution pattern of the filtered and unfiltered platelets since it is known that damaged platelets are rapidly removed by the liver (Goodwin *et al.* 1978; Saba, 1970; Kaplan & Saba, 1978). Our results showed no such differences (Table II). The *in-vitro* labelling procedure that we employ thus does not seem to influence the distribution and localization of In-111-labelled platelets significantly.

These results demonstrate that our techniques of labelling platelets with In-111-oxine does not damage platelets permanently and that the 'collection injury' is reversible. In-111-labelled platelets are thus suitable for computerized scintillation camera studies and the results of quantitative studies are valid. The labelling procedure may have a slight effect on the shape of platelet survival curves and this should be taken into account when platelet survival data are interpreted.

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KINETICS, REDISTRIBUTION, AND SITES OF SEQUESTRATION OF
NORMAL PLATELETS

H. F. Kotzé, P. Wessels, and H. Pieters

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I. INTRODUCTION

Blood platelets were first described by Donné in 1842 as "globules" in the circulation. Since the classical monograph by Bizzozero in 1881¹ much attention has been given to the function of platelets. Platelet kinetics, the term to describe the production, distribution, survival, and sites of destruction, could be studied reliably only after an efficient procedure to label platelets with ⁵¹Cr-disodium chromate was developed in the 1950s and 60s.²⁻⁷ The cornerstone of early studies of platelet kinetics was the determination of the disappearance curve of ⁵¹Cr-labeled platelets from the blood.³⁻⁸ In isolated cases, surface counting of organ radioactivity was also done.³⁻⁶ In this regard, Aster and Jandl,³ Kotilainen,⁵ and Davey⁶ contributed much to the understanding of the *in vivo* distribution and sites of sequestration of ⁵¹Cr-labeled platelets. Harker and Finch⁷ added significantly to this when they elucidated thrombokinetis in humans in detail.

In 1976, ¹¹¹In-oxine was introduced as a new and superior platelet label.⁹ Since then, ¹¹¹In has become the label of choice, mainly because of its high labeling efficiency, appropriate half-life, and high gamma photon yield. Heyns et al.¹⁰ exploited these characteristics by applying the technology of the scintillation camera and computer-assisted image analysis to visualize and quantify the *in vivo* distribution and sites of sequestration of ¹¹¹In-labeled platelets. This group later refined the method of quantification to measure the *in vivo* platelet distribution more accurately.¹¹ Thus, although platelets were discovered over a century ago, the study of platelet kinetics is relatively young.

Platelet production and survival are reviewed in detail in other contributions in this volume. A brief description of these, however, is necessary for the purposes of understanding platelet kinetics in the context of the present review. After this, platelet kinetics will be discussed under the headings of platelet distribution, platelet sequestration, and the fate of the radio-nuclide after platelet sequestration.

Blood platelets are produced by the megakaryocytes of the bone marrow to replenish utilized circulating platelets.¹² There is convincing evidence suggesting that platelet production is regulated by a humoral factor, thrombopoietin,¹³ maintaining a relatively constant peripheral blood platelet count.¹⁴ Thrombocytopenia stimulates the megakaryocytes to produce more platelets.¹⁵ The feedback mechanism that suppresses further platelet production, thus maintaining a normal platelet count, is unknown.¹²

Platelet survival is that aspect of platelet kinetics that has been studied most intensively. Much useful information has been derived from the study of the shape of the clearance curve of labeled platelets from the circulation.^{3-8,16} In normal subjects, platelets are removed from the blood by an intrinsic mechanism related to platelet senescence.⁸ The survival curve of a randomly labeled platelet population isolated from the blood thus fits a linear function best. Disappearance of labeled platelets from the circulation is, however, never strictly linear. This may be because a fraction of the platelets are randomly utilized to maintain vascular integrity.¹⁷ This curvilinear disappearance may also be explained by biological variations in the intrinsic life span of the individual members of the platelet population.^{3,8} When platelets are removed from the circulation by a mechanism related to their hemostatic function, or are destroyed by an extrinsic (i.e., immunologic) mechanism, the survival curve fits an exponential function best. This is due to the random removal of platelets of all ages from the circulation.⁸

II. PLATELET DISTRIBUTION

The *in vivo* distribution of viable platelets in the body is best assessed shortly after reinjection of the labeled platelets when equilibrium has been reached.

The attainment of equilibrium between injected labeled platelets and circulating platelets

is influenced by a number of events: the mixing of the labeled platelets with circulating platelets; the size of the exchangeable splenic platelet pool; sequestration of irreversibly damaged platelets from the circulation in the reticuloendothelial system (RES); and re-entry of reversibly damaged platelets from the RES to the circulation.^{3,5,6,18-20} In vitro platelet handling also influences the attainment of equilibrium. Storage of platelets at 4°C for 24 hr results in an increased permanent sequestration,²¹ as does in vitro treatment of platelets with thrombin.²² The anticoagulant used to collect blood for platelet labeling is also important.^{3,5,6}

Thus, following intravenous injection of the labeled platelets as a bolus, there is a rapid increase in circulating radioactivity. Equilibrium radioactivity in the circulation would be reached within 2 to 3 min in the absence of an extravascular platelet pool.^{5,6} Thus, after complete mixing of labeled platelets with those in the circulation, pooling of platelets results in a continuous decrease in blood radioactivity, lasting for approximately 10 min. Usually blood radioactivity then increases 3 to 5%. This increase is ascribed to re-entry of reversibly damaged platelets from the RES to the circulation.^{3,5,6,8,19,23}

Changes in surface counts, reflecting organ radioactivity, have been studied with ⁵¹Cr-labeled platelets. Aster,^{3,4,19} Kotilainen,⁵ and Davey^{6,23} found that maximum splenic radioactivity is attained approximately 15 min after bolus injection of labeled platelets. Thereafter, splenic radioactivity remained relatively constant, or fluctuated markedly for up to 2 hr.^{5,6,23}

After injection of ⁵¹Cr-labeled platelets, liver radioactivity also increased, and this increase was more pronounced when EDTA (Ethylenediaminetetraacetic acid), rather than acid-citrate-dextrose was used as anticoagulant.^{3,6} The labeled platelets accumulate only temporarily in the liver. This is demonstrated by the finding of a subsequent decrease in hepatic radioactivity.³ A steady state is attained approximately 15 min after injection.^{5,6,23} The transient uptake of labeled platelets in the liver was initially termed "transient segregation",^{5,6} but is now known as "transient sequestration".^{8,10,16} The uptake and release of the labeled platelets is ascribed to the "collection injury" sustained by the platelets during in vitro labeling.^{3,8,10}

Although the organ and blood radioactivity tends to stabilize after 15 min, slight changes may be observed for up to 24 hr following the reinjection of the labeled platelets.^{3,8,19,20,23} It is therefore apparent that in vitro handling causes reversible "damage" to the platelets and that the time for recovery of these platelets may vary significantly.

The dynamic changes observed until equilibrium between pooled and circulating radioactivity is reached, are illustrated in Figure 1. Mean radioactivity/time curves for ¹¹¹In-labeled platelets and ¹¹¹In-labeled red blood cells observed in six normal humans are shown. The heart, liver, and spleen were imaged anteriorly with a scintillation camera. Images were acquired every 30 sec for the first 15 min after reinjection and then every 3 min for the following 75 min. A region of interest was selected for each of the heart, liver, and spleen, and the mean organ count rate (counts per pixel per minute) determined. The mean count rates were normalized to the percentage of whole body radioactivity present in the organ at equilibrium. The distribution at equilibrium was quantified with the geometrical mean method.¹¹ These techniques are fully described elsewhere in this volume (Appendix 3).

Heart—Heart radioactivity decreased rapidly to a steady state of approximately 8% of whole body radioactivity 16 to 20 min after reinjection of the ¹¹¹In-labeled platelets (Figure 1A). Moreover, the rate of decrease of radioactivity over the heart was the same as that of the blood (Figure 1B). The blood radioactivity decreased to a nadir approximately 20 min after reinjection. This was followed by an increase of approximately 5% and then remained constant. These exchanges in blood radioactivity are due to a transient sequestration of the platelets reversibly damaged by in vitro handling.^{3,5,8,10,24}

Spleen—Splenic radioactivity increased as a single exponential function at a rate constant of $0.177 \pm 0.057/\text{min}$. Equilibrium was reached approximately 20 min after reinjection of

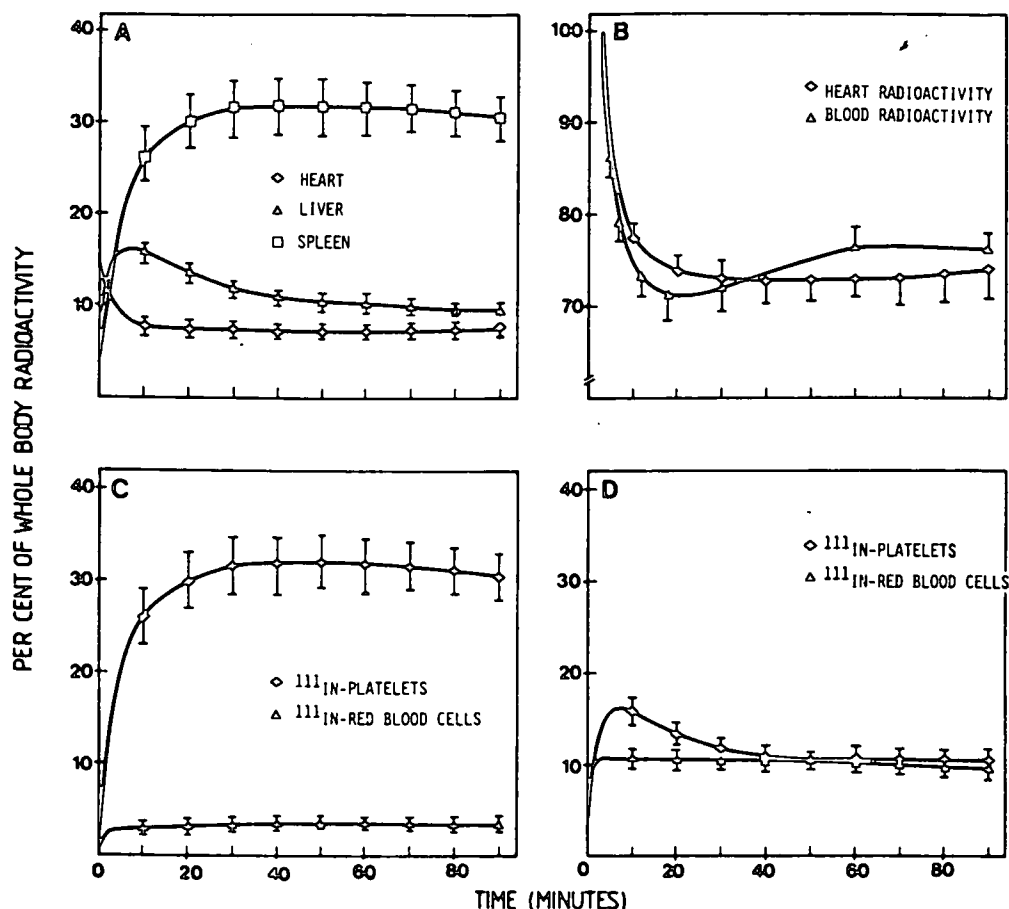


FIGURE 1. The distribution of ^{111}In -labeled platelets in normal humans. (A) Pooling of platelets in the spleen and transient hepatic uptake is evident; (B) Blood and heart radioactivity changes occur in parallel. A comparison between the distribution of ^{111}In -labeled platelets and ^{111}In -labeled red blood cells (C) in the spleen and (D) the liver clearly demonstrates the different handling of the two blood components by these organs. Radioactivity is expressed as a percent of whole body radioactivity, mean $\pm$ 1 SEM.

the ^{111}In -labeled platelets. Spleen ^{111}In activity at this time was $31 \pm 6\%$ of whole body radioactivity (Figure 1A). As is evident in Figure 1C, the spleen handles platelets and red blood cells differently. In contrast to the relatively slow accumulation of ^{111}In -labeled platelets, ^{111}In -labeled red blood cells reached an equilibrium (3 to 4% of whole body radioactivity) approximately 4 min after reinjection.

Liver — Liver radioactivity also increased rapidly to a maximum of approximately 16% of whole body radioactivity 6 to 8 min after reinjection of the ^{111}In -labeled platelets. This was followed by a gradual decrease to a steady state of 9 to 10% of whole body radioactivity, reached after approximately 45 min (Figure 1A). This transient accumulation of ^{111}In -labeled platelets is illustrated in Figure 1D. In contrast to that of the platelets, the hepatic ^{111}In -labeled red blood cell concentration was maximal after 3 to 4 min and then remained relatively constant, at approximately 10% of whole body radioactivity. The steady state hepatic radioactivity of labeled platelets and red blood cells is virtually the same. Thus, it appears that the ^{111}In -labeled platelets in the liver are in the perfusing blood and not in a pool as has been suggested.^{10,16,25}

The radioactivity/time curves in Figure 1 demonstrate that the spleen and liver are important determinants of equilibrium distribution of labeled platelets: the spleen because it is a platelet

pool and the liver because it is a site of transient accumulation of reversibly damaged platelets.

Although various investigators have studied the *in vivo* distribution of ^{111}In -labeled platelets,^{10,25-28} the mechanism of platelet pooling in the spleen remains unknown. This aspect is discussed in detail elsewhere in this volume and will only be referred to in general terms.

The transit time of platelets through the spleen is 8 to 10 min,^{16,19,25,26} and the rate constant of pooling of labeled platelets in the spleen is between 0.135 and 0.177/min.^{10,25} There is a direct relationship between the size of the spleen and the platelet pool; significantly more platelets are pooled in splenomegaly.^{19,20,29,30}

The transient accumulation of reversibly damaged platelets by the liver suggests that this organ recognizes minor alterations in platelets, removes them from the circulation, and releases them after recovery. This phenomenon has also been described after the administration of protamine sulfate.³¹ The thrombocytopenia induced by hypothermia is also associated with transient accumulation of platelets in the liver.^{32,33} However, this capacity of the liver seems limited.¹⁸ The mechanisms of transient hepatic clearance of damaged platelets is unknown. It is possible that activated platelets are retained in the liver until they recover. This may be influenced by the pH of the labeling medium. A pH between 5.5 and 6.5 prevents surface-platelet and platelet-platelet interactions.^{3,34} Adequate acidification therefore minimizes the transient accumulation of ^{51}Cr -labeled platelets in the liver.³ The slight activation of platelets labeled with ^{111}In -oxine in saline is reversed by incubating the product for 30 min at 37°C in autologous plasma.³⁵ *In vitro* activated platelets will recover from the "collection injury" caused by labeling in Ringers-citrate-dextrose, after reinjection into the circulation.³⁶ It is important to note that this minor degree of platelet activation does not influence the *in vivo* and *in vitro* function of labeled platelets.^{10,34-38}

It is known that platelets attach to the macrophages of the spleen and these adhesive properties may delay their transit through this organ.^{26,39} Therefore, one may speculate that activated platelets attach to a receptor on the Kupffer cell membranes. This happens with serum albumin microparticles.⁴⁰ It is also possible that the expression of the glycoprotein-receptors on the membrane of activated platelets facilitates this temporary platelet attachment.^{41,42} After a time, which depends on the degree of activation, the platelet membranes may lose their stickiness and the platelets will return to the circulation.

This transient platelet sequestration may also be mediated via activation of the complement system. C5b-9 complexes form on the membranes of activated platelets and the alternate complement pathway is critical.⁴³ Acidification of serum activates the C3-complement system.⁴⁴ Complement activation is not unique to platelets. A similar mechanism is known to cause temporary accumulation of red blood cells in the liver.^{45,46}

Equilibrium of reinjected labeled platelets is reached after 30 to 60 min.^{5,6,10,25,37} It is well known that approximately two thirds of reinjected labeled platelets are recovered in the circulation. The remainder are present in an exchangeable splenic platelet pool. The number of platelets present in the different organs may be determined semiquantitatively with a scintillation probe.^{3-6,21,47} A more quantitative approach is to determine the radioactivity in human spleens removed surgically or organ radioactivity of sacrificed animals.^{29,37,47}

Significant progress was made with the introduction of the ^{111}In -labeling technique which permits *in vivo* imaging (Figure 2) and quantification of platelet distribution.^{10,11} The interpretation of results of *in vivo* quantification of organ radioactivity is critically dependent on the viability of the ^{111}In -labeled platelets. The organ radioactivity value is a composite of viable pooled and/or circulating platelets on the one hand, and irreversibly sequestered platelets on the other hand. The separation of these two components may be done by deconvolution analysis.¹⁶ There are also important pointers that will indicate whether labeled platelets are viable or not. *In vitro* aggregation of the labeled platelet population must be normal.¹⁰ The recovery of labeled platelets in the circulation is a more important indicator.^{10,16,24,34}



FIGURE 2. An anterior whole body scan of the distribution of ¹¹¹In-labeled platelets at equilibrium. Pooling of platelets in the spleen and blood flow in the liver, heart, and large vessels are prominent.

To our knowledge, the effect of the "collection injury" and possible irreversible damage to ¹¹¹In-labeled platelets on platelet kinetics, morphology, and function has been investigated in only two studies. Badenhorst et al.³⁸ compared the survival, in vivo distribution, and

Table 1
THE DISTRIBUTION OF ¹¹¹IN-LABELED
PLATELETS IN THE BODY AT EQUILIBRIUM

Species	n	Recovery in the circulation (%)	Equilibrium organ radioactivity ^a		Ref.
			Spleen	Liver	
Humans	6	72 ± 16	26 ± 10	16 ± 2	10
	8	57 ± 11	38 ± 10	13 ± 4	49
	13	57 ± 11	35	12	50
	6	69 ± 5	31 ± 6	10 ± 1	51
	5	70 ± 21	42 ± 7	9 ± 2	52
Mean ^b	25	64 ± 14	34 ± 7	12 ± 4	
Baboons	6	87 ± 6	17 ± 3	16 ± 2	51
	10	80	10	13	53
Mean ^b	6	87 ± 6	17 ± 3	16 ± 2	
Dogs	5	45 ± 23	35 ± 4	20 ± 4	27
	6	48 ± 4	38 ± 6	25 ± 5	38
Mean ^b	11	47 ± 16	37 ± 5	23 ± 5	
Rabbits	9	75 ± 8	5	30	28

^a Per cent of whole body radioactivity, mean ± 1 SD.

^b The mean was calculated from the above quoted results.

sites of sequestration of canine platelets that were freshly labeled with ¹¹¹In-oxine with those "filtered" in a matched dog. The biologic filter served to remove platelets damaged by the labeling procedure. The mean platelet life spans of the two groups did not differ significantly, and the in vivo distribution and sites of sequestration of filtered and unfiltered platelets were the same. In the other study, Schmidt et al.³⁶ found no apparent functional, morphologic, and morphometric differences between labeled and unlabeled platelets. Moreover, after reinjection of the labeled platelets into rabbits some of the morphologic changes were reversed. This reversal was similar to that seen when labeled platelets are incubated in plasma before reinjection into the recipient.^{34,35,48}

It may therefore be concluded that careful isolation and labeling of platelets will not have an adverse influence on the in vitro and in vivo behavior of platelets.

A relatively simple, indirect method to assess irreversible damage to labeled platelets is by measuring their recovery in the circulation.^{8,10} Low recovery values in subjects with normal-sized spleens indicate permanent sequestration of irreversibly damaged labeled platelets. This phenomenon is illustrated in the study by Goodwin et al.²⁴ Platelets that were labeled in a saline medium had a low recovery (5.4% ± 5.5%) in the circulation and the damaged platelets were rapidly sequestered by the liver. The interpretation of organ ¹¹¹In radioactivity is thus impossible and misleading.

The in vivo distribution of ¹¹¹In-labeled platelets has been quantified in a variety of species (Table 1). Although different methods of quantification were used, the results are in reasonably close agreement. It should be noted that in three studies,^{10,27,38} in vivo quantification was estimated from anterior scintillation camera images. This method underestimates splenic radioactivity and overestimates hepatic radioactivity.^{11,27} The size of the splenic pool and the fraction of whole body radioactivity in the liver differ from species to species (Table 1). The physiological significance of these differences is not entirely clear and cannot be readily explained. It may be the result of histological differences, the size of the organs, or blood flow to these organs.^{5,19,20,29,34,35}

At equilibrium approximately 12% of the whole body ¹¹¹In radioactivity was quantified in the liver of normal humans (Table 1), a value comparable with the 10 to 14% blood

Table 2
THE EFFECT OF
INTERMITTENT PLATELET
PHERESIS (12 PASSES PER
SUBJECT) ON THE SPLENIC
PLATELET POOL IN 4 NORMAL
HUMANS

	¹¹¹ In-labeled platelets ^a	
	Whole body	Spleen
Prepheresis	100.0 ± 0.0	32.1 ± 5.5
Postpheresis	65.4 ± 5.0	20.9 ± 5.2
% Difference	34.6 ± 5.0	34.9 ± 6.5
Postpheresis ^b	100.0 ± 0.0	30.9 ± 5.9

^a Per cent of whole body radioactivity. Mean ± 1 SEM.

^b The % ¹¹¹In-labeled platelets in the spleen is expressed as a percent of whole body radioactivity remaining after pheresis.

reservoir of the liver.^{56,57} The labeled platelets present in the liver thus reflect that in the perfusing blood pool. This view is strengthened by the radioactivity/time curves for ¹¹¹In-labeled platelets and red blood cells (Figure 1D). It has also been shown in rats that, 2 hr after reinjection, the distribution of ⁵¹Cr-labeled platelets and red blood cells is similar.⁴⁷

The mechanism by which platelets are pooled in the spleen is unknown. It has been proposed that platelets attach to the macrophages of the spleen and that this may delay their transit through this organ.^{37,39} It is, however, known that platelets in the splenic pool are exchangeable and in equilibrium with circulating platelets. Adrenalin, in a pharmacological dose, causes an increase in the peripheral blood platelet count and number of radiolabeled platelets.^{19,20,29,30} There is a corresponding decrease in splenic radioactivity.^{19,29} Ten to fifteen minutes after discontinuing a constant infusion of adrenalin, the labeled platelets have redistributed to baseline values.¹⁹

The exchange between platelets pooled in the spleen and those in the circulation is illustrated in Table 2. Platelets from four normal humans were labeled with ¹¹¹In-oxine and reinjected. After 18 hr, quantification of the organ distribution of the labeled platelets was done with the geometrical mean method.^{11,58} Platelets were then removed by intensive intermittent platelet pheresis. After 12 passes on a Haemonetics® cell separator whole body radioactivity was again quantified. Prior to platelet pheresis, the splenic pool consisted of approximately 33% of the total ¹¹¹In-labeled platelets. Platelet pheresis decreased whole body radioactivity by approximately 35%, equivalent to the decrease in blood radioactivity (35.5 ± 7.4%). This was accounted for by the 30.9 ± 3.0% of labeled platelets removed by the pheresis procedure. Spleen radioactivity also decreased by approximately 35% during pheresis. This was equivalent to the decrease in whole body radioactivity. After pheresis, the splenic platelet pool was approximately 31% of whole body ¹¹¹In-labeled platelets. The relative sizes of the pre- and postpheresis pools did not differ significantly ($p > 0.10$). This demonstrates the dynamic equilibrium between platelets in the spleen and those in the circulation.

The size of the splenic platelet pool, quantified in vivo with ¹¹¹In-labeled platelets, is 34 ± 7% in normal humans. This estimate corresponds to that determined from the recovery of ¹¹¹In-labeled platelets in the circulation, approximately 36% (Table 1). This finding is not in support of the suggestion that there is a nonsplenic platelet pool in normal humans.^{6,30,59}

The evidence for a nonsplenic platelet pool was based on the finding that exercise or the infusion of adrenalin into asplenic persons increased the number of circulating platelets significantly. This increase was of the same extent as that in normal subjects.^{19,29,30,50} However, in normal humans and rats the increase in circulating ⁵¹Cr-labeled platelets was isochronous with a decrease in splenic radioactivity.^{6,19}

The site of the nonsplenic platelet pool is unknown. The lungs have been suggested as a possible source,^{60,61} but contradictory results have been reported.^{10,47} Our experience with dual isotope scintillation camera imaging of ¹¹¹In-labeled platelets and ^{99m}Tc-labeled red blood cells, injected simultaneously into humans, could demonstrate no excess platelets in the lungs.⁷⁹ It has also been proposed that there is a marginal vascular pool of platelets³⁰ and that this is mobilized by exercise or infusion of adrenalin. This view suggests that some platelets are distributed in an analogous manner as granulocytes.⁶²

III. PLATELET SEQUESTRATION

Platelets normally have a life span of 9 to 11 days in humans,^{8,10,16,34,50} 5 to 7 days in dogs,^{27,38,63} 5 to 6 days in baboons,⁶⁴⁻⁶⁶ approximately 3 days in rabbits,^{34,67} and 3 to 4 days in rats.^{34,47} In those studies where random samples of platelets are labeled with a radio-nuclide,^{8,16,34,35} platelets of all ages are tagged. Therefore, assuming a mean platelet life span of 10 days in humans, sequestration of platelets will commence on the day of injection and continue at a constant rate of about 10% per day.

In this review platelet sequestration may be defined as the final irreversible accumulation of nonviable platelets in an organ or region. Organ radioactivity will be the sum total of labeled platelets present in the blood pool, a true platelet pool, and those platelets sequestered. It is not possible to separate these components by simple quantitative imaging. The number of labeled platelets sequestered in an organ can thus only be determined by quantification of organ radioactivity at the end of platelet life span. There are however mathematical procedures available for separating pooling and sequestration of organ ¹¹¹In radioactivity.¹⁶

The sites of sequestration of labeled platelets may be determined by different methods. In earlier studies in humans, sequestration of ⁵¹Cr-labeled platelets was studied by external counting of organ radioactivity with a scintillation probe positioned over the organ concerned. Attempts have been made to obtain quantitative data with this technique. Some investigators reported the count rate over the liver and spleen as a fraction of the injected platelet-bound ⁵¹Cr radioactivity^{3,68} and others related the organ count rate to precordial radioactivity.^{6,23} The organ count rate has also been expressed as a fraction of whole body radioactivity.⁶⁹ Other approaches to quantify organ radioactivity have been to express the spleen/heart and liver/heart radioactivity ratio,^{29,70,71} or the spleen/liver ratio^{68,72} or determining the excess count rates in organs.⁵ The potential errors inherent in these approaches have been discussed.⁵

The determination of the sites of platelet sequestration with ¹¹¹In-labeled platelets has been facilitated by the application of the technology of the scintillation camera and computer-assisted image analysis.^{10,27} This technique was later applied and modified by various investigators.^{11,28,38,50-54} These different methods are discussed in detail elsewhere in this volume.

Results obtained with ¹¹¹In-labeled platelets and the geometrical mean method of quantification in six normal humans, is graphically illustrated in Figure 3. Mean whole body radioactivity decreased by approximately 3% at the end of the platelet life span (9.4 ± 1.0 days), this was not significant. The radioactivity in the liver and spleen increased significantly ($p < 0.03$) during the platelet life span. Similar results have been reported for ¹¹¹In-labeled platelets in baboons⁵¹ and ⁵¹Cr-labeled platelets in rats.⁴⁷ However, increases in liver and spleen radioactivity in quantification studies with ¹¹¹In-labeled platelets have not been found by some investigators. Robertson et al.⁵² reported an increase in liver radioactivity but not

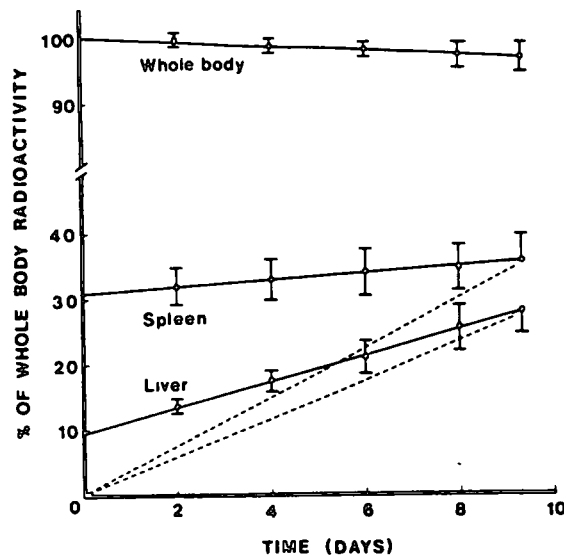


FIGURE 3. The redistribution of ^{111}In -labeled platelets during their survival in normal humans. Organ radioactivity is the sum total of circulating, pooled, and sequestered platelet radioactivity (solid line). Sequestered platelet radioactivity is illustrated by the broken lines. Only at the end of platelet survival does organ radioactivity represent only platelet sequestration.¹⁶ Values are expressed as a percentage of whole body radioactivity ± 1 SEM.

in the spleens of normal humans. Other investigators failed to demonstrate increases in either liver or spleen radioactivity in normal humans^{49,50} and rabbits.²⁸

The reason for these discrepancies is unknown, especially since there was good agreement in the estimates of the distribution of labeled platelets at equilibrium (Table 1). The conflicting results reported for the relative importance of the components of the RES to sequester senescent platelets may possibly be ascribed to the different methods of *in vivo* quantification of organ radioactivity. Only a few of these methods have been validated by comparing *in vivo* and *ex vivo* organ radioactivity.^{11,27,28}

In normal humans, ^{111}In -labeled platelets are mainly sequestered in the liver and spleen (Figure 4). At the end of the platelet life span, between 65 and 72% of the labeled platelets are sequestered in these two organs.^{10,51,58} The corresponding values reported for ^{51}Cr -labeled platelets vary between 60 and 85%.^{3,4,6,69,73} It was only after studies with ^{111}In -labeled platelets that the important role of the bone marrow in platelet sequestration became apparent. Heyns et al.¹⁰ showed that the bone marrow sequesters significant numbers of senescent platelets and this group later validated this finding in baboons.⁵¹ Similar results have been reported in rabbits.²⁸

Role of the spleen — The spleen is an important site of sequestration of senescent platelets and sequesters approximately 35% of ^{111}In -labeled platelets in normal humans (Table 3). This value is in close agreement with the estimates (30 to 55%) previously reported for ^{51}Cr -labeled platelets.^{69,73} The relative importance of the spleen to sequester platelets differs from species to species (Table 3). In normal dogs, most platelets are sequestered in the spleen, whereas in rabbits the spleen sequesters a relatively small number. In normal rats, approximately 44% of ^{51}Cr -labeled platelets are sequestered in this organ.⁴⁷

Role of the liver — The liver is regarded as an important site of platelet sequestration^{10,51,58} but there is some disagreement.^{49,50} In our study with baboons,⁵¹ the liver sequestered $35.2 \pm 6.5\%$ of the ^{111}In -labeled platelets. This was measured *in vivo* and confirmed by *ex vivo*

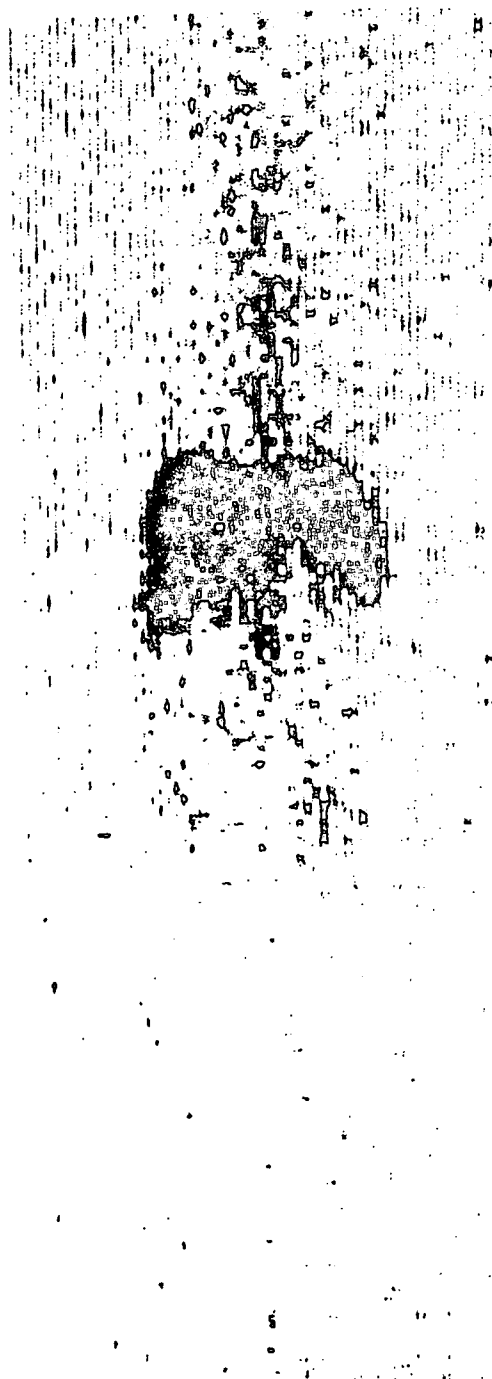


FIGURE 4. An anterior whole body scan demonstrating the sites of sequestration of ^{111}In -labeled platelets. The liver and spleen are prominent, and bone marrow radioactivity can be visualized.

determination of organ radioactivity. These values are in close agreement with hepatic radioactivity determined in normal humans.^{10,51,58} The importance of the liver as a site of platelet sequestration has also been confirmed by the finding that approximately 34% of

Table 3
THE SITES OF SEQUESTRATION OF
SENESCENT ¹¹¹IN-LABELED PLATELETS

Species	Organ ¹¹¹ In radioactivity at the end of platelet life span ^a			Ref.
	Spleen	Liver	Remainder	
Humans	33 ± 13	39 ± 9	28 ± 14	10
	35 - 40	20 - 30	35 - 40	50
	36 ± 10	29 ± 8	35 ± 7	51
Baboons	34 ± 3	35 ± 7	30 ± 5	51
	13	19	-	53
Dogs	59 ± 9	14 ± 4	26 ± 7	27
	45 ± 10	33 ± 6	22 ± 13	38
Rabbits	14 ± 4	40 ± 7	41	28

^a Percent of whole body radioactivity. Mean ± 1 SD.

⁵¹Cr-labeled platelets in rats⁴⁷ and approximately 40% of ¹¹¹In-labeled platelets in rabbits²⁸ are sequestered in this organ.

Role of the bone marrow — In rats, the bone marrow sequesters only about 3% of ⁵¹Cr-labeled platelets.⁴⁷ Only after the study with ¹¹¹In-labeled platelets by Heyns et al.¹⁰ did the importance of this organ as a site of platelet sequestration become apparent. The diffuse nature of the bone marrow makes accurate *in vivo* quantification of the radioactivity in this component of the RES virtually impossible. It has therefore been proposed that platelets not sequestered in the liver and spleen, i.e., 28 to 35%, should be regarded as sequestered in the bone marrow.^{10,51,58} It should however, be recognized that bone marrow sequestration is slightly overestimated by this approach. Thus, in baboons and rabbits (Table 4), 13 to 16% of ¹¹¹In-labeled platelets are not sequestered in either the spleen, liver, or the bone marrow. This component of platelets may be utilized to maintain vascular integrity¹⁷ or may be destroyed in the macrophages lying outside the three organs mentioned.

IV. SUBSEQUENT FATE OF THE RADIONUCLIDE

The extent to which organ radioactivity levels reflect platelet sequestration depends on: the capacity of the reticuloendothelial tissues to retain the radionuclide after ingestion of labeled platelets; the avidity with which the reticuloendothelial cells clear nonplatelet bound radionuclide from the circulation;⁵⁰ and the rate of excretion of nonplatelet bound radionuclide.⁴⁷ Ideally, therefore, the radionuclide should not elute or show a slow rate of elution from the reticuloendothelial cells after platelet phagocytosis.¹⁶

In rats, ⁵¹Cr elutes from the liver and spleen at rates of 3.3 and 2.9%/day respectively.⁴⁷ The elution rates in humans vary between 3 and 6%/day.^{3,74} The nonplatelet bound ⁵¹Cr is excreted by the kidneys.^{3,47}

Very little is known about the *in vivo* elution of ¹¹¹In from platelets and the ultimate fate of the radionuclide following platelet sequestration. The loss of ¹¹¹In from the whole body appears to be negligible, since whole body radioactivity does not decrease significantly during the platelet survival time.^{10,27,51,58} Interestingly, whole body ¹¹¹In radioactivity decreased significantly in asplenic subjects, a phenomenon that could not be explained by the authors.⁵⁴ Because very little ¹¹¹In is excreted in the urine and feces,^{24,53,75} and plasma ¹¹¹In radioactivity is low during the time that ¹¹¹In-labeled platelets are circulating,^{9,10,34} it may be concluded that the radionuclide is retained in the RES after phagocytosis of senescent platelets. This is shown by the results in Table 5. Venesection and drainage of blood have

Table 4
THE SITES OF SEQUESTRATION
OF SENESCENT ^{111}In -LABELED
PLATELETS IN BABOONS AND
RABBITS²⁸

Organ	^{111}In -labeled platelets sequestered (% whole body radioactivity, mean $\pm$ 1 SD)	
	Baboons	Rabbits ²⁸
Spleen	33.6 $\pm$ 4.1	14.3 $\pm$ 3.1
Liver	36.7 $\pm$ 7.1	40.0 $\pm$ 6.6
Skeleton	14.4 $\pm$ 1.7	27.5 $\pm$ 9.4
Muscle and skin	6.7 $\pm$ 1.4	—
Muscle	—	3.8 $\pm$ 1.4
Intestines	4.9 $\pm$ 1.6	—
Kidneys	2.5 $\pm$ 0.6	2.2 $\pm$ 0.6
Lungs	0.9 $\pm$ 0.6	1.0 $\pm$ 2.7
Heart	0.5 $\pm$ 0.2	—
Blood	0.5 $\pm$ 0.3	2.5 $\pm$ 0.9
Bone	—	3.9 $\pm$ 1.4
Total	100.7 $\pm$ 1.5	95.3 $\pm$ 11.8

Note: Five baboons were sacrificed and dissected 8 days after reinjection of the labeled platelets. The radioactivity in the organs were determined in a whole body counter and expressed as a percent of the sum total of radioactivity in all the organs.

Table 5
THE EFFECT OF EXSANGUINATION ON THE ^{111}In
RADIOACTIVITY DISTRIBUTION IN 5 BABOONS

Time	Percent of whole body radioactivity (mean $\pm$ 1 SD)		
	Whole body	Liver	Spleen
Prebleeding	100.0 $\pm$ 0.0	35.9 $\pm$ 3.5	32.8 $\pm$ 3.6
Postbleeding	99.1 $\pm$ 0.4	35.9 $\pm$ 4.0	32.2 $\pm$ 3.5
p-value*	<0.05	>0.10	>0.10

* Students *t*-test for paired data.

little effect on organ and whole body radioactivity. At the end of the platelet life span, less than 1% of the radioactivity is present in the blood. The radioactivity in the liver and spleen were retained by the reticuloendothelial cells and could not be eluted by the loss of blood. However, this does not mean that long term elution from the liver and spleen, similar to that reported with ^{51}Cr ,^{3,47} does not occur.

After clearance of heat-damaged ^{111}In -labeled red blood cells from the circulation, the radionuclide accumulated in the liver at a rate of 5%/day and was lost from the spleen at 2%/day.⁷⁶ Intravenously injected ^{111}In -chloride is taken up principally by liver and bone marrow, and to a much lesser extent by the spleen.^{77,78} This may explain why ^{111}In accumulated, although slowly, in the liver after clearance of the heat-damaged red blood cells.⁷⁶

The elution of ^{111}In from the spleen and redistribution to the liver may have an influence on the final organ sequestration patterns of senescent platelets. This aspect needs to be investigated further.

V. SUMMARY

The study of platelet kinetics provides information on the production, distribution, and destruction of blood platelets.

The in vivo distribution of reinjected labeled platelets is determined at equilibrium. This equilibrium is influenced by accumulation or pooling of platelets in the liver and the spleen. Normally, approximately one third of all platelets are pooled in the spleen of humans. Platelets in this pool are in equilibrium with circulating platelets and may be mobilized on demand.

The liver apparently removes defective or activated circulating platelets and this clearance may be permanent or transient. Reversible damage of platelets, whether sustained during labeling, after intravenous protamine sulfate administration, or hypothermia, accumulate temporarily in the liver. After recovery these viable platelets are released back into the circulation and will have a normal life span. The liver effectively sequesters severely damaged platelets.

As labeled platelets age under normal conditions in the circulation so they are selectively sequestered by the macrophages of the spleen, the liver, and the bone marrow. A number of platelets may also be utilized to maintain vascular integrity or may be sequestered by the macrophages lying outside the liver, spleen, and bone marrow.

The study of the in vivo distribution and sites of sequestration of labeled platelets is seriously affected by: the susceptibility of platelets to sustain damage during in vitro labeling and the inability of the in vivo quantification method to distinguish between ^{111}In -labeled platelets in the circulation, pooled ^{111}In -labeled platelets, and sequestered ^{111}In -labeled platelets. Both of these factors must be taken into consideration when the results are interpreted.

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SITES OF SEQUESTRATION AND RADIATION DOSE OF IN-111-LABELLED PLATELETS

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ABSTRACT

The sites of sequestration of In-111-labelled platelets were quantitatively determined in humans and baboons. Approximately 61% of the senescent platelets were sequestered by the macrophages of the liver and the spleen. In 5 baboons that were sacrificed 8 days after the injection of labelled platelets, about one-half of the non-hepatosplenic radioactivity was in the bone marrow. The remainder was evenly distributed in the rest of the tissues. Although relatively large amounts of In-111 were used for the platelet kinetic studies, the radiation dose to the body and organs was well within the safety limits for radiation.

INTRODUCTION

In normal humans, blood platelets have a mean life span of approximately 10 days, whereafter they are selectively sequestered by the reticuloendothelial system of the spleen, liver and bone marrow (1). Since a random population of platelets are labelled with In-111, senescent In-111-labelled platelets are removed from the circulation at a rate of approximately 10% per day, starting on the day of injection. Thus, during the time that labelled platelets are circulating, organ radioactivity represents a composite of circulating radioactivity, pooled radioactivity and sequestered radioactivity. It is only after labelled platelets are cleared from the circulation that organ radioactivity represents only sequestered radioactivity. The sites of sequestration of In-111-labelled platelets must therefore be determined at the end of the mean platelet life span. Sequestration may be defined as the final, irreversible accumulation of non-viable (senescent) labelled platelets in an organ or region. Heyns *et al* (2) was the first to use In-111-labelled platelets and the scintillation camera, interfaced with a computer-assisted imaging system, to quantify the *in vivo* distribution and sites of sequestration of platelets. Various other investigators soon followed (3 - 5). In later studies by Heyns and his associates, they refined their method of quantification, and it became possible to predict the percentage of labelled platelets in an organ to within 5% of its actual value (6 - 8).

The accurate quantification of the *in vivo* distribution and sites of sequestration of In-111-labelled platelets requires images with relatively high count rates. It is thus necessary to use relatively large amounts of In-111,

i.e. 15 - 23 MBq. This invariably increases the danger of radiation to the whole body and organs. In a recent report it was shown that the In-111-product may be contaminated with In-114M (9). This makes it even more important to determine the radiation dose that is received from In-111, and the contribution of In-114M to the total radiation dose. Since In-114M is a radionuclide with a half life of 49.5 days, the radiation from this radioisotope may increase the radiation danger considerably.

METHODS

Human Volunteers and Experimental Animals

Six healthy volunteers, aged 18 - 25 years, gave written consent to partake in the study. In addition, 11 healthy baboons (*Papio ursinus*), mean mass 17.6 ± 5.4 kg, were also studied. Both projects were approved by the Ethical Committee of the University of the Orange Free State. The baboons were sedated with intramuscular ketamine hydrochloride, 10mg/kg body mass, when necessary.

Platelet Labelling and Survival

Autologous blood were collected in acid-citrate-dextrose NIH, formula A (8.5:1.5) by a two syringe technique. A fully representative population of platelets were isolated from the blood, and labelled with In-111-oxine. These methods are described in detail elsewhere (1, 10). Platelet survival studies were done as recommended by the ICSH (11).

In vivo Quantification of the Distribution of Labelled Platelets

Imaging of the In-111-labelled platelets was done with a large field of view scintillation camera with scanning facilities. The images were analysed with a A²-MDS data processing system. The whole body and organ In-111-radioactivities were quantified with the geometrical mean method (12). In 5 baboons the in vivo distribution of the labelled platelets was quantified once a day for 11 days. The normal life span of baboon platelets is 5.8 ± 0.5 days (10). Thus, the in vivo redistribution of In-111 following sequestration of labelled platelets was studied.

Ex vivo Determination of the Sites of Platelet Sequestration

Five baboons were sacrificed 8 days after In-111-labelled platelets were injected. The baboons were deeply anaesthetized and exsanguinated by severing their femoral arteries. The baboons were dissected and the radioactivity in each organ determined as described previously (6). Organ radioactivity was expressed as a percentage of total radioactivity.

Radiation Dose

The radiation dose from 1MBq In-111 to the whole body and organs was calculated

from the results that were obtained from the quantification of the in vivo distribution of In-111-labelled platelets. The recommendations of the ICRP were followed (13). For purposes of comparison, the contribution of 0.08% contaminating In-114M to the radiation dose was also calculated.

RESULTS

Sites of Sequestration of Platelets

The results are given in Table 1. Remainder is the radioactivity that was not quantified in the liver and the spleen at the end of the mean platelet life span.

TABLE 1. The sites of sequestration of In-111-labelled platelets. Values are given as a mean percentage of whole body radioactivity $\pm$ 1 SD.

Organ	Humans (n = 6)	Baboons (n = 11)
Spleen	35.6 $\pm$ 9.7	23.3 $\pm$ 4.6
Liver	28.7 $\pm$ 8.3	37.6 $\pm$ 6.0
Remainder	30.3 $\pm$ 7.8	31.8 $\pm$ 6.3

Five baboons were sacrificed to determine the sites of sequestration of the platelets that were not sequestered by the liver and spleen. The results are given in Table 2.

TABLE 2. The distribution of In-111-labelled platelets in the 5 baboons that were sacrificed 8 days after the injection of labelled platelets.

	Quantification in exised organs	<u>In vivo</u> quantification
Whole body	100.0	100.0
Spleen	33.6 $\pm$ 4.1	32.8 $\pm$ 3.6
Liver	36.7 $\pm$ 7.1	35.9 $\pm$ 3.5
Skeleton	14.4 $\pm$ 1.7	-
Muscle and skin	6.7 $\pm$ 1.4	-
Intestines	4.9 $\pm$ 1.6	-
Kidneys	2.5 $\pm$ 0.6	-
Lungs	0.9 $\pm$ 0.6	-
Heart	0.5 $\pm$ 0.2	-
Blood	0.5 $\pm$ 0.3	-

The spleen, liver and bone marrow sequestered $84.4 \pm 3.6\%$ of the senescent labelled platelets. The remaining $15.5 \pm 4.0\%$ were sequestered in various other tissues. Splenic tissue sequestered $0.55 \pm 0.25\%$ of the labelled platelets per gram. The liver sequestered $0.10 \pm 0.07\%$ per gram.

The organ redistribution of In-111 after labelled platelets was sequestered was studied in 5 baboons. The relevant results are given in Figure 1. In-111 was excreted from the body with a half life of 60 days. The biological half life of In-111 in the spleen was 18 days.

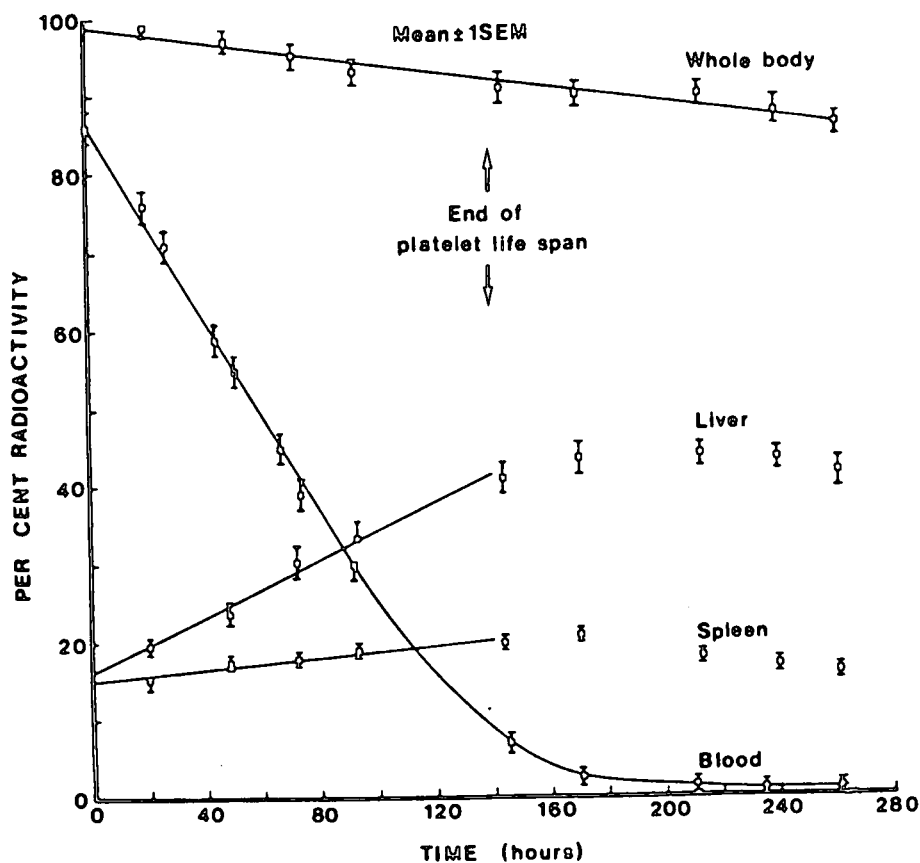


FIGURE 1. The organ redistribution of In-111 after the sequestration of In-111-labelled platelets.

Radiation Dose

The results of the in vivo distribution of radioactivity in the humans and the 5 baboons that were studied for 11 days were used to calculate the radiation dose (Table 3). The changes in whole body and organ radioactivity in the baboons were used to extrapolate the results to humans in order to calculate the radiation dose in humans.

TABLE 3. The radiation dose (mGy) received from 1 MBq In-111 and 0.8 Bq In-114M

	Humans (n = 6)			Baboons (n = 5)		
	In-111	In-114M	Total	In-111	In-114M	Total
Whole body	0.16	0.01	0.17	0.16	0.01	0.17
Spleen	7.77	0.48	8.25	4.41	0.24	4.65
Liver	0.69	0.11	0.80	1.10	0.16	1.26
Bone marrow	0.16	0.00	0.16	0.16	0.00	0.16
Gonads	0.11	0.00	0.11	0.13	0.01	0.14
Total weighted dose	0.64	0.04	0.68	0.45	0.04	0.49

In-114M increased the total weighted radiation dose with 6 to 8%. The increase in radiation to the liver, 15%, is of importance. The contribution to splenic radiation was approximately 5%.

DISCUSSION

The spleen and liver of normal humans sequestered approximately 64% of the In-111-labelled platelets; that of baboons 61%. These values agree with reported results (2 - 5). Hence, the macrophages of the spleen and liver are the major sites where senescent platelets are sequestered. On a weight basis, the spleen of baboons sequestered 5 to 6 times as many platelets as the liver.

The diffuse nature of the bone marrow makes it virtually impossible to quantify the number of platelets that is sequestered in this organ. Therefore, it was proposed that non-hepatosplenic sequestration, i.e. 26%, represents bone marrow sequestration (2, 12). This may be an overestimation. In the sacrificed baboons approximately one-half of the non-hepatosplenic radioactivity was present in the bone marrow. The remainder were distributed amongst the other tissues (Table 2). We have no ready explanation for this distribution of radioactivity. It is possible that these platelets were used to maintain vascular integrity (14), or were sequestered by the macrophages lying outside the spleen, liver and bone marrow. The lungs were not a site where senescent platelets was sequestered; only about one per cent of all the radioactivity was present in this organ (Table 2).

It is possible that post-survival redistribution of In-111 may affect the quantification of the sites of sequestration of In-111-labelled platelets. The

biological half life of In-111 in the spleen of the baboons was 18 days. However, the rate of elution of In-111 from the spleen was small. The resultant effect on the quantified values was therefore negligible. The elution does have a marked effect on the radiation dose of the spleen. Had the radionuclide been retained by the macrophages, the total radiation dose (In-111 + In-114M) to the spleen would have been 13% higher than the value given in Table 3.

In-114M contamination (0.08%) increased the total weighted radiation dose with approximately 6%. The biggest contribution was to the liver, i.e. 16%. The redistribution of the radionuclide (Figure 1) had little effect on the radiation from the shortlived In-111. The effect on radiation from the longlived In-114M was marked. If the radionuclide was retained by the macrophages, the total weighted radiation dose would have increased by 10%.

In summary, the macrophages of the spleen, liver and bone marrow are the major sites of sequestration of senescent platelets. On a weight basis, the spleen was the most effective. As a result, the spleen received the highest radiation dose. However, the radiation dose is still within the safety limits laid down by the ICRP(13). In fact, one can use up to 61 MBq In-111. Thus, the amounts of In-111 that are used, 15 to 23 MBq, may be regarded as safe for patients.

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4.14

Evaluation of Platelets and Thrombopoiesis

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4.14.1 INTRODUCTION

Blood platelets or thrombocytes are small cellular elements in the blood that originate from megakaryocytes in the bone marrow. They

circulate as disk-shaped fragments, 1–3 μm in diameter and in a concentration of $150\text{--}400 \times 10^9 \text{ L}^{-1}$. Although by far the smallest of all blood cells, platelets are biologically amongst the most active and play a pivotal part in the

maintenance of normal hemostasis and vascular integrity as well as in various thrombotic processes.

Several volumes, rather than a single chapter, would be required to do justice to this fascinating blood cell. At best this overview will be superficial and the reader is referred to the reviews indicated in the text. The chapter is divided into three parts: platelet kinetics, platelet morphology, and platelet function. Each part includes a section on the evaluation of the particular aspect of platelet physiology discussed.

4.14.2 PLATELET KINETICS

The term platelet kinetics incorporates all the processes of platelet production, distribution, survival, utilization, and destruction as well as the homeostatic mechanisms which control them. A clear knowledge of these processes is useful in understanding the mechanisms of thrombocytopenia and the part platelets play in vascular diseases such as thrombosis, hypercoagulability, and interaction with foreign surfaces such as prostheses.

4.14.2.1 Platelet Production

4.14.2.1.1 Megakaryocyte development

Platelets are produced from distinctive giant cells in the bone marrow called megakaryocytes which normally comprise 0.02–0.05% of the bone marrow nucleated cell population.¹ Since the mid-1980s the study of megakaryocyte development has largely shifted away from studies based on morphology towards cell cultures.

The megakaryocyte is a nondividing, end-stage cell that is derived from an undifferentiated pluripotent hematopoietic stem cell.² Once lineage commitment occurs, the stem cell develops into a megakaryocyte by a process of endomitosis where the DNA is replicated without nuclear division; the result is that the nucleus becomes bigger and multilobular in shape. The cytoplasm develops simultaneously and more or less doubles its mass with each DNA replication (Table 1).

The regulators of megakaryocyte development can be divided into those that act early in development upon progenitor cells, the so-called megakaryocyte colony stimulatory factors (Meg-CSFs), and those that act late upon terminally differentiated cells, namely thrombopoietins. The Meg-CSFs comprise lineage nonspecific cytokines such as interleukin (IL)-3

Table 1 Hierarchical line of platelet formation.

Undifferentiated pluripotent hemapoietic stem cell
Megakaryocyte progenitor cells
(i) Burst-forming megakaryocyte unit
forms multiple clusters of more than 100 megakaryocytes
expresses the stem cell marker, CD 34 antigen
is HLA-DR antigen negative
(ii) Colony-forming megakaryocyte unit
forms single clusters of approximately 12 megakaryocytes
expresses CD 34 and HLA-DR antigens
(iii) Light density colony-forming megakaryocyte unit
has little proliferative capacity
is CD 34 antigen negative
Promegakaryoblast
has the size and morphology of lymphocytes
expresses membrane Glycoprotein Ib
contains von Willebrand factor and platelet factor 4 in cytoplasm
Megakaryocyte
unable to form clones
endoreplicate and mature to polyploid megakaryocytes
Platelets

Source: Gewirtz and Hoffman;³ Chatelain *et al.*;⁴ Vainchenker *et al.*;⁵ Zucker-Franklin.⁶

and granulocyte-macrophage colony stimulating factor (GM-CSF) and the final maturation of the committed megakaryocyte progenitors is supported by thrombopoietin.^{7,8} Other factors such as IL-1 α , IL-6, IL-11, granulocyte colony stimulating factor (G-CSF), and stem cell factor (the c-kit ligand or KL), appear to have little or no effect by themselves, but act synergistically with IL-3 and GM-CSF.

In vitro data indicate that recombinant thrombopoietin functions primarily as a differentiation factor and has limited ability to promote progenitor growth. In liquid culture, thrombopoietin augments terminal differentiation in megakaryocytes, as demonstrated by a significant increase in their DNA content and by expression of the platelet-specific glycoproteins (GP)Ib and IIb/IIIa.⁹ Thrombopoietin, used in combination with early acting growth factors such as IL-3, strongly stimulates the generation of megakaryocyte colonies. Issues that are still unresolved include the site and regulation of thrombopoietin production and modulation of receptor expression,⁹ as well as the factor that commits the pluripotent hemapoietic stem cell to become a megakaryocyte progenitor.

There are indications that megakaryocytopoiesis might also be subjected to negative regulatory influences, although the nature of these putative factors remains poorly defined.¹ Candidates include a 12–17 kDa secreted platelet glycoprotein, transforming growth factor, platelet factor 4, and β -thromboglobulin.

4.14.2.1.2 Thrombopoiesis

It is widely accepted that platelets are produced in the bone marrow but there is controversy over the site of platelet release into the blood stream. Although platelets may be released in the bone marrow, there is evidence that megakaryocytes can enter the circulation and lodge in the pulmonary capillaries where platelets are released.⁷ The possibility therefore remains that platelets are released in both the bone marrow and lungs.

Two general hypotheses have been suggested to explain the release of platelets by megakaryocytes into the peripheral blood.^{1,7} The main difference between them concerns the part played by the demarcating membrane system (DMS). The promegakaryoblast develops a DMS by the invagination of the plasma membrane. As the megakaryocyte matures, the DMS enlarges to increase the total membrane surface area by as much as 2600%.⁷ According to one hypothesis, the DMS is organized into discrete platelet domains by partial fusion of parallel channels of DMS. Such a fusion leads to the formation of fenestrated sheets of membrane. Megakaryocytes, located adjacent to bone marrow sinusoids, extend pseudopodia into the sinusoids where these fracture to release variable-sized segments of megakaryocyte cytoplasm, or "proplatelets," into the circulation. These pieces then further fragment into individual platelets. The other hypothesis, the "flow model," suggests that the DMS represents a storage form of highly redundant, invaginated megakaryocyte plasma membrane. The DMS does not delineate platelet territories but is crucial for platelet formation and release. During platelet release, the redundant membrane is extruded from the megakaryocyte in the form of pseudopodia. Shear forces induced by blood flow in the sinusoids cause constrictions to develop in the pseudopodia which eventually attenuate enough to break, thereby releasing platelets.

4.14.2.2 Mean Platelet Lifespan, Distribution, and Destruction

The mean platelet lifespan (MPLS), *in vivo* distribution, and sites of destruction of platelets

are measured by labeling of a representative platelet population, isolated from blood by differential centrifugation, with a radioisotope. Indium-111¹⁰ has replaced ⁵¹Cr¹¹ as label of choice because of its high labeling efficiency (>80%), appropriate half-life (2.8 d), high gamma photon yield (89% at 173 keV and 94% at 247 keV), and acceptable radiation dose levels to patients.^{12,13} We shall not review the methods of platelet labeling, calculation of platelet recovery and MPLS, scintillation camera imaging, and quantification of the *in vivo* distribution of labeled platelets. An in-depth review by Heyns *et al.* is available.¹⁴

Labeled platelets, reinjected into normal volunteers, circulate for 10–12 d.¹⁵ Measurement of MPLS is a valuable and sensitive indicator of the presence and extent of *in vivo* processes involving platelets. It has contributed significantly to our understanding of the role of platelet interactions with thrombogenic surfaces, the evaluation of optimal antiplatelet drug therapy, and prediction and management of patients with thromboembolic, atherosclerotic, and immune diseases.¹²

Removal (sequestration) of platelets from the circulation either takes place as an age-dependent process or as a result of increased destruction or random utilization. Age-dependent removal of platelets occurs at the end of their lifespan and the resulting survival curve, constructed from circulating radioactivity determined at time-intervals over the survival period, will best fit a linear function. If platelet removal is age-independent, platelets of all ages are randomly removed and the survival curve will best fit an exponential function.^{12,14,16} Information relating to the mechanism of platelet utilization and destruction can therefore be derived from the shape of the survival curve.

Circulating platelets are distributed in two compartments. Approximately one-third are pooled in the spleen and the remaining two-thirds are circulating.^{12,17} The mechanism of pooling of platelets in the spleen is unknown, but may be related to the circulation and size of the spleen. Many platelets apparently enter the splenic cords and flow through the sinuses. This delays their transit through the organ by 8–10 min and causes platelet accumulation out of proportion to the splenic plasma and red cell volume.^{18,19} Platelets present in the pool are in dynamic equilibrium with platelets in the circulation, and are freely exchangeable.²⁰

When platelets reach the end of their lifespan in the circulation, the macrophages of the liver, spleen, and bone marrow recognize them as "old" and remove them from the circulation.^{12,21} The spleen, the major site of removal

of senescent platelets, sequestrates 38–40% and the liver approximately 28% of the senescent platelets. It is generally accepted that the remainder of senescent platelets are sequestered by the bone marrow.¹⁵ In baboons, however, approximately 12–15% of the non-liver/nonspleen platelets were present in the muscle, skin, intestines, and kidneys,¹⁶ thereby indicating that the bone marrow was responsible for the sequestration of approximately 15% of senescent platelets. It is conceivable that the platelets not present in the macrophages are utilized to maintain vascular endothelial integrity.²²

The mechanism whereby the macrophages recognize senescent platelets is not known. There are indications that loss of sialic acid content of the platelet membrane may play a part,²³ and that recognition of senescence may well be based on immunologic mechanisms.²¹ A possible mechanism is that platelets lose sialic acid from their membranes during their circulation in the blood.²³ Since the membrane glycoproteins are enriched with sialic acid, the loss of sialic acid could expose senescent cell antigens.²⁴ An antibody, presumably an anti-Gal antibody, recognizes and binds to the exposed antigens to form an antigen–antibody complex. Once sufficient IgG is bound to the platelet membrane, it signals that the platelet is old. This signal is recognized by the macrophages of the spleen, liver, and the bone marrow and the senescent platelets are phagocytosed. The hypothesis suggests that the macrophages recognize senescent cells as “foreign” while performing their normal task of surveillance against foreign cells in the circulation.²¹

4.14.2.3 Evaluation of Platelet Kinetics

The first step in the evaluation of platelet kinetics is to determine the platelet count (described in Section 4.14.4.5.1). The reference range for platelets generally is considered to be between 150 and $400 \times 10^9 \text{ L}^{-1}$. Perturbations of the count activate compensatory mechanisms to restore it to the normal range, suggesting that platelet numbers are regulated.²⁵

In thrombocytosis the platelet count is increased as a result of increased production and unchanged peripheral destruction. It is generally asymptomatic but may cause thrombosis in a small proportion of cases, especially in elderly patients and those with symptomatic disease, a propensity for thrombosis or immobility.²⁶

The development of thrombocytopenia can either result from a defect in platelet production or increased peripheral destruction of platelets. Increased destruction can be caused by

increased consumption as a result of platelet participation in thromboembolic episodes or it can be immunologic in nature, as seen in immune thrombocytopenic purpura (ITP).¹²

Platelet production can be assessed by determining the total megakaryocytic mass.²⁷ This technique is complex and requires simultaneous examination of bone marrow sections and iron turnover. Platelet production is therefore usually measured indirectly. In a steady state, platelet production is equal to platelet destruction (turnover). The production can therefore be calculated from the MPLS and platelet count, correcting for the splenic platelet pool.^{12,27}

In patients with arterial disease, aortic aneurysms, arterial grafts, hypercholesterolemia, and after coronary artery bypass surgery the MPLS is usually shortened, and platelet turnover increased. In these situations platelet production increases sufficiently to maintain a normal platelet count.¹² In these patients the most useful information is obtained from combining the interpretation of the MPLS with imaging of the *in vivo* distribution of the ¹¹¹In-labeled platelets. For example, the MPLS is markedly and comparably shortened in patients with aortic aneurysms and vascular grafts, suggesting equivalent platelet utilization by aneurysms and grafts. Taking the shortening in MPLS into account, compartmental analysis of the kinetics of deposition of labeled platelets onto the aneurysms and grafts, clearly demonstrates that thrombus formation differs in these two cases.²⁸ In the patients with aneurysms maximum uptake of labeled platelets is significantly higher than in the patients with the grafts; $3.3 \pm 1.6\%$ of whole body radioactivity vs. $1.6 \pm 1.1\%$. Maximum uptake is also attained more rapidly in the aneurysm patients; $2.3 \pm 0.8 \text{ d}$ vs. $3.5 \pm 1.3 \text{ d}$. These results strongly suggest that the surface of an aneurysm is more thrombogenic and that the residence time of platelets in the aneurysm is shorter than in the graft.

The value of MPLS measurements in arterial thromboembolic diseases is reflected by the fact that they have helped to establish some basic principles of platelet participation in arterial disease.²⁹ First, these patients have excessive platelet consumption, whereas in patients with venous thrombosis there are both fibrin formation and increased platelet consumption. This underlines the importance of platelets in arterial-type thrombogenesis as opposed to venous-type thrombosis. Second, antiplatelet drugs can interrupt this increase in platelet consumption. Third, increased platelet consumption persists for weeks or months after the clinical event, suggesting that a thrombus

is not static, but that platelets are continuously activated on the thrombus surface. This assumption is supported by the results of platelet participation in aneurysms and vascular grafts.²⁸

Calculation of platelet production (turnover) also has proved useful in patients with ITP. In this disease IgG antiplatelet antibodies are directed against a specific platelet-associated antigen.³⁰ The MPLS is characteristically very short and platelets coated with antibody are rapidly sequestered by the macrophages of the spleen, liver, and bone marrow. Based on platelet production calculations, these patients can be divided into those with effective production and those with depressed production. The patients having effective production are those with less severe disease, a slightly higher platelet count, a slightly longer MPLS, and platelet sequestration predominantly in the spleen. Patients with depressed production have more severe disease, a lower platelet count, very short MPLS, and platelet sequestration distributed diffusely in the monocyte-macrophage system.³¹

Scintillation camera imaging of ¹¹¹In-labeled platelets can also be used to evaluate platelet participation in disease processes *in vivo*. For example, there is prominent accumulation of radiolabeled platelets during acute rejection of a kidney transplant and not during chronic rejection.³² Imaging of the labeled platelets can also be used to evaluate the thrombogenicity of biomaterials such as oxygenators³³ and vascular graft material.³⁴

4.14.3 PLATELET MORPHOLOGY

4.14.3.1 Light Microscopic Appearance

Platelets circulate in the bloodstream as anucleate cytoplasmic fragments and appear as small bluish-gray bodies on Romanowsky-stained blood films. They are irregular in outline with fine purple-red granules that may be scattered or centralized in the cell. In the latter case the central granule-containing area is called the granulomere and the peripheral, light blue cytoplasm the hyalomere. The granules correspond with the α -granules detected by electron microscopy. A small number of larger platelets, up to 5 μ m in diameter may be seen in normal films.³⁵

4.14.3.2 Electron Microscopic Appearance

The ultrastructure of platelets has been well established.^{6,36-38} In order to combine morphological features with functional considerations

the platelet structure will be described under the headings: surface membrane, organelles, membrane systems, and cytoskeletal elements (Figure 1).

4.14.3.2.1 Surface membrane

The exterior coat or glycocalyx is an irregular layer ranging from 10 nm to 50 nm in thickness. This "fuzzy" layer is mainly formed by the protruding carbohydrate chains of the membrane glycoproteins and glycolipids. It is rich in sialic acid, which contributes to a negative surface charge. Many plasma proteins are found in this layer where they are either held by electrostatic charges or weakly bound to receptors as part of a process of internalization. It seems that small amounts of practically all plasma proteins are taken up into α -granules and the concentration of these proteins in platelets is determined by their plasma concentration.³⁹ The glycoproteins are important structural components that serve as receptors for a wide variety of inhibiting and stimulating agents. Although they have been designated by Roman numerals, GPI to GPIX,⁴⁰ many of them have now been cloned and sequenced. Their genes belong to one of several major gene families (Table 2).

The unit membrane consists mainly of a phospholipid bilayer with transmembrane protein components randomly dispersed in it. Cholesterol and small amounts of glycolipids are also embedded in the phospholipid bilayer. The phospholipid molecules are distributed asymmetrically on the two sides of the bilayer (phosphatidylcholine and sphingomyelin in the outer layer and phosphatidylinositol, phosphatidylethanolamine, and phosphatidylserine in the inner layer).⁴¹ Upon stimulation, the negatively charged phospholipids in the inner layer are repositioned to the outer layer where they provide the catalytic surface for the formation of the "tenase" and "prothrombinase" coagulation complexes. The capacity of activated platelets to enhance prothrombin activation has been termed platelet factor 3 (see Section 4.14.4.4). Other important components of the platelet unit membrane include the membrane endoenzymes; those enzymes involved in membrane transport, cyclic AMP, and prostaglandin metabolism are of particular interest.

4.14.3.2.2 Organelles

Several different types of organelles are found in the cytoplasm of platelets. They are randomly distributed in nonactivated platelets,

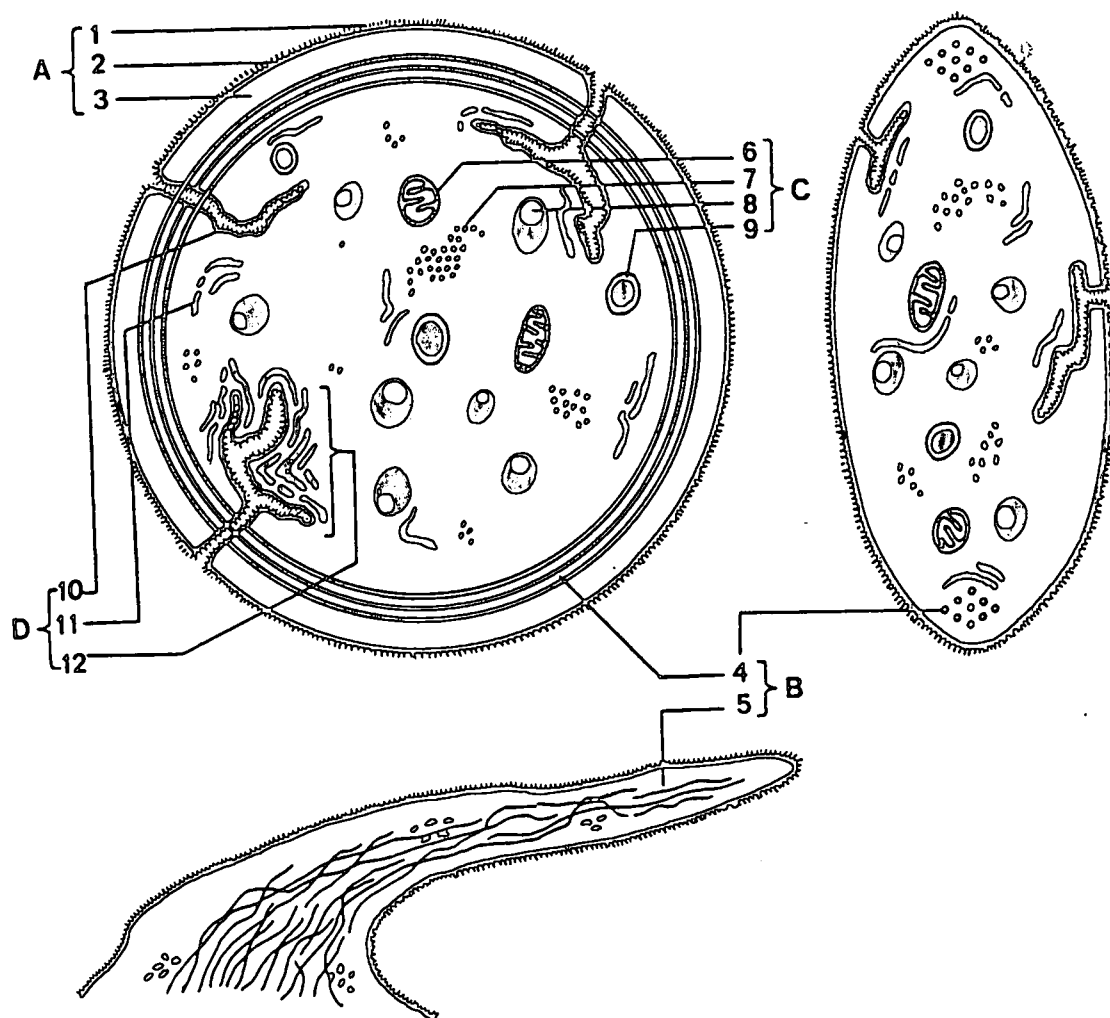


Figure 1 (This page) A diagrammatic representation to illustrate the ultrastructural features of a non-activated platelet cut in the equatorial plane (top left) and in cross-section (top right), as well as in a pseudo-podium of an activated platelet (bottom). The surface membrane (A) consists of the exterior coat (1), the unit membrane (2), and the submembrane area (3). The cytoskeletal elements (B) consist of the microtubules (4) and the microfilaments (5). The organelles (C) consist of mitochondria (6), glycogen (7), α -granules (8), and dense bodies (9). The membrane systems (D) consist of the connected open canalicular system (10), the dense tubular system (11), and the membrane complexes (12). (Next page) An electron micrograph of a platelet cut in the equatorial plane. Components include the exterior coat (EC), mitochondria (M), microtubules (MT), dense bodies (DB), α -granules (G), and open canalicular system (OCS).

but centralize upon activation. The dense bodies contain a nonmetabolic (storage) pool of calcium, pyrophosphate, serotonin, ADP, ATP, and possibly antiplasmin, all of which are secreted during the release reaction. The non-dense granules are the most abundant platelet organelles and can be divided into α -granules, lysosomes, and peroxysomes.⁴² The α -granules contain platelet-specific proteins (platelet factor 4, β -thromboglobulin, and platelet basic protein), adhesive glycoproteins (fibrinogen, von Willebrand factor (VWF), fibronectin, thrombospondin, and vitronectin), coagulation factors (factor V, protein S, and factor XIII), mitogenic factors (platelet-derived growth

factor, transforming growth factor β , endothelial cell growth factor, and epidermal growth factor), fibrinolytic inhibitors (α 2-plasmin inhibitor and plasminogen activator inhibitor-1), and plasma proteins (albumin and immunoglobulins).⁴²

The lysosomes contain several hydrolases, elastase, collagenase, and cathepsin D and E, and the peroxysomes contain catalase that contribute to lipid metabolism. The mitochondria are easily recognized by their characteristic double membranes and cristae and are involved in oxidative energy metabolism. Glycogen particles occur singly or in relatively large masses.

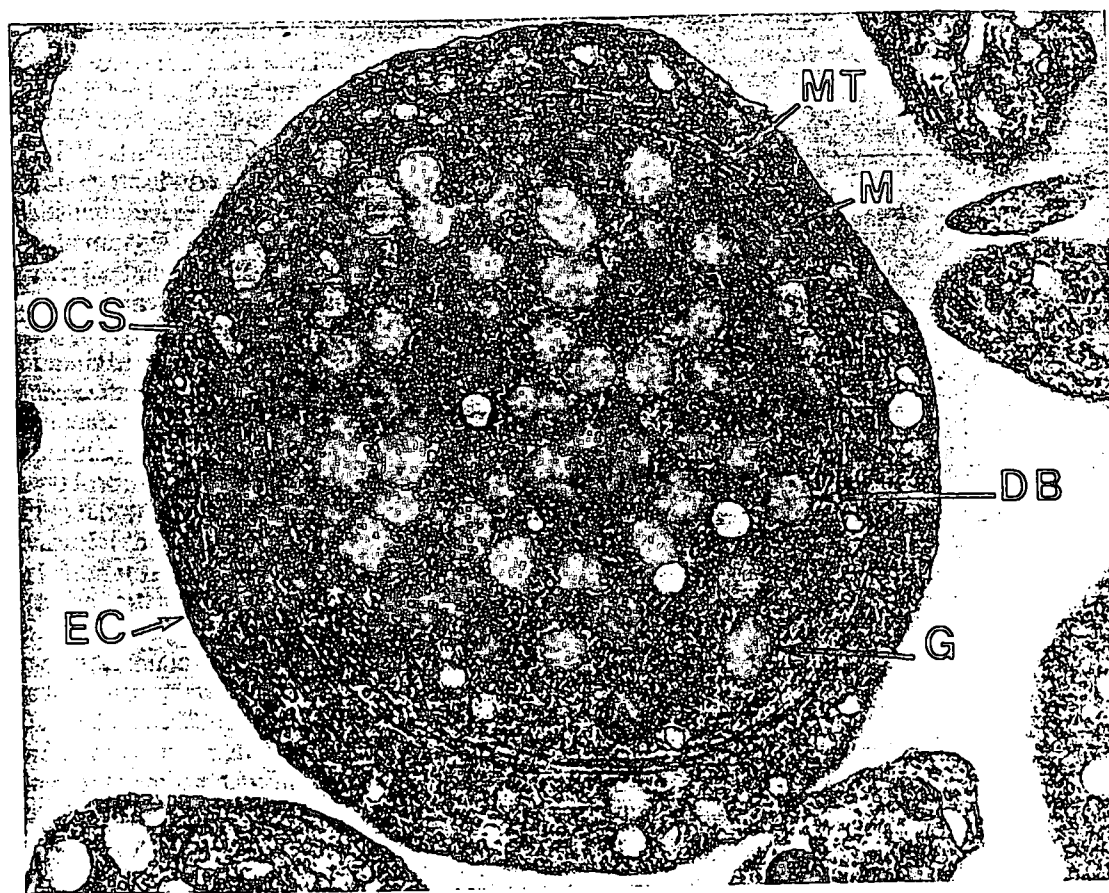


Figure 1 (continued)

4.14.3.2.3 Membrane systems

The surface connected open canalicular system consists of tortuous invaginations of the surface membrane into the interior of the platelet. The channels of this system form an anastomosing network spreading throughout the cytoplasm. These channels greatly enlarge the platelet surface area and enable access of plasma-borne substances to the inner parts of the platelets. They also serve as conduits for substances released by the platelets as well as an extensive internal store of membrane. During the formation of pseudopodia and platelet spreading, both of which require a large increase in surface membrane, internalized platelet membranes are externalized. At the same time additional functional sites may be exposed.

The dense tubular system is derived from the smooth endoplasmic reticulum and forms a continuous network of narrow canaliculi. It is the major storage site for calcium which is mobilized to regulate contractile events. Prostaglandin metabolism also appears to be localized in the dense tubular system.

4.14.3.2.4 Cytoskeletal elements

The microtubules form a circumferential band located in the equatorial plane just beneath the cell wall in the nonactivated platelet. The microtubules are composed of tubulin, polymerized into a single tubule of approximately 24 nm in diameter, which is coiled to form a group of 10–24 circular profiles. The microtubules appear to participate in maintaining the platelet diskoid shape and to be involved in the internal contraction of platelets. Since the microtubules themselves cannot contract, the contractile force must be provided by the microfilaments.

The membrane cytoskeleton consists of a sub-membrane web of short actin filaments with a delicate lacy appearance. Its apparent attachment to the microtubules contributes to maintaining the diskoid shape of the platelet. It is presumably also attached to the cytoplasmic domains of the transmembrane glycoproteins, including GPIb/IX, GPIa/IIa, and GPIIb/IIIa. Occupation of these receptor sites leads to clustering and cytoskeletal reorganization which involves a wide range of molecules,

Table 2 Platelet glycoprotein receptors and their ligands.

<i>Glycoprotein</i>	<i>Ligand</i>
Integrins	
α_{IIb}/β_3 (GPIIb/IIIa)	fibrinogen, VWF, fibronectin, and vitronectin
$\alpha_v\beta_3$ (α_v /GPIIIa)	vitronectin, VWF, fibronectin, fibrinogen, and thrombospondin
$\alpha_5\beta_1$ (GPIc/IIa)	fibronectin
$\alpha_6\beta_1$ (GPIc/IIa)	laminin
$\alpha_2\beta_1$ (GPIa/IIa)	collagen
Leucine-rich glycoprotein (LRG) family	
GPIb/IX	VWF and thrombin
Selectin family	
GMP-140 (PADGEM)	platelet-leukocyte interaction
Immunoglobulin supergene family	
PECAM-1	heparin
Seven transmembrane receptors (G protein linked)	
	thrombin, thromboxane A ₂ , epinephrine, and vasopressin
Miscellaneous	
GPIV	thrombospondin

VWF = von Willebrand factor.

including talin, vinculin, paxilin, and tyrosine kinases (see Section 4.14.4).⁴³

Platelets contain large amounts of actin that can polymerize into microfilaments, in addition to proteins that can either facilitate or inhibit the process. The state of the microfilaments in unstimulated platelets is not clear. There may be a continuous alteration in the state between actin filaments and their globular monomeric precursors called G-actin. Alternatively, polymerization into filaments may occur as a result of platelet activation. The microfilaments must be seen as the essential element of the contractile mechanism of platelets. They are arranged in bundles in the pseudopodia and form an extensive network in spreading platelets.

4.14.3.3 Evaluation of Platelet Morphology

Platelet abnormalities consist of either an increase or decrease in their number or size. The assessment of platelets on a blood film is discussed in Section 4.14.4.5.1. As a general rule, increased platelet consumption or destruction is accompanied by a compensatory increased production and the appearance of

megathrombocytes or stress platelets in the peripheral blood. This has led to the assumption that young platelets are larger and metabolically more active than older platelets.^{44,45} This explains why many patients with ITP and severe thrombocytopenia can have a normal bleeding time.⁴⁶ However, there is clear evidence that platelets do not decrease in volume with aging^{47,48} and it is believed that platelet size is a feature of platelet production and may be ascribed to megakaryocyte ploidy.⁴⁹ Abnormally large platelets are also found in situations where the megakaryocytes are affected by underlying disease processes such as the myeloproliferative and myelodysplastic syndromes.

A number of congenital thrombocytopenias associated with giant platelets have been described. These include the Bernard-Soulier syndrome,⁵⁰ May-Hegglin anomaly,⁵¹ Alport syndrome,⁵² Fechtner syndrome,⁵³ Montreal platelet syndrome,⁵⁴ and Mediterranean macrothrombocytopenia.⁵⁵ Although distinctive ultrastructural abnormalities have been described,⁵⁶ the diagnosis of these disorders is based rather on the inheritance pattern, associated clinical features, and platelet function tests.

There are three disorders in which the platelet granules are affected. In the Chédiak-Higashi syndrome⁵⁷ the granules are unusually large and resemble those in bovine platelets. In the storage pool disease the platelets look normal on light microscopy but the dense granules are either undetectable or markedly decreased when examined by electron microscopy.⁵⁸ Platelets of patients with the gray platelet syndrome lack ultrastructurally demonstrable α -granules,⁵⁹ and appear blue or gray on stained blood smears. However, agranular platelets have also been described in myelodysplastic and myeloproliferative syndromes, after cardiopulmonary bypass, hairy cell leukemia, and platelet stimulation due to difficult venepuncture.⁶⁰

4.14.4 PLATELET FUNCTION

Blood platelets form the first line of defense when normal blood vessels are injured. The exposed endothelium causes a series of platelet reactions that ultimately culminate in the formation of a platelet plug that prevents excessive blood loss. Intact endothelial cells at the edge of the vascular lesion produce inhibitors of blood coagulation and platelet aggregation that limit the size of the hemostatic plug.⁶¹ However, the induction of excessive platelet activation by biomaterials or structures such as ulcers and fissures in the fibrous cap of

atheroma, overcomes these thromboregulatory mechanisms. The platelets then become major prothrombotic offenders predisposing to arterial vasooclusive disease.⁶² Quantitative or qualitative abnormalities in platelet function manifest themselves in excessive bleeding at mucocutaneous sites, resulting in ecchymoses, petechiae, epistaxis, gingival hemorrhage, and menorrhagia.⁶³

Various factors probably control the reaction of the platelets to the exposed subendothelium. The depth of the lesion is important; deeper damage exposes more platelet-reactive material and tissue factor.⁶⁴ Increased numbers of red cells enhance platelet interactions with the vessel wall by forcing platelets to the periphery of the blood stream.⁶⁵ The blood flow rate and the size of the vessel determines the numbers of platelets passing by in a given time, the time that the platelet has to interact with the injured vessel, the rate of dilution of the platelet-activating factors and the forces tending to dislodge the platelets from the vessel wall or other platelets.⁶⁶

The platelet membrane glycoprotein receptors (see Section 4.14.3.2.1) and their ligands both play an important part in platelet adhesion (platelet-subendothelial interactions) and aggregation (platelet-platelet interactions), as described in Chapter 13, this volume. Most of the receptors are specific for their respective ligands, while different ligands can bind to GPIIb/IIIa (Table 2). The binding of these

ligands to GPIIb/IIIa is mutually exclusive, being partly mediated by the amino acid sequence, Arg-Gly-Asp, present in their primary structures.⁶⁷

4.14.4.1 Platelet Adhesion

The subendothelium contains various adhesive proteins and trace amounts of tissue factor. When exposed, the platelets attach to these proteins. The shear rate, determined by blood flow rate and size of the blood vessel, affects platelet adhesion differentially. At high shear rates, VWF-dependent adhesion is most important, probably because a high shear rate causes conformational changes to VWF and/or GPIb. VWF, bound to collagen, binds to the GPIb/IX receptor on platelets causing adhesion (Figure 2).⁴² Although it is generally accepted that the binding of VWF to GPIb/IX results in an outside-in transduction signal, the mechanism remains obscure.⁴² At low shear rates, fibronectin-dependent adhesion and binding of collagen to the GPIa/IIa receptor on the platelet membrane appears to be important. The contribution of the binding of laminin to GPIc/IIa to adhesion is still unclear.^{68,69} It must be noted that platelet adhesion, in contrast to shape change, spreading, release, and aggregation, does not require metabolic energy.⁶⁹

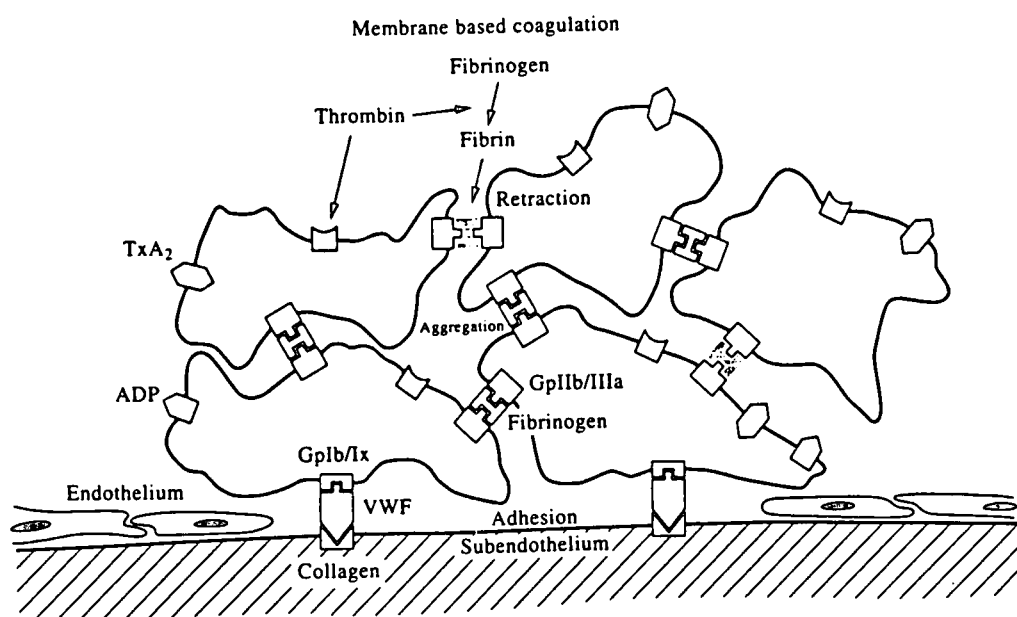


Figure 2 A schematic representation of the important participants in thrombus formation. The wall shear rate determines if fibrinogen, VWF, fibronectin or vitronectin will bind to GPIIb/IIIa to result in platelet aggregation (see Section 4.14.4). The important receptors for platelet agonists include receptors for thrombin, ADP, and thromboxane A₂ (TxA₂).

4.14.4.2 Shape Change, Spreading, and Secretion

The smooth disk shape of nonactivated platelets is not conducive to close cell-to-cell contact. Shape change is necessary to ensure greater cohesion and to allow the formation of a tight plug. After activation, the diskoid platelets change into irregular spheres with numerous projections or pseudopodia which markedly increase the platelet surface area for adhesion and aggregation. Platelet shape change is also associated with structural changes that suggest active contractions. The submembranous bundle of microtubules dissolves, the granules move to the center of the platelet and the microtubules reform centrally to surround the granules. The driving force underlying these striking conformational changes remains obscure but involves internal contraction-relaxation phenomena. An increase in cytoplasmic calcium and activation of myosin light chain kinase through calmodulin, and interaction of phosphorylated myosin with actin cause contraction.⁷⁰ Polymerization and bundling of actin in the pseudopodia also play a part.²

Platelet shape change is a reversible process, provided the activating stimulus is not too strong. In such an event the platelets revert back to their original shape, probably as a result of reuptake of calcium by the dense tubular system, and the platelet clump falls apart. When sufficient amounts of calcium are released into the cytoplasm of the platelets, the platelets secrete their granular contents. Thrombin, ADP, collagen, thromboxane A₂, and epinephrine are physiologically the most relevant of the many platelet agonists capable of inducing the release reaction. When these agonists bind to their respective receptors on the platelet membrane a signal is transferred to the interior of the cell (see Section 4.14.4.3).

Moderate stimulation of platelets only cause a slight increase in calcium levels, just sufficient to activate phospholipases, which in turn, cause the release of arachidonic acid which is rapidly transformed into thromboxane A₂. Thromboxane A₂ not only activates surrounding platelets, but also induces further mobilization of calcium ions. When platelet activation is intense, for example, with thrombin or high concentrations of collagen or ADP, sufficient amounts of calcium ions are mobilized to cause the release reaction independently of thromboxane A₂.⁴²

4.14.4.3 Platelet Aggregation

The adherence of platelets to each other is essential for the formation of a stable hemo-

static plug. A critical event in this process is the assembly on the platelet surface membrane of a receptor formed by the GPIIb/IIIa complex which has the capacity to bind fibrinogen, VWF, fibronectin, or vitronectin (Figure 2).^{42,68-70}

A variety of platelet agonists are able to activate platelets. These include collagen, thrombin, thromboxane A₂, epinephrine, and ADP. Collagen and thrombin are regarded as primary agonists that act at sites of vascular injury, while thromboxane A₂, epinephrine, and ADP help to recruit platelets into the growing plug. Specific receptors for these agonists exist on the platelet surface (Table 2). These receptors belong to the seven transmembrane domain family of receptors and are linked to G-proteins inside the cell.^{42,71} The details of the action of the G-proteins are still emerging. Some interact with target proteins coupled to ion-permeable channels in the platelet membrane, modulating the inward movement of calcium. Others are linked to protein kinases that phosphorylate other sites on the receptor protein itself. Binding of the agonist to its receptor activates the G-protein which in turn activates phospholipase C to hydrolyze phosphatidylinositol biphosphate in the membrane to produce diacylglycerol (DAG) and inositoltriphosphate (IP₃). DAG and IP₃ serve as second messengers within the cell. IP₃ binds to a receptor in the dense tubular system and causes mobilization of intracellular calcium leading to promotion of calcium-dependent enzyme activity. DAG activates protein kinase C activity which promotes protein phosphorylation of serine and threonine residues, granule secretion, and fibrinogen (GP IIB/IIIa) receptor expression. Once initiated, platelet activation is amplified by several endogenous mechanisms, which include the formation of several stimulatory eicosanoids (prostaglandin G₂, and thromboxane A₂) which pass through the membrane and bind to their G-protein-linked receptors to amplify platelet activation through one or more of the second messengers.⁷²

The activated GPIIb/IIIa complex is able to bind to fibrinogen, VWF, fibronectin, and vitronectin.^{42,68-70} There are indications that fibrinogen is most important at low shear rates, and VWF at higher shear rates which are usually obtained in stenosed arteries.⁶⁸ In resting platelets GPIIb/IIIa binds fibrinogen to a limited extent only and is unable to bind VWF. When platelets are activated the 40 000-50 000 GPIIb/IIIa receptors are changed into high affinity binding sites for fibrinogen and VWF. The receptor recognizes at least two binding sites on the fibrinogen molecule; an

Arg-Gly-Asp amino acid sequence in each of the two A-alpha chains and a dodecapeptide on the carboxy terminal of the gamma chain.⁶⁸⁻⁷⁰ The binding of fibrinogen to GPIIb/IIIa appears to be a multistep process.⁴² The initial event is cation-dependent and most likely involves the gamma chain. Subsequent interaction, apparently not cation-dependent, renders the binding irreversible.

Activated luminal GPIIb/IIIa receptors on adherent platelets may bind fibrinogen and/or VWF. These then await the arrival of and interaction with GPIIb/IIIa on the surfaces of circulating platelets that have been activated by released ADP and thromboxane A₂ in their neighborhood. Alternatively, circulating platelets may be activated and bind fibrinogen or VWF. In this case the platelet-ligand complex can bind to activated GPIIb/IIIa receptors on the luminal surfaces of adherent platelets. This process can repeat itself several times, each time adding an additional layer of platelets to form the hemostatic plug.⁴²

There are several factors that inhibit platelet activation and so prevent excessive platelet deposition.⁷¹ Flowing blood is probably the most important in removing proaggregatory substances from the site of thrombus formation, diluting their local concentration. Stimulated by thrombin, endothelial cells in the proximity can release prostacyclin and nitric oxide. Both are important inhibitors of platelet aggregation. Endothelium also has an ecto-ADPase that can limit the effects of ADP.

GPIIb/IIIa is capable of signaling in both directions of the platelet membrane.⁴² Inside-out signaling activates the GPIIb/IIIa to become a high affinity receptor for fibrinogen and VWF. This takes place after activation of platelets by other agonists. The mechanism of activation of the receptor remains obscure. Indications are that the cytoplasmic domains of GPIIb and GPIIIa play a role and that additional molecules or signaling pathways may be necessary to activate GPIIb/IIIa. Outside-in signaling occurs when fibrinogen and/or VWF bind to GPIIb/IIIa. This binding induces changes in the association of GPIIb/IIIa with the cytoskeleton and in enzymes, including the kinases, involved in the downstream signaling leading to clustering of the GPIIb/IIIa receptors.

4.14.4.4 Procoagulant Activity of Platelets

There is irrefutable evidence that platelets promote thrombin formation, but the mechanism remains controversial.⁷³ Activation of factor X by factors IXa and VIIIa and activation of prothrombin by factors Xa and Va are

both dramatically accelerated by activated platelets. Platelets are also able to accelerate the activation of factor VIII by thrombin.⁷⁴ It appears that factor VIIIa on platelets acts as a binding site for factor IXa and that factor Va acts as a binding site for factor Xa. What remains unclear is whether factors VIIIa and Va bind to receptors or to negatively charged phospholipids, particularly phosphatidylserine, on the activated platelet membrane.⁴² Platelet activation also results in the formation of microparticles rich in factor V and phospholipid coagulant activity.⁷⁵

Along with enhancing the activation of coagulation factors, platelets are also involved with coagulation in other ways.⁴² These include their ability to bind fibrinogen where it is strategically situated on the platelet surface for interactions with locally produced thrombin. The presence in the α -granules of adhesive proteins, coagulation factors, and inhibitors of coagulation and fibrinolyses (see Section 4.14.3.2.2) further illustrates the involvement of platelets in coagulation.

4.14.4.5 Evaluation of Platelet Function

The platelet count, inspection of the blood film, and the bleeding time are first-line laboratory tests to evaluate platelet function. The second-line tests include determination of the turbidometric aggregation in response to platelet agonists, spontaneous aggregation, the platelet aggregate ratio, plasma and platelet levels of β -thromboglobulin, and thromboxane B₂ generation by maximally stimulated platelets. We shall not describe the methodology of the different tests, and refer the reader to detailed discussions.^{35,75,76}

4.14.4.5.1 Platelet count

Before the advent of fully automated blood cell analyzers, platelets were assessed on the blood film. The manual technique using a phase contrast microscope and hemocytometer is time consuming and unpractical for large volumes of work. Films prepared from EDTA blood give an even distribution and less clumping of platelets than films prepared directly from venous blood or from a finger prick. Both the number and morphology of the platelets are evaluated. The platelet estimate is performed in each of ten 100 $\times$ oil immersion fields (OIF). Normally there are 7-25 platelets per OIF. The platelet estimate is reported as decreased, low normal, normal, high normal, or increased, and the decreased and increased graded as slight,

moderate, or marked. The presence of platelet clumps or giant platelets is also noted.

Modern medical practice necessitated the use of automated hematology analyzers. These instruments count platelets by either electronic impedance or light scatter technology and the results are generally accurate. However, certain artifacts or the presence of giant platelets may cause faulty results (Table 3). This means that regardless of whether or not an official estimate of the platelet count is done, verification of the instrument platelet count should be included in the final full blood count report.

4.14.4.5.2 Bleeding time

The bleeding time measures platelet function and vascular integrity. A standard incision is made on the volar surface of the forearm. The time it takes to stop bleeding is dependent on extravascular tissue, platelet adhesion and aggregation, and the presence of certain coagulation factors.⁷⁷ A defect in any of these will prolong the bleeding time, which varies between 2 and 10 min in normal humans. If the bleeding time suggests impaired primary hemostasis, further tests should be performed in a systematic way to determine the cause. These tests include *in vitro* platelet aggregation studies (see Section 4.14.4.5.4), factor VIII:coagulant and factor

VIII:VWF antigen activity, platelet coagulant activity, dense granule contents (ADP and ATP), and α -granule contents (platelet factor 4 and β -thromboglobulin).⁷⁸

4.14.4.5.3 Platelet adhesion tests

The most widely used test measures platelet retention after a single passage of whole blood through a glass bead column. Although many attempts have been made to standardize the method, the results remain variable. Platelet retention not only measures adhesion but can also be affected by the plasma VWF levels, fibrinogen coating of the glass surface, and platelet aggregation. The test is therefore not widely used for diagnostic purposes and is not recommended. The Baumgartner flow chamber is perhaps a more accurate measure of platelet adhesion to different substrates.⁶⁵ However, this test is expensive, time consuming and technically difficult, and is therefore mainly used for research purposes.

4.14.4.5.4 Platelet aggregation tests

Platelet-rich plasma is stirred in a cuvette at 37°C between a light source and a photo cell. When an agonist (ADP, collagen, thrombin, arachidonic acid, epinephrine, ristocetin, etc.) is added, the platelets aggregate and absorb less light. This results in an increase in light transmission which is measured and converted to a tracing made on a chart recorder (see Chapter 13, this volume). The addition of different agonists, at a range of concentrations, allows the detection of certain aggregation defects or hyper-reactive platelets.^{76,79} The length of the lag phase, the aggregation rate and the amplitude of the aggregation response may be measured. Alternatively, threshold concentrations or the ED₅₀ (the concentration of agonist that will cause 50% of the maximum aggregation response) can be calculated.

The interpretation of results of platelet aggregation defects is summarized in Table 4. Platelet hyper-reactivity can, for example, be assessed by the aggregation of platelets in response to low doses (0.25 M) of ADP.⁷⁹

4.14.4.5.5 Platelet adenine nucleotides

Measurement of platelet ADP and ATP and their release from platelets can be used to diagnose a storage pool and other defects.⁸⁰ Platelets contain two separate pools of nucleotides, a metabolic and a storage pool in the

Table 3 Causes of inaccurate automated platelet counts.

Cause	Blood cell analyzer
<i>Falsely low platelet counts</i>	
Partial clotting of specimen	all
Platelet activation during venepuncture with consequent aggregation	all
EDTA-induced platelet aggregation	all
Platelet satellitism	all
Giant platelets	all
<i>Falsely high platelet counts</i>	
Red cell fragments or microscopic red cells counted as platelets	all
White cell fragments counted as platelets	all
Hemoglobin H disease	Coulter
Cryoglobulin	Coulter and Technicon H1 series

Source: Bain.⁶⁰

Table 4 Patterns of abnormal platelet aggregation.

Disease	ADP	Coll	Rist 1.25 mg mL ⁻¹	Rist 0.5 mg mL ⁻¹	SA	Cal
VWD	N	N	O/A	O	N	N
VWD*	N	N	N/A	H	N	N
BSS	N	N	O	O	N	N
GLANZ	O	O	P	O	O	?
SPD	P	A	N/P	O	A	A
COX	A/N	A	N	O	A	N
TXSYN	A/N	A	N	O	A	N
TXREC	A/N	A	N	O	A	N
EHL DAN	N	A	N	O	N	N
CAL	A	A	N	O	A	A
MEMB	N/A	A	N/A	O	N	N

Source: Chanerlin⁷⁶ and Roberts.⁷⁸

VWD = von Willebrand's disease (* = subtype 2B). BBS = Bernard-Soulier syndrome. GLANZ = Glanzman's thrombasthenia. SPD = storage pool disease. COX = cyclooxygenase defect. TXSYN = thromboxane A₂ synthetase defect. TXREC = thromboxane A₂ receptor defect. EHL DAN = Ehler's Danlos defect. CAL = calcium binding/contractile protein abnormality. MEMB = other membrane receptor defect. COLL = collagen. Rist = ristocetin. SA = sodium arachidonate. Cal = calcium ionophore A23187. N = normal, H = increased, P = primary wave only, A = abnormal/reduced, O = absent.

dense bodies. The latter pool cannot interchange with the metabolic pool and is only released after platelet activation. Approximately 70% of the ADP is in the storage pool, whereas most of the ATP is in the metabolic pool. Decreased levels of platelet ADP and ATP accompanied by decreased release in response to collagen stimulation suggest a storage pool defect or *in vivo* platelet release due to platelet activation by thrombogenic surfaces. Decreased release in the presence of a normal pool size can indicate a platelet enzyme or membrane defect or a drug-induced effect.⁷⁶

4.14.4.5.6 Tests for *in vivo* activation

Numerous tests have been developed to demonstrate platelet hyperactivity or to show that circulating platelets have been activated by some prothrombotic process.⁷⁶ These tests include the determination of the circulating platelet aggregate ratio, the ADP aggregation threshold, spontaneous platelet aggregation, the plasma and platelet levels of β -thromboglobulin and platelet factor 4, and the ability of platelets to form thromboxane A₂. Of these, the determination of the plasma levels of platelet factor 4 and β -thromboglobulin, the circulating platelet aggregate ratio, spontaneous platelet aggregation and the aggregation of platelets in response to low concentrations of ADP appear to be the most sensitive to detect *in vivo* platelet activation.^{77,81}

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S-19

Influence of Platelet Membrane Sialic Acid and Platelet-Associated IgG on Ageing and Sequestration of Blood Platelets in Baboons

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Summary

Platelets were isolated from blood of baboons and treated with neuraminidase to remove platelet membrane sialic acid, a process which artificially ages the platelets. The platelets were then labelled with ^{111}In and their mean life span, in vivo distribution and sites of sequestration were measured. The effect of removal of sialic acid on the attachment of immunoglobulin to platelets were investigated and related to the sequestration of the platelets by the spleen, liver, and bone marrow. Removal of sialic acid by neuraminidase did not affect the aggregation of platelets by agonists in vitro, nor their sites of sequestration. The removal of 0.51 (median, range 0.01 to 2.10) nmol sialic acid/ 10^8 platelets shortened their life span by 75 h (median, range 0 to 132) h ($n = 19$, $p < 0.001$), and there was an exponential correlation between the shortening of the mean platelet life span and the amount of sialic acid removed. The increase in platelet-associated IgG was 0.112 (median, range 0.007 to 0.309) fg/platelet ($n = 25$, $p < 0.001$) after 0.79 (median, range 0.00 to 6.70) nmol sialic acid/ 10^8 platelets was removed ($p < 0.001$). There was an exponential correlation between the shortening of mean platelet life span after the removal of sialic acid and the increase in platelet-associated IgG. The results suggest that platelet membrane sialic acid influences ageing of circulating platelets, and that the loss of sialic acid may have exposed a senescent cell antigen that binds IgG on the platelet membrane. The antibody-antigen complex may then provide a signal to the macrophages that the platelet is old, and can be phagocytosed and destroyed.

Introduction

When somatic blood cells reach the end of their natural life span, the macrophages of the monocyte-macrophage system recognise them as "old" and phagocytose and destroy them. The proposed mechanism of recognition of the old cells is that there is a senescent cell antigen in the glycoproteins of somatic cell membranes (1, 2), and that asialoglycoconjugates on the cell surface may determine the survival time of red cells (2). The hypothesis to explain red cell senescence states that circulating cells lose sialic acid from their glycoproteins, and this exposes the senescent cell antigen. IgG-molecules attach to these antigens, and the antigen-antibody complex is the signal for phagocytosis and destruction of old cells by the macrophages (1, 2). A similar mechanism of recognition may exist for senescent platelets. A senescent cell antigen was isolated from old platelets (1), platelets lose membrane sialic acid from glycoproteins during ageing in the circulation (3, 4), and enzymatic removal of sialic acid shortens the mean platelet life span (MPLS) (5–9). Platelets from patients with Bernard-Soulier syndrome have

lower concentrations of sialic acid (10), and their mean platelet life span is shorter than normal (11).

In this study we investigated the mechanism of ageing and sequestration of senescent platelets in baboons. Platelets were artificially aged by treatment with neuraminidase to remove sialic acid from the platelet membrane. The in vitro aggregation response of platelets to agonists and the kinetics of treated and untreated ^{111}In -labelled platelets were measured. Sequestration was investigated by calculating the correlation between the shortening of MPLS after sialic acid was removed and the amount of sialic acid removed, the correlation between the amount of sialic acid removed and the increase in platelet associated immunoglobulin (PA-Ig), and the correlation between the shortening in MPLS and the increase in PA-Ig.

Methods

Experimental Animals

Normal healthy baboons (*Papio ursinus*), weighing between 9 and 12 kg, were used in a project that was approved by the Ethics Committee of the Provincial Administration and the University of the Orange Free State. The baboons were anaesthetised with ketamine hydrochloride (10 mg/kg body mass) to enable handling. They did not receive medication that could affect platelet function for at least 2 weeks before and during the experiments. Their peripheral blood platelet counts, $360 \pm 60 \times 10^9/\text{l}$, were within the reference range (10, 11).

Harvesting, Neuraminidase Treatment, and Labelling of Platelets with ^{111}In -tropolone

Harvesting. Blood, 42.5 ml in 7.5 ml ACD A, was collected from the femoral vein after discarding the first 5 ml. Platelets were harvested by differential centrifugation and repeated washing to prepare platelet-rich plasma (ACD-PRP) (12–14). More than 90% of the platelets in each sample were harvested. Aliquots of the ACD-PRP were removed to measure the in vitro aggregation response of platelets to agonists and the sialic acid concentration on the platelet membrane.

Neuraminidase treatment. Platelets in the ACD-PRP were concentrated to $300\text{--}320 \times 10^9/\text{l}$. Neuraminidase from *Clostridium perfringens* (Sigma Chemical Company, St Louis, MO) was added to give a final concentration of 0.02 U/ml, and the mixture was incubated for 30 min at 37°C. Following incubation, aliquots were again removed for the measurements of aggregation and sialic acid. The platelets in the remaining mixture were labelled with ^{111}In .

Platelet labelling. Platelets were labelled with ^{111}In -tropolone as previously described (14). Briefly, platelets were concentrated by centrifugation at $900 \times g$ for 20 min. The supernatant was removed and the platelet pellet resuspended in 0.2 ml ACD-platelet-poor plasma. Tropolone (0.01 ml of a 1 g/l solution) and ^{111}In -chloride (25 MBq, CSIR, Pretoria) was added. After incubation for 5 min, 10 ml of ACD-platelet-poor plasma were added to remove unbound ^{111}In . The platelets were again concentrated by centrifugation. The labelled platelets were finally resuspended in 5 ml ACD-platelet-poor plasma for reinjection. Labelled platelets were not reinjected unless aggregates consisting of 10 or more platelets were formed in response to ADP (11). In all instances macroscopically visible platelet aggregates formed after ADP was added.

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The labelling efficiency with ^{111}In -tropolone was $65 \pm 14\%$. Red cell, white cell and plasma protein contamination of the labelled platelet product was less than 5%.

Concentration of Sialic Acid in the Platelet Membrane

The two aliquots (pre- and post-neuraminidase) of ACD-PRP were centrifuged at $900 \times g$ for 15 min to form a platelet pellet. The pellet was resuspended in $0.1 \text{ N H}_2\text{SO}_4$ to a platelet count of $500 \times 10^9/\text{l}$. The sialic acid was hydrolysed from the platelet membrane by incubation at 80°C for 1 h. The samples were centrifuged, and the sialic acid in the supernatant was measured by the thiobarbituric acid assay of Warren (15).

Kinetics of the ^{111}In -labelled Platelets

The mean life span of the labelled platelets, both treated and untreated, was measured in the same baboons, that of the normal platelets within a month of that of treated platelets. In the studies where the platelets were treated with neuraminidase, 2 ml of blood was collected in EDTA at 5, 20, 40, 60, 90 and 180 min after injection of the labelled platelets and then twice daily for 3 days. For untreated platelets, 2 ml of blood was collected at five and 180 min after injection and then daily for 6 days. The radioactivity in the blood samples was measured in a well-type scintillation counter (Packard, Packard Instrument Company, Downers Grove, IL).

MPLS was calculated by fitting a gamma function to the disappearance of the labelled platelets from the circulation (16). The recovery of labelled platelets in the circulation at equilibrium was estimated from the radioactivity/ml injected, correcting for splenic platelet pooling and by back-extrapolation of the survival curve to zero time (12). The count rates of the blood samples collected during the first 3 h after injection were not used to calculate recovery and MPLS because, under normal conditions, mixing between injected labelled and native platelets is incomplete (16).

In vivo distribution and sites of sequestration. Seventy min after injection of the treated ^{111}In -platelets, anterior and posterior images (256×256 word mode) of the whole body were acquired with a large field of view scintillation camera (13). The images were stored on a A²-MDS data processing system for analysis. Images were acquired twice daily for 2 days.

The *in vivo* distribution of the labelled platelets was quantified by the geometrical mean method and their distribution at equilibrium and sites of sequestration estimated by linear least square analysis (13, 17). The *in vivo* distribution at equilibrium was not estimated if the MPLS was shorter than 20 h.

Platelet Associated Immunoglobulins: PA-IgG and PA-IgM

PA-IgG and PA-IgM were measured before and after treatment of the platelets with neuraminidase using a slightly modified ELISA-method (18). Briefly, washed platelets, $200 \times 10^9/\text{l}$, in 0.2 M Tris-HCl buffer containing 5% calf serum, were added to microplate wells coated with human serum. Known amounts of rabbit anti-human IgG or IgM antibodies (Dakopath Immunoglobulins, Denmark) were added, and incubated with the platelets for 90 min at 37°C . The free antibodies were washed from the platelets and O-dianisidine colour reagent was added. The optical density was determined in a spectrophotometer (Biotek Instruments, Winoski, VT) and the concentration of free antibodies calculated. PA-IgG and PA-IgM were calculated by the difference in the amount of antibodies added and those remaining in the supernatant after incubation of the platelets with the antibodies.

In Vitro Platelet Aggregation Studies

In vitro platelet aggregation studies were done before and after treatment with neuraminidase. An additional 18 ml of blood was collected in 2 ml of sodium citrate (3.8%). The blood was centrifuged at $2,000 \times g$ for 20 min to prepare citrate-platelet-poor plasma for the aggregation studies. The treated and untreated aliquots of ACD-PRP were centrifuged at $900 \times g$ for 15 min to form a platelet pellet. The supernatant was removed, and the platelets resuspended in the citrate-platelet-poor plasma to a concentration of 250×10^9 platelets/l.

The aggregation studies were done on a Chrono-log aggregometer. The agonists used to induce platelet aggregation were ADP: 20, 10 and $5 \mu\text{M}$ final concentration; collagen: 0.05 and 0.005 g/l final concentration

and adrenaline: $0.01 \mu\text{M}$ final concentration. The aggregation response of the platelets was expressed as the percent change in light transmission after 4 min.

Statistical Analyses

Differences between the measurements of treated and untreated platelets were tested with Student's *t*-test for paired or unpaired data. If the data were not normally distributed, the Wilcoxon signed rank test was used to test for differences. Least squares regression analysis was used to determine possible correlations.

Results

The Effect of Removal of Sialic Acid on In Vivo Platelet Kinetics

In 19 studies, platelets had a mean of $3.04 \pm 1.24 \text{ nmol sialic acid}/10^8$ platelets. Treatment with neuraminidase removed 0.79 (median, range 0.00 to 2.10) $\text{nmol sialic acid}/10^8$ platelets.

Recovery and mean platelet life span. The recovery of injected labelled platelets in the circulation was 84% (median, range 55 to 101%) for untreated platelets and 69% (median, range 52 to 100%) for treated platelets.

The mean disappearance curves of untreated and treated labelled platelets differed in the same baboons (Fig. 1). Those of the untreated platelets fitted a linear function best, indicating that they were removed from the circulation as a result of senescence (12, 16). The survival curves of the treated platelets were divided into two groups and are presented as two curves (Fig. 1). The one curve represents the disappearance of platelets where less than 20% of the sialic acid was removed ($n = 10$) and the other curve include data where more than 20% of the sialic acid was removed ($n = 9$). This was done because there was a linear correlation between the reduction in circulating radioactivity and the amount of sialic acid removed,

Reduction in radioactivity = $2 + 29$ (sialic acid removed)
 $r = 0.7360$, $p < 0.001$, and $n = 19$.

The shape of the survival curve of the platelets treated with neuraminidase had two components, indicating that there were two subpopulations of labelled platelets (Fig. 1). In the case where less than 20% of the sialic acid were removed, 8% (median, range 0 to 46%) of the treated platelets were cleared from the circulation in the first 3 h after their reinjection.

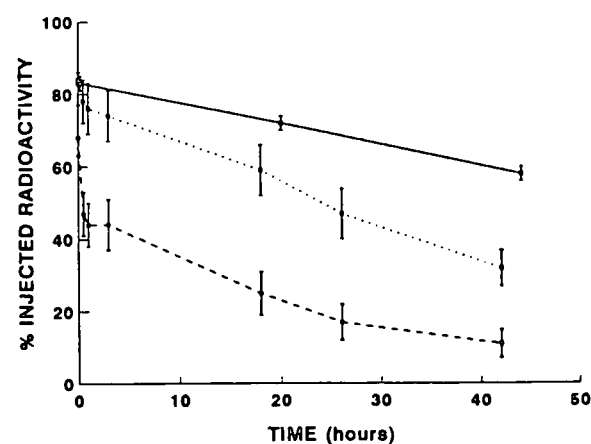


Fig. 1 The mean disappearance curves ($\pm 1 \text{ SEM}$) of ^{111}In -labelled platelets, untreated and treated with neuraminidase, from the circulation in 19 baboons. The disappearance curves of the treated platelets divided into a group where less than 20% of the sialic acid was removed from the platelets ($n = 10$, dotted line), and a group where more than 20% of the sialic acid was removed ($n = 9$, broken line)

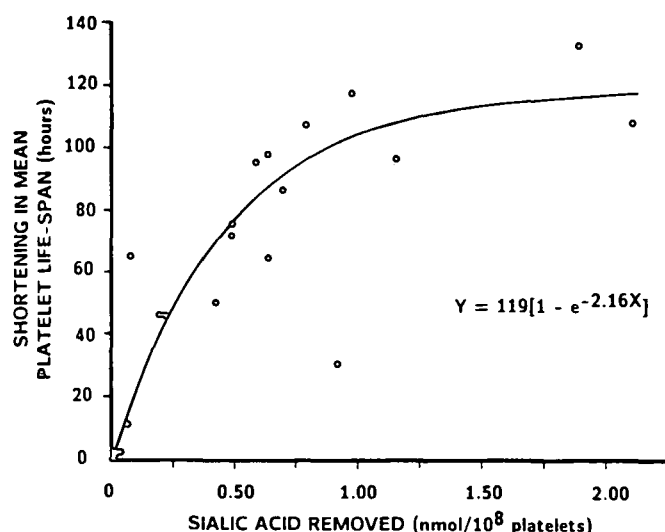


Fig. 2 Exponential relationship between the shortening of MPLS after the removal of sialic acid and the amount of sialic acid removed by treatment with neuraminidase

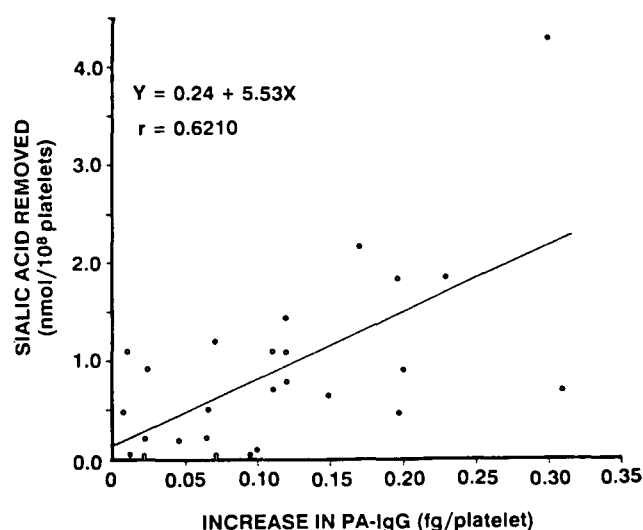


Fig. 3 Linear correlation between the amount of sialic acid removed with neuraminidase and the increase in IgG attachment of the treated platelets

(decrease in circulating radioactivity from 5 to 180 min). When more than 20% was removed 37% (median, range 12 to 82%) of the treated platelets were cleared in the first 3 h. The corresponding reduction for untreated platelets was about 4%.

Those treated platelets that remained in the circulation after 3 h had an almost linear, but shortened, survival curves (Fig. 1). The MPLS of the platelets that lost less than 20% of their sialic acid was 64 h (median, range 39 to 108 h), and the platelets that lost more than 20% sialic acid survived for 17 h (median, range 10 to 66 h). In both cases, the MPLS was substantially shorter than the 116 ± 15 (mean $\pm$ 1 SD) h of the untreated platelets. The correlation between the amount of sialic acid removed and the shortening of MPLS is given in Fig. 2.

The equilibrium distribution and sites of sequestration were investigated in nine baboons. The results are shown in Table 1. At equilibrium significantly more treated platelets were found in the spleen. The fraction in the liver was slightly higher than

normal. These increases probably represents those platelets that were removed from the circulation in the first 3 h after injection.

The sites of sequestration was not adversely affected by the removal of sialic acid (Table 1). The liver sequestered slightly, but significantly ($p = 0.05$), more treated platelets. Platelet sequestration in the spleen and bone marrow was unchanged.

The Effect of the Removal of Sialic Acid on Immunoglobulin (Ig) Attachment to Platelets

Twenty-five studies were done to determine the association between the removal of sialic acid and attachment of Ig to platelets. The platelets contained 0.028 (median, range 0.001 to 0.099) fg IgM/platelet and 0.197 (median, range 0.048 to 0.400) fg IgG/platelet before treatment. Treatment with neuraminidase removed 0.79 (median, range 0.06 to 6.70) nmol sialic acid/ 10^8 platelets. PA-IgM after treatment, 0.038 (median, range 0.009 to

Table 1 Platelet sialic acid removed, in vivo distribution, and sites of sequestration of platelets treated with neuraminidase. The reference values were measured in a matched group of 11 baboons (13)

Baboon	Sialic acid removed (nmol/ 10^8 platelets)	In vivo distribution of platelets				
		At equilibrium		Final		
		Liver	Spleen	Liver	Spleen	Remainder
1 ²	0.42	20.5	20.5	38.8	29.1	31.1
2	0.49	15.1	22.9	36.2	28.9	34.9
3 ²	0.58	27.7	20.6	50.3	22.6	27.1
4	0.63	12.7	20.0	35.6	23.2	41.2
5 ²	0.78	-	-	52.6	19.7	27.7
6	0.97	-	-	45.7	21.9	32.4
7 ²	1.15	26.8	19.4	51.1	17.6	31.3
8 ²	1.88	-	-	68.1	13.7	18.2
9 ²	2.10	-	-	53.7	14.3	32.0
Mean	1.00	20.6	20.7	48.6	21.2	30.2
$\pm$ 1 SD	0.60	6.7	1.3	10.6	5.6	6.8
Reference	-	15.8	16.0	40.8	24.7	34.5
$\pm$ 1 SD	-	2.9	1.9	6.0	4.2	5.5
³ p-value	-	0.06	<0.001	0.05	0.14	0.16

¹ Percentages of that of whole body radioactivity.

² In vitro aggregation studies were done on these six baboons.

³ Student's t-test for unpaired data.

Table 2 Effect of treatment with neuraminidase on the in vitro aggregation response of platelets to agonists. Values are the mean percentage change (± 1 SD) in light transmission of six studies. A median of 1.22 (range 0.42 to 2.10) nmol sialic acid/ 10^8 platelets was removed

Agonist	Agonist concentration	Pre-treatment response	Post-treatment response	¹ p-value
ADP	20 μ M	43 $\pm$ 11	48 $\pm$ 9	0.16
	10 μ M	27 $\pm$ 13	34 $\pm$ 15	0.10
	5 μ M	19 $\pm$ 12	24 $\pm$ 13	0.006
Collagen	0.05 g/l	66 $\pm$ 13	75 $\pm$ 11	0.11
	0.005 g/l	3 $\pm$ 4	19 $\pm$ 14	0.08
Adrenaline	0.01 μ M	11 $\pm$ 2	20 $\pm$ 6	0.003

¹Student's *t*-test for paired data

0.081) fg/platelet, was not different ($p > 0.50$) from that before treatment. PA-IgG increased significantly ($p < 0.001$) to 0.322 (median, range 0.076 to 0.680) fg/platelet after treatment with neuraminidase. The increase ranged from 0.007 to 0.309 fg/platelet. There was a significant correlation between the amount of sialic acid removed and the increase in PA-IgG (Fig. 3).

The Effect of the Increase in PA-IgG on the Shortening of MPLS

This was investigated in 15 baboons. Treatment with neuraminidase removed 0.20 (median, range 0.01 to 0.93) nmol sialic acid/ 10^8 platelets. This resulted in a shortening of 46 (median, range 0 to 86) h of the MPLS and an increase of 0.046 (median, range 0.007 to 0.309) fg IgG/platelet. The shortening of MPLS and the increase in PA-IgG was significant ($p < 0.001$ in both cases). The correlation between the shortening of MPLS and the increase in PA-IgG is shown in Fig. 4.

In Vitro Platelet Aggregation

In six studies, the in vitro aggregation response of platelets to agonists was not adversely influenced by the removal of sialic acid with neuraminidase (Table 2). Untreated and treated platelets were handled in an identical way, and the results were therefore comparable. A median of 1.22 (range 0.42 to 2.10) nmol sialic acid/ 10^8 platelets was removed. The treated platelets tended to be somewhat more sensitive to agonists, especially at lower concentrations.

Discussion

The relevance of removing membrane sialic acid to study senescence of blood cells has been questioned (20, 21). It was therefore necessary to prove that the platelets treated with neuraminidase were viable. This we did in two ways. First, the sites of sequestration of treated platelets did not differ from those of untreated platelets (Table 1). Secondly, platelet function was not adversely affected by treating the platelets with neuraminidase, their in vitro aggregation response to agonists was not significantly different from that of platelets not so treated (Table 2). Treatment of the platelets with neuraminidase therefore had no apparent adverse effects, and the measured differences in MPLS may thus be attributed to the removal of sialic acid.

The shape of the mean survival curve of the treated labelled platelets (Fig. 1) indicated two components, implying two subpopulations. The first was cleared from the circulation within 3 h after injection. These platelets were most likely sequestered by the macrophages of the monocyte-macrophage system, and were

permanently removed from the circulation. This is so because slightly more asialo-platelets than untreated platelets were found in the spleen and liver at equilibrium (Table 1), and there was a positive correlation between the reduction in circulating radioactivity from 5 to 180 min and the percentage sialic acid removed. It is therefore reasonable to assume that enough sialic acid was removed to make them recognised as "old" and ready for permanent sequestration by the macrophages.

The second subpopulation had a nearly linear survival curve, and their disappearance almost paralleled that of untreated platelets (Fig. 1), implying that their removal was age-dependent (22). Their MPLS was also shorter than that of the untreated platelets in the same baboons. The linear survival curve is consistent with the view that equal amounts of sialic acid were removed from platelets of all ages. The removal of sialic acid therefore induced accelerated ageing of the platelets. Similar results were obtained in studies on rabbits (5-9). It was, however, necessary to remove roughly 15% to cause clearance of the platelets within 1 h after injection (8). In the baboons it was only after more than 40% of the sialic acid was removed that MPLS was shorter than 10 h. The difference may be species specific. There seems to be an evolutionary reciprocity in the expression of the antigenic structures of the senescence-related anti-Gal antibody by old platelets of mammals (1, 23, 24). It is therefore possible that the differences in MPLS after treatment with neuraminidase is the availability of the antigen on the platelets of the two species. The discrepancy between our results and those reported in the rabbits may also be explained by the number of blood samples collected within the first 20 h after the injection of the labelled platelets. We collected seven blood samples, whereas only two samples were collected from the rabbits.

The exponential correlation between the shortening of MPLS and the amount of sialic acid removed (Fig. 2) probably has little resemblance to the normal process of in vivo platelet aging. It is probable that treatment with neuraminidase removes more sialic acid than is necessary to signal senescence (2, 21). The correlation nevertheless implies that sialic acid plays a part in the determination of MPLS, and thus in the mechanism of platelet senescence. The fact that the correlation was exponential further implies that it is necessary to remove only a critical amount of sialic acid to signal senescence.

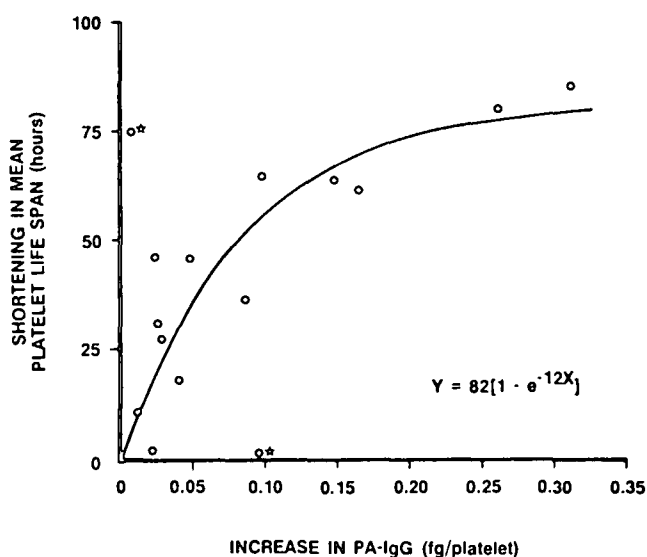


Fig. 4 Exponential correlation between the shortening of MPLS and removal of sialic acid with neuraminidase and the subsequent increase in PA-IgG (*data points not used)

The mechanism of recognition of senescent cells by the macrophages of the monocyte-macrophage system is still not known. An autoimmune mechanism seems likely. Antigenic structures that react with senescent-related anti-Gal antibodies has been identified on "old" platelets (1). The IgG may bind to the senescent cell antigen to form antigen-antibody complexes (1, 2, 23, 24), or the macrophages may recognise the antigen itself (25). Our results favour the binding of IgG to the antigen. This is indicated by the linear correlation between sialic acid removed and the increase in PA-IgG (Fig. 3), and the exponential relationship between the shortening of MPLS and the increase in PA-IgG (Fig. 4). It is not possible to estimate the amount of IgG that must be attached to signal phagocytosis of platelets by the macrophages. It is probably not much. Treatment with neuraminidase probably removes more sialic acid than is necessary to signal senescence (2, 21). This assumption is supported by the linear correlation between the reduction in circulating radioactivity in the first 3 h following the injection of treated platelets and the amount of sialic acid removed. Also, the amount of antibody that binds to the senescent cell antigen, anti-Gal, represents only about 1% of the total IgG (19, 24). It is, however, highly likely that a critical number of antigen-antibody complexes have to form before the platelets will be recognised as "old" and sequestered by the macrophages, the exponential correlation between the increase in PA-IgG and shortening of MPLS (Fig. 4) supports such an assumption.

In conclusion, the differences in the shape of the survival curves and in the mean life span of the platelets implies that the removal of sialic acid from platelets is analogous to the normal process of ageing of platelets in the circulation. First, a subpopulation of treated platelets was removed from the circulation within 3 h after injection. It is likely that enough sialic acid was removed to make these platelets recognisable for phagocytosis and destruction by the macrophages. Secondly, the linear, but shorter than normal, disappearance of the treated platelets that remained in the circulation after the first 3 h suggests that the removal of sialic acid shortened the MPLS of these platelets and that subsequent sequestration of platelets by the macrophages mimics senescence-related mechanisms. Thirdly, the almost normal numbers of senescent platelets that were sequestered in the spleen, liver and bone marrow is consistent with the view that the senescent platelets were sequestered by "normal" mechanisms.

We were also able to identify possible mechanisms involved in platelet senescence. Platelets lose sialic acid from their membrane during their circulation in the blood (3, 4). Because the membrane glycoproteins are enriched with sialic acid (10), it is reasonable to assume that the loss may be the result of sublethal interactions of the platelets with diseased or foreign surfaces. The loss of sialic acid exposes senescent cell antigens (1, 2). An antibody, presumably the anti-Gal antibody (26), recognises the exposed antigen and binds to it to form an antigen-antibody complex. Once sufficient IgG is bound to the platelet membrane, this signals that the platelet is old. The signal to phagocytose and destroy the old platelet is then recognised by the macrophages of the liver, spleen and bone marrow (13). This hypothesis suggests that macrophages recognise senescent cells as "foreign", and are therefore only performing their normal task of surveillance against foreign cells in the circulation.

Acknowledgements

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INFLUENCE OF PLATELET MEMBRANE SIALIC ACID AND PLATELET ASSOCIATED IgG ON AGEING AND SEQUESTRATION OF BLOOD PLATELETS IN BABOONS

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INTRODUCTION

Blood platelets circulate for approximately 10 days in normal humans. The macrophages of the monocyte-macrophage system then recognise them as old and phagocytose and destroy them. The mechanism through which the macrophages recognise the "old" platelets is not known. A hypothesis to explain red cell ageing and sequestration implicates that membrane sialic acid may play a role. According to this view, red cells lose sialic acid while they are circulating. This exposes a senescent cell antigen on the red cell membrane. IgG recognises and binds to the antigen. Once sufficient antibody-antigen complex is formed, the macrophages recognise the red cells as "foreign" and phagocytose and destroy them¹.

We investigated if a similar mechanism of recognition exists for senescent platelets. Enzymatic removal of platelet membrane sialic acid shortens their mean platelet lifespan markedly², and a senescent cell antigen, similar to that identified for red cells, was identified in old platelets¹.

METHODS

Normal, healthy baboons were used in this study. Blood was collected, and the platelets harvested by differential centrifugation. An aliquot of the platelet rich plasma was removed to determine platelet sialic acid and platelet associated IgG. The platelets were then treated with neuraminidase to remove sialic acid (0.002 U/ml for 30 min at 37°C). An aliquot was again removed to determine platelet sialic acid and platelet associated IgG. The treated platelets were then labelled with ¹¹¹In-oxine, reinjected and their recovery from the circulation and mean lifespan determined^{3,4}. In all baboons, the lifespan of normal untreated platelets was determined within a month after the studies with treated platelets.

RESULTS

The treated, labelled platelets were viable. Their recovery in the circulation, $81 \pm 24\%$, was not significantly different from that of the untreated platelets, $85 \pm 12\%$. Also, in vitro aggregation in response to ADP and collagen was not different from that of untreated platelets.

The disappearance of treated and untreated labelled platelets from the circulation is given in Figure 1. This was determined in 19 baboons. A mean of $20 \pm 15\%$ of the sialic acid was removed. There were two subpopulations of treated labelled platelets. The first, approximately 22%, was removed from the circulation within the first 3 hours after their injection. It is likely that enough sialic acid was removed to signal that this subpopulation was old, and must be destroyed by the macrophages. The remaining subpopulation had a linear, but shorter than normal survival curve. This indicates that these platelets were removed as a result of senescence. The linearity further implicates that equal amounts of sialic acid was removed from platelets of all ages.

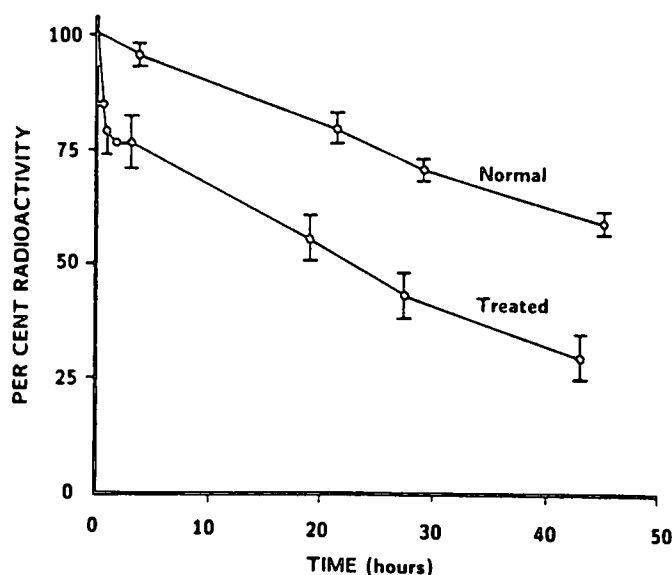


Figure 1. Platelet survival curves of normal (untreated) and neuraminidase-treated ^{111}In -labelled platelets. The untreated platelets disappeared from the circulation in an age-dependent manner; this is reflected by the linear survival curve. The disappearance curve of the treated platelets shows two components; an exponential rapid clearance, and a second phase parallel to that of normal platelets.

Figure 2 illustrates the relationship between the shortening in platelet lifespan as a result of the removal of sialic acid and the amount of sialic acid removed. The exponential relationship is consistent with the view that only a given amount of sialic acid have to be removed to signal platelet senescence and thus permanent removal of the platelets from the circulation.

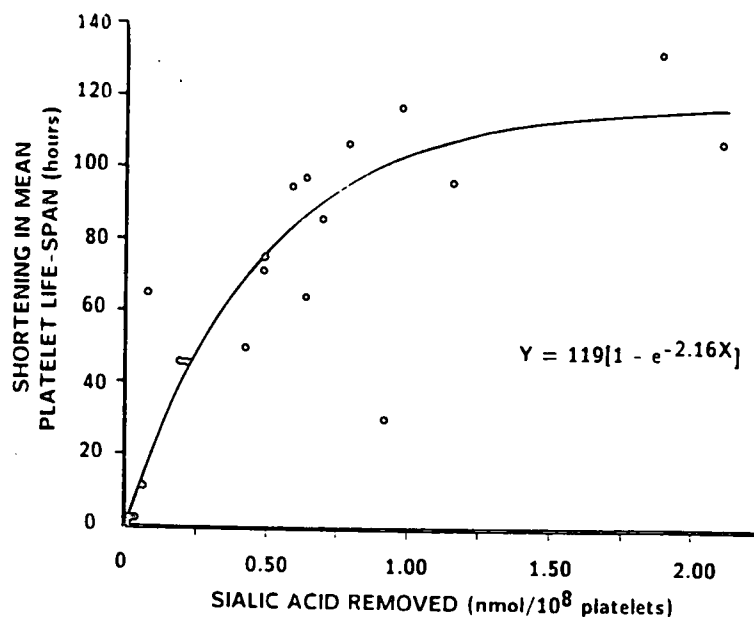


Figure 2. The relationship between decrease in the sialic acid content of the platelet membrane after treatment and the shortening in platelet survival as a result of the removal of sialic acid indicates that the removal of sialic acid shortens platelet survival.

Removal of sialic acid resulted in an increase in platelet associated IgG. In 26 studies, the linear relationship between the amount of sialic acid removed and the increase in platelet-associated IgG was

$$\text{sialic acid removed} = 0.14 + 0.068 \times \text{increase in IgG} \quad (r = 0.621)$$

The relationship between the shortening in mean platelet lifespan after sialic acid was removed and the increase in platelet-associated IgG is illustrated in Figure 3. The exponential relationship is consistent with the view that only a given amount of IgG have to attach to platelets to signal senescence, and thus sequestration and permanent removal of the platelets from the circulation.

CONCLUSIONS

The results of this study strongly suggest that platelet sialic acid and platelet-associated IgG plays a role in platelet ageing and subsequent sequestration by the macrophages. We hypothesize that platelets loose sialic acid from their membranes while they are circulating. The loss of sialic acid exposes a neo-antigen, presumably similar to the senescent cell antigen described in red cells. An antibody recognises the exposed antigen and binds to it to form an antigen-antibody complex. Once sufficient IgG is bound to the platelet membrane, this signals that the platelet is aged. This signal to phagocytose and destroy the "old" platelets is recognised by the macrophages of the monocyte-macrophage system, especially those located in the spleen, liver and bone marrow.

This hypothesis is logical. It suggests that the monocyte-macrophage system will recognise an aged cell in the circulation as "foreign". It is therefore not necessary to postulate a new mechanism specifically aimed at the recognition of senescent platelets. Macrophages will only be performing their normal task of surveillance against foreign cells when they remove senescent platelets from the circulation.

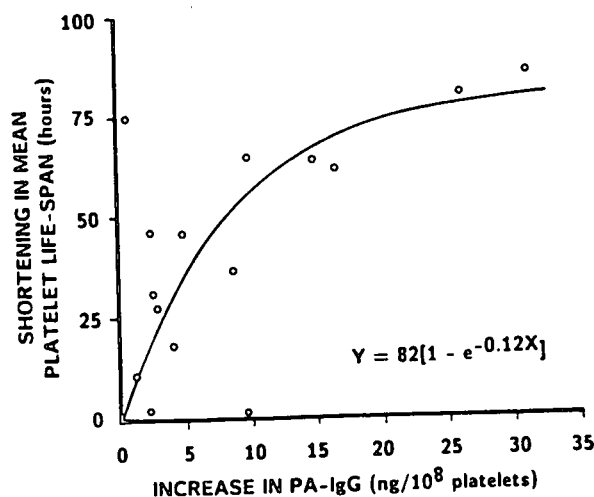


Figure 3. There is a correlation between the increase in platelet-associated IgG after removal of sialic acid and the shortening in mean platelet lifespan. This supports the view that removal of sialic acid and the resultant increase in platelet-associated antibody induces sequestration of platelets.

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Blood platelet kinetics in normal subjects modelled by compartmental analysis

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Abstract. The purpose of this study was to describe the function of platelets throughout their life span by expressing their in vivo distribution and kinetic behaviour in mathematical terms by using multicompartmental analysis. The distribution of indium-111 labelled platelets in five normal subjects was imaged and quantified with a scintillation camera image processing system. Serial blood samples were also obtained. The data were modelled using the SAAM (Simulation Analysis and Modelling) compartmental computer program. Five models were entertained to evaluate the role of platelets that were either functional or injured during collection and their interaction with the liver, spleen and vascular endothelium. Models were evaluated by comparing *F* values calculated from the least squares estimate obtained from each model. The Dornhorst function was used to describe the sequestration of platelets in the compartmental model. Results indicated that the data could not be satisfactorily simulated when compartments were included that simulated only functional and sequestered platelets (model 1). It was necessary to include compartments that simulated the kinetics of collection-injured platelets in the liver (model 2) and spleen (model 3). The model that simulated the interaction with the vascular endothelium (model 5) showed a visual but not significant improvement in the fitting of the observed data compared to model 3. The mean organ uptake and range indicated in parentheses were calculated at equilibrium. There were 20% (15%–27%) of the injected platelets in the spleen, 10% (8%–11%) in the liver and 70% (64%–75%) in the circulation. The relatively high accumulation of activity in the spleen was as a result of the slow transit time of the functional platelets through the spleen of 5.1 (3.5–6.0) min compared with the transit time through the liver of 0.33 (0.19–0.50) min. The 9% (5%–12%) collection-injured platelets in the spleen and 10% (5%–16%) in the liver had longer transit times than functional platelets. Platelet sequestration was well simulated with

the compartmental model. The mean platelet survival time estimated by the compartmental model and standard curve fitting techniques did not differ significantly. A multicompartmental model and reference range for platelet kinetics have been established and may prove to be clinically useful in platelet disease.

Key words: Blood platelets – Indium 111 – Tropolone – Scintillation camera – Platelet kinetics – Compartmental modelling

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Introduction

Blood platelets play an essential role in haemostasis and the vascular response to injury. Many techniques have been developed to study their function and to quantify the behaviour of platelets in the circulation. One approach involves the assessment of platelet function by in vitro methods. This does not reflect the true in vivo behaviour; as stated by Peters (1988), the kinetics of distribution immediately following injection are probably more sensitive to cell damage, especially when the platelets are activated in vivo (Powers et al. 1983; Isaka et al. 1991). The other approach is to study the in vivo behaviour of indium-111 labelled platelets. Mean platelet survival time can be determined from the rate of disappearance of the labelled platelets from the circulation. In vivo function of the labelled platelets can also be studied by imaging their distribution in the body. The limitation of imaging is that within an organ it does not distinguish platelets that are functional from those that have been injured during collection or have been sequestered. Compartmental modelling can be used to overcome this limitation. Heyns et al. (1980b), Peters et al. (1980, 1984), Peters and Lavender (1982), Kotzé et al. (1986) and Wadenvik et al. (1987) used compart-

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mental modelling to investigate the kinetics of functional platelets in the spleen. They used a simplified two-compartmental model which did not simulate the behaviour of collection-injured platelets. Inspection of the accumulation of organ radioactivity shows a transient uptake of in vitro labelled platelets in the liver (Isaka et al. 1991; Peters et al. 1980, 1984; Peters 1988, 1991; Lötter et al. 1986). These collection-injured platelets may, however, also interact with the spleen and vascular endothelium. In addition, two-compartmental modelling simulates only the first 15, 30 or 90 min after reinjection of the platelets (Heyns et al. 1980b; Peters et al. 1980, 1984; Peters and Lavender 1982; Kotzé et al. 1986; Wadenvik et al. 1987) and therefore excludes modelling platelet sequestration.

For the purpose of this study we defined functional platelets as normal unactivated platelets, collection-injured platelets as platelets injured during in vitro labelling and sequestered platelets as those removed permanently from the circulation.

The purpose of this study was to use multicompartmental analysis to describe the kinetic behaviour of platelets by expressing their in vivo distribution in mathematical terms. This makes possible the calculation of the relative contributions of collection-injured, functional and sequestered platelets to the observed organ radioactivity. The transient uptake of collection-injured platelets in the liver is also quantified. The mean platelet survival time can be estimated using the simultaneous disappearance of platelets from the circulation and the appearance of platelets in the organs of the reticulo-endothelial system.

Methods

Platelet labelling. Platelets were harvested and labelled according to the method described by Kotzé et al. (1991). Briefly, 85 ml of blood was drawn into syringes containing 15 ml ACD A (Baxter Travenol, Baxter Laboratories). Platelets were harvested by differential centrifugation and repeated washing to get rid of red cells. The platelets were concentrated by centrifugation and resuspended in 0.3 ml plasma. Tropolone and 17 MBq of ^{111}In -chloride were added and then incubated for 4 min at room temperature. After incubation the platelets were again centrifuged and the unbound radionuclide removed by withdrawing the supernatant. The labelled platelets were finally resuspended in platelet-poor plasma for reinjection. Viability of labelled platelets was assessed in vitro by their aggregation response to ADP and they were not reinjected unless aggregates consisting of ten or more platelets were observed.

In vivo quantification (Heyns et al. 1983; Kotzé et al. 1986). The in vivo distribution of the ^{111}In -labelled platelets was imaged with a General Electric Starcam 400AC scintillation camera and data processing system. After reinjection, dynamic anterior images including the heart, liver and spleen were acquired in two phases; 30 images of 30 s duration followed immediately by 25 images of 3 min each. Static anterior and posterior images that included the whole body were acquired at 2 h after reinjection of the labelled platelets and then daily for 7 days.

The images acquired during the sequestration phase were processed as follows. Using the chest image, regions of interest were selected over the liver, spleen and heart. A region of interest was also selected for the area excluding the liver, spleen and heart. Regions were also selected on the other images to include the whole body. Integrated counts were obtained for the anterior and corresponding posterior regions of interest. The geometrical mean counts for each region of interest was calculated as the square root of the product of the anterior and posterior integrated counts. The corrected activity in the region of interest was expressed as a percentage of the geometrical mean-corrected whole body counts. All the corrected counts were summed to represent the whole body counts. The corrected counts for the liver, heart and spleen were expressed as a percentage of the whole body counts. Radioactivity not in the spleen or liver at the end of the life span of the platelets was assumed to be in the rest of the reticulo-endothelial system (Heyns et al. 1983).

Only anterior images were acquired during the equilibration phase. Regions of interest over the liver, spleen and heart were selected on the dynamic anterior images to generate time-activity curves. To normalize these curves to percentage organ activity, the geometrical mean distribution data obtained at 2 h were used.

The geometrical mean-corrected time-activity curves of the spleen, liver, heart and blood were subjected to compartmental modelling. Average time-activity curves for the five patients were calculated. The error bars represent one standard deviation of the mean of the observed data points in all the graphs. For ease of interpretation, only every tenth observed data point for the first 15 min and every fourth data point for the next 75 min are shown on the graphs for the liver and spleen during the equilibration phase. Data for the heart resembled those of the blood; therefore these data are not included in the figures.

Determination of the mean platelet survival time by the standard curve fitting technique. Blood samples were obtained at 5, 10, 20, 40, 60, 90, and 150 min after reinjection of the labelled platelets and then twice daily for 6 days. The mean platelet survival time was calculated by fitting the Dornhorst mathematical function to the blood clearance curve (Lötter et al. 1986, 1988). The Dornhorst function was used because it was incorporated in the multicompartment computer model.

Normal subjects. The particulars of the five normal healthy subjects and reference values for our laboratory are given in Table 1. The following reference values were used as the criteria to assess whether the platelet kinetics of the subjects were normal: platelet count, mean platelet survival time and percentage recovery of injected labelled platelets in the circulation at equilibrium. The patients were instructed not to take any antiplatelet drugs for at least 2 weeks before and throughout the study.

Informed consent was obtained and the study was approved by the Ethics Committee for Human Experimentation of the Orange Free State Provincial Administration and University of the Orange Free State.

Compartmental modelling. In compartmental model building a model is "tentatively entertained" rather than assumed (Box and Hunter 1965). In this study a basic model for normal subjects, model 1 (Fig. 1), was entertained and then improved upon by entertaining four other models of increasing complexity (Figs. 2–5). The method used to assess the process of model optimization, i.e. the lack of fit or the goodness of fit of an entertained model, was by minimising the least squares estimate. To decide whether the differences among models was significant, a standard *F*-test was

Table 1. Subject information

Subject number	Age (yr)	Sex	PC ($10^{-9}/l$)	MPST (h)	Recovery (%)
1	23	Male	260	203	59.3
2	31	Male	303	221	50.3
3	22	Female	232	250	62.1
4	34	Male	393	240	46.1
5	32	Male	248	222	59.4
Mean $\pm$ SD	28 $\pm$ 5		287 $\pm$ 65	227 $\pm$ 18	55.4 $\pm$ 6.9
Reference values			150–421 ^a	226 $\pm$ 25 ^b	61 $\pm$ 12 ^c

PC, Platelet count; MPST, mean platelet survival time

^a Wessels et al. (1985)

^b Lötter (1985)

^c Wessels et al. (1985); Heyns et al. (1985)

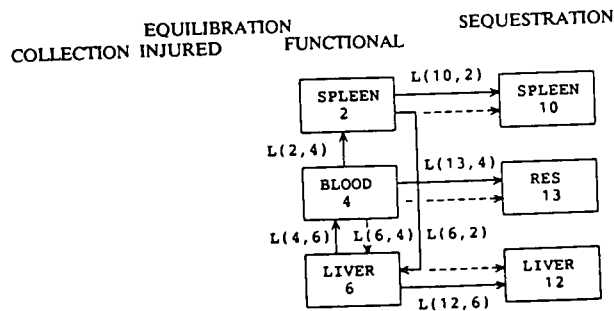


Fig. 1. Model 1: the most basic compartmental model investigated to model the kinetics of functional platelets in the spleen (compartment 2), circulation or blood (compartment 4) and the liver (compartment 6) during the first 7 h after reinjection of ^{111}In -labelled platelets. Compartments 10, 13 and 12 modelled the sequestration of platelets in the spleen, remainder of the reticulo-endothelial system (RES) and liver throughout the life span of the platelets

applied to the least squares estimate of each model (Thakur 1983; Mendenhall 1971). Graphs were, however, highly regarded throughout the fitting and optimisation procedure (Box and Hunter 1965). The computer program Simulation, Analysis and Modelling (SAAM) was used in this study (Kuikka et al. 1991; Foster and Boston 1983).

The parameters calculated during compartmental modelling are expressed as a mean and range (given in parentheses). Platelet kinetics were modelled during the equilibration and sequestration phases.

Equilibration phase. This phase included the first 7 h after platelet reinjection. During this period equilibrium was established between the injected labelled platelets and circulating platelets. Model 1 (Fig. 1) was entertained to simulate labelled functional and sequestered platelets. Compartments 10, 12 and 13 (which simulated sequestration) has little influence during the equilibration phase and will be discussed later. When reinjected, the labelled platelets were in the blood (compartment 4). They then redistributed to the spleen compartment 2 and the liver compartment 6. The rate of redistribution was determined by the rate constants. The rate constant $L(2,4)$ determined the fraction of platelets that flowed per minute from the blood compartment 4 to the spleen compartment 2. The rate

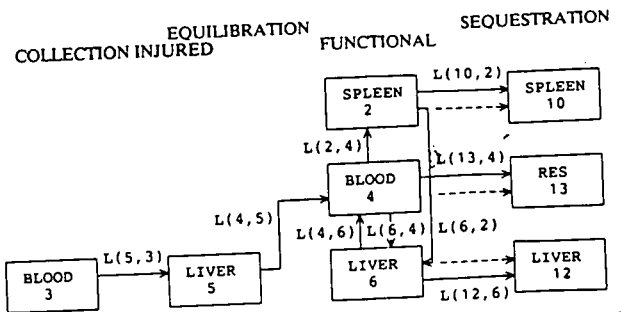


Fig. 2. Model 2: this model also simulates the kinetics of collection-injured platelets in the liver. The collection-injured reinjected platelets were initially in the blood (compartment 3), and then transiently accumulated in the liver (compartment 5)

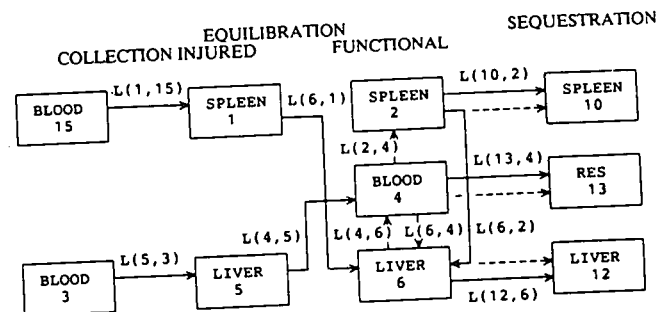


Fig. 3. Model 3: includes additional compartments 15 and 1 for simulation of the transient accumulation of collection-injured platelets in the spleen

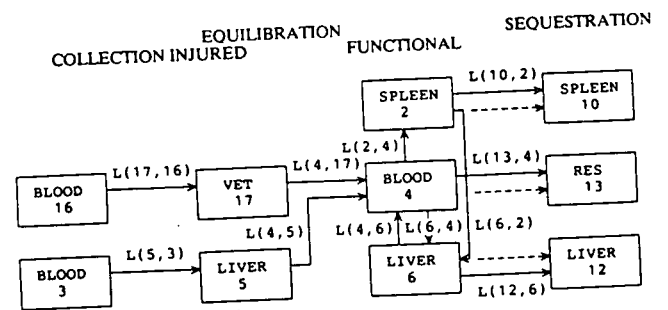


Fig. 4. Model 4: simulates the interaction of collection-injured platelets with the vascular endothelium (VET) (compartments 16 and 17). The simulation of collection-injured labelled platelet interaction with the spleen was excluded

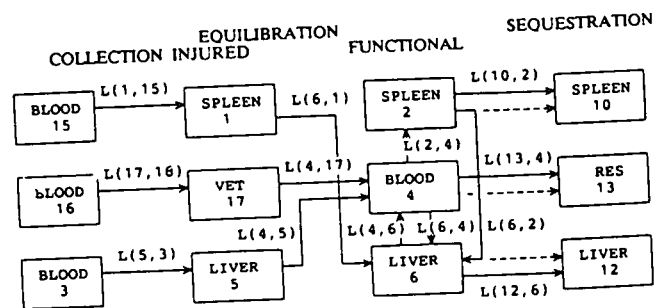


Fig. 5. Model 5: includes all the compartments that were entertained in this study, i.e. the interaction of collection-injured platelets with the liver, spleen and vascular endothelium was simulated

constant $L(6,2)$ determined the rate of flow of platelets from the spleen to the liver. The transit time of platelets through the spleen was calculated from the inverse of the rate constant $L(6,2)$.

In model 2, two compartments were added (Fig. 2). Compartment 3 simulated collection-injured platelets in the blood that transiently accumulated in the liver, compartment 5.

Similarly, in model 3 the compartment introduced to model the collection-injured platelets in the blood that went to the spleen was compartment 15 and compartment 1 simulated these platelets in the spleen (Fig. 3).

Compartments 16 and 17 were introduced in model 4 (Fig. 4) to simulate the kinetics of the interaction for collection-injured platelets with the vascular endothelium. Collection-injured platelets in the spleen were not simulated and compartments 15 and 1 were excluded from this model.

All the compartments were included in model 5 to simulate the interaction of collection-injured platelets with the liver, spleen and vascular endothelium (Fig. 5).

Sequestration phase. The sequestration phase was defined as the time interval from equilibration until the end of the platelet life span. The mathematical function selected for this phase had to simulate the survival of a population of labelled platelets that were sequestered by random and age-related processes. The Dornhorst mathematical function was used for this purpose (Dornhorst 1951; Lötter et al. 1986). Only one compartmental model for the sequestration phase was used (Figs. 1–5). Compartments 10, 12 and 13 simulated the platelets that were sequestered in the spleen, liver and the remainder of the reticulo-endothelial system. The rate constants $L(10,2)$, $L(13,4)$ and $L(12,6)$ determined the rate of random sequestration in the spleen, reticulo-endothelial system and liver respectively.

The rate of age-dependent sequestration for each organ remained constant and therefore could not be simulated in a compartmental model by the use of rate constants. These rates were calculated as follows: from the Dornhorst equation (Dornhorst 1951) the number of labelled functional platelets that are sequestered per hour as a result of age is given by

$$K = \frac{Ake^{-kT}}{(1 - e^{-kT})}, \quad (1)$$

where T = the potential age-dependent life span in hours, that is the life span in the absence of random destruction; A = the total number of functional platelets; and k = the total rate constant for random platelet destruction, calculated as the weighted sum of the rate constants $L(10,2)$, $L(13,4)$ and $L(12,6)$.

In Figs. 1–5 the dashed lines represent the age-dependent sequestration in each organ. The following parameters were obtained by solving the compartmental model using SAAM: the fraction of total age-dependent sequestration for each organ, the potential survival time (T), the total number of functional platelets (A) and the rate constants $L(10,2)$, $L(13,4)$ and $L(12,6)$.

The mean platelet survival time (MPST) is determined from the Dornhorst function as

$$\text{MPST} = \frac{1 - e^{-kT}}{k}. \quad (2)$$

Results

Equilibration phase

Figure 6 shows the graphs of the mean observed and simulated data points calculated with model 1 (Fig. 1).

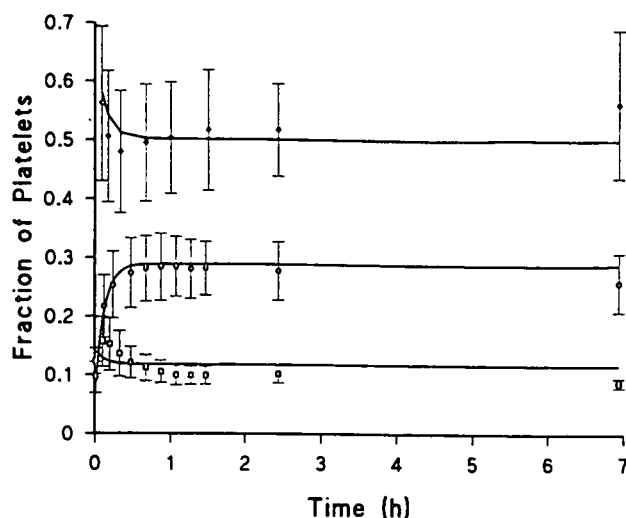


Fig. 6. The mean radioactivity-time curves of ^{111}In -labelled platelets during the equilibration phase simulated with model 1 (solid lines). The symbols represent the mean and standard deviation of the observed data for the blood (diamonds), spleen (circles) and liver (squares). This model does not simulate the observed data well

With this model the kinetics of the functional platelets were simulated. The platelet activity in the liver and the circulation or blood during the 1.5 h following platelet reinjection was not adequately modelled. This is also reflected by the large least squares estimate value for the liver (Table 2).

The behaviour of collection-injured platelets in the liver was simulated with model 2 (Fig. 2). A better fit to the data was obtained with this model and the least squares estimate for all organs was less than the least squares estimate obtained with model 1 (Table 2). The reduction in the least squares estimate was significant for all patients (Table 3). This model was regarded as the standard with which the other models were compared. The improvement in the fit of the liver data with model 2 is shown by the mean radioactivity-time curve (Fig. 7).

The transient accumulation of collection-injured platelets in the spleen was modelled with model 3 (Fig. 3). The reduction in the least squares estimate (Table 2) with model 3 in comparison with model 2 resulted in a simulation that was statistically better in four of the five subjects (Table 3). The simulated data for the liver and spleen fitted the observed data well (Fig. 8). The blood data were not as well simulated.

The purpose of model 4 (Fig. 4) was to investigate whether the blood data could be better simulated by modelling the interaction of the collection-injured platelets with the vascular endothelium and liver, but not with the spleen. The improvement in the simulation of the blood data and the deterioration in the simulation of the spleen data are shown in Fig. 9. Three of the five patients had a significantly better fit with model 4 than was obtained with model 2 (Table 3).

Table 2. Least squares estimate obtained from models 1, 2, 3, 4 and 5

Subject number	Spleen	Blood	Liver	Heart
<i>The least squares estimate obtained from model 1</i>				
1	0.43	0.18	3.19	0.04
2	0.06	0.05	2.27	0.13
3	0.07	0.07	1.82	0.10
4	0.10	0.05	1.91	0.17
5	0.12	0.05	0.98	0.03
Mean $\pm$ SD	0.16 $\pm$ 0.15	0.08 $\pm$ 0.06	2.03 $\pm$ 0.80	0.09 $\pm$ 0.06
<i>The least squares estimate obtained from model 2</i>				
1	0.06	0.03	0.18	0.07
2	0.16	0.02	0.35	0.03
3	0.07	0.06	0.06	0.05
4	0.06	0.05	0.19	0.13
5	0.11	0.00	0.04	0.03
Mean $\pm$ SD	0.09 $\pm$ 0.04	0.03 $\pm$ 0.02	0.16 $\pm$ 0.12	0.06 $\pm$ 0.04
<i>The least squares estimate obtained from model 3</i>				
1	0.05	0.01	0.09	0.06
2	0.07	0.01	0.20	0.03
3	0.04	0.04	0.08	0.05
4	0.06	0.02	0.15	0.13
5	0.05	0.01	0.06	0.03
Mean $\pm$ SD	0.05 $\pm$ 0.01	0.02 $\pm$ 0.01	0.12 $\pm$ 0.06	0.06 $\pm$ 0.04
<i>The least squares estimate obtained from model 4</i>				
1	0.05	0.01	0.07	0.03
2	0.13	0.02	0.12	0.04
3	0.15	0.02	0.06	0.08
4	0.08	0.01	0.10	0.03
5	0.10	0.00	0.04	0.02
Mean $\pm$ SD	0.10 $\pm$ 0.04	0.01 $\pm$ 0.01	0.08 $\pm$ 0.03	0.04 $\pm$ 0.02
<i>The least squares estimate obtained from model 5</i>				
1	0.05	0.00	0.08	0.03
2	0.06	0.01	0.23	0.03
3	0.04	0.02	0.07	0.07
4	0.06	0.01	0.12	0.02
5	0.07	0.00	0.07	0.02
Mean $\pm$ SD	0.06 $\pm$ 0.01	0.01 $\pm$ 0.01	0.11 $\pm$ 0.07	0.03 $\pm$ 0.02

In model 5 the interaction of the collection-injured platelets with the vascular endothelium, liver and spleen was simulated (Fig. 5). The visual comparison of the results obtained with model 5 (Fig. 10) and those obtained with model 4 (Fig. 9) shows an improvement in the simulation of the spleen data without a deterioration in the simulation of the other organ data. Model 5 fitted the observed data better than model 2 in four of the five subjects (Table 3). Model 5 therefore did not simulate platelet kinetics better than model 3.

The kinetic parameters of the functional and collection-injured platelets obtained during the equilibration phase for models 1–5 are shown in Table 4. The mean and the range of the percentage distribution, transit times and flow rates for the functional and collection-

Table 3. *F* values calculated for the comparison of the least squares estimate obtained from models 1, 2, 3, 4 and 5

Subject number	Spleen	Blood	Liver	Heart	Mean <i>F</i>
<i>Model 2 compared to model 1</i>					
1	315.38	16.08	874.99	−20.15	296.57* ¹
2	−32.56	4.24	290.71	181.83	111.06* ¹
3	−1.45	0.64	1516.97	47.38	390.89* ¹
4	39.56	0.15	483.45	15.45	134.65* ¹
5	7.23	29.90	1250.58	−3.75	320.99* ¹
<i>Model 3 compared to model 2</i>					
1	7.66	8.65	55.00	8.44	19.94* ²
2	69.38	1.63	35.12	2.91	27.26* ²
3	52.17	1.28	−10.68	0.18	10.74* ²
4	−3.35	2.80	10.82	3.30	3.39
5	55.48	−0.24	−17.61	8.65	11.57* ²
<i>Model 4 compared to model 2</i>					
1	4.30	2.38	41.63	35.16	20.87* ³
2	5.88	0.26	49.59	−6.76	12.24* ³
3	−13.08	1.06	−1.82	−8.36	−5.55
4	−7.06	2.20	22.83	104.48	30.61* ³
5	3.10	−0.01	−3.24	11.22	2.77
<i>Model 5 compared to model 2</i>					
1	3.98	4.31	21.53	15.88	11.43* ⁴
2	27.23	0.80	7.81	2.09	9.48* ⁴
3	14.39	0.78	−1.86	−4.09	2.30
4	−0.60	2.02	8.66	30.33	10.10* ⁴
5	10.74	0.03	−6.66	9.93	3.51* ⁴

*¹ Statistically significant ($P > 0.95$, $F > 4.04$)

*² Statistically significant ($P > 0.95$, $F > 4.04$)

*³ Statistically significant ($P > 0.95$, $F > 3.19$)

*⁴ Statistically significant ($P > 0.95$, $F > 2.8$)

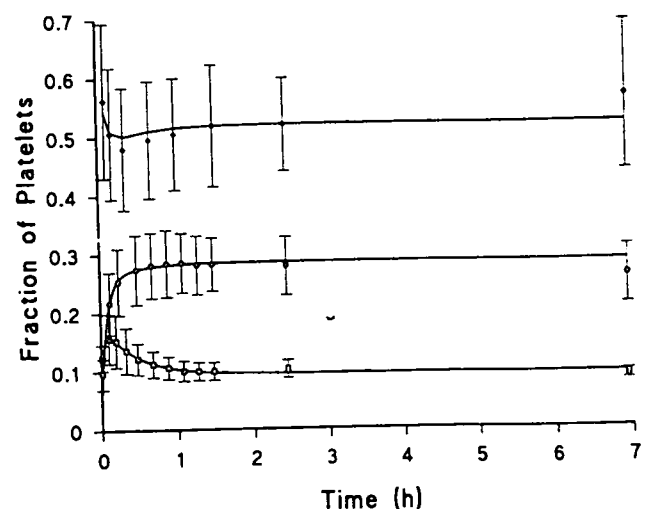


Fig. 7. The mean radioactivity-time curves of ¹¹¹In-labelled platelets during the equilibration phase simulated with model 2 (solid lines). The symbols represent the mean observed data for the blood (diamonds), spleen (circles) and liver (squares). Model 2 simulated the liver observed data accurately.

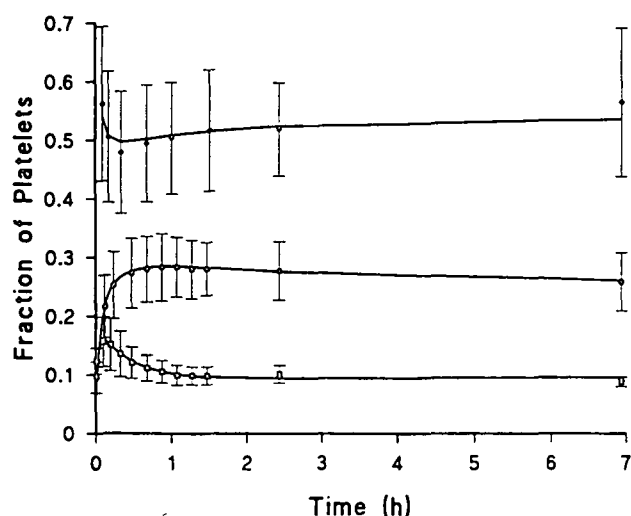


Fig. 8. The mean observed data simulated with model 3 (solid lines). The symbols represent the mean observed data for the blood (diamonds), spleen (circles) and liver (squares). Model 3 simulated the liver and spleen data well

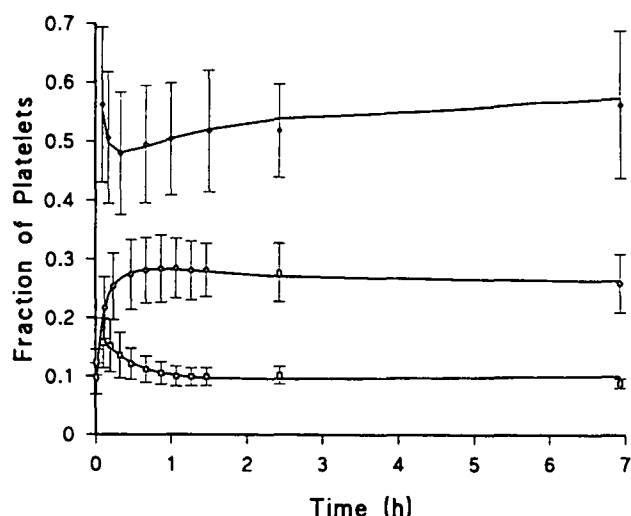


Fig. 10. The mean observed data simulated with model 5 (solid lines). The symbols represent the mean observed data for the blood (diamonds), spleen (circles) and liver (squares). Model 5 simulated the blood, spleen and liver observed data well

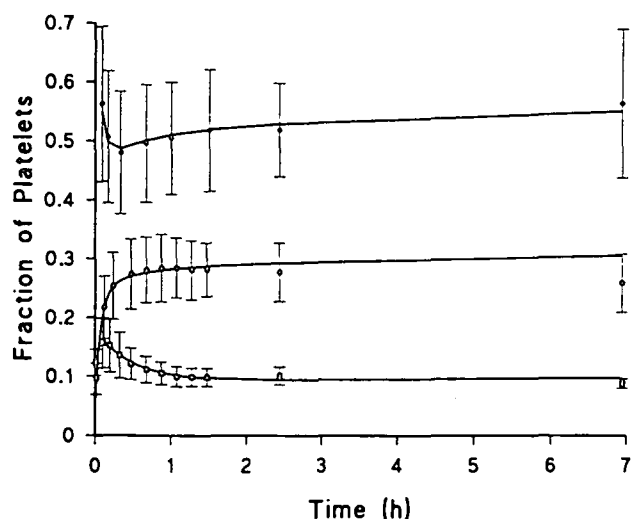


Fig. 9. The mean observed data (diamonds: blood; circles: spleen; squares: liver) and mean simulated data obtained with model 4 (solid lines). Model 4 simulated the blood and liver data well but the spleen was not well simulated

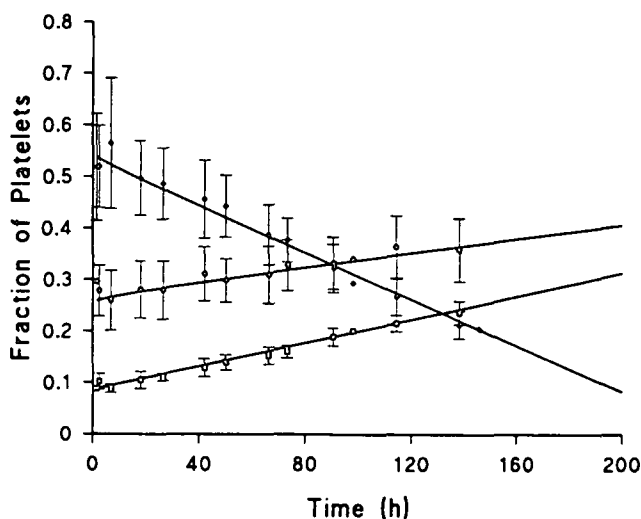


Fig. 11. Mean radioactivity-time curves of ^{111}In -labelled platelets during the sequestration phase. The plot of the mean observed data for the blood (diamonds), spleen (circles) and liver (squares) with error bars (one standard deviation). The simulated data are represented by solid lines

injured platelets in the liver, spleen and vascular endothelium are presented.

Sequestration phase

A good fit was obtained between the simulated and the observed data during the sequestration phase (Fig. 11). The mean platelet survival time and platelet production for the standard curve fitting technique and compartmental modelling are shown in Table 5. The estimates obtained with the two methods were not significantly different ($P > 0.05$, Student's t -test for paired data). This

indicated that compartmental modeling conforms to the conditions of the Dornhorst function. A mean of 30% (3%–39%) of the labelled platelets were randomly sequestered.

The distribution of the labelled platelets simulated by the multicompartmental model at the end of sequestration was 42% (36%–47%) in the spleen, 33% (26%–39%) in the liver and 23% (14%–35%) in the reticulo-endothelial system. Random sequestration did not occur in the liver, but resulted in platelet accumulation of 19% (1%–36%) in the spleen and 11% (2%–26%) in the rest of the reticulo-endothelial system.

Table 4. The mean and range of the platelet kinetic parameters obtained from multicompartmental modelling when models 1, 2, 3, 4 and 5 were applied to the normal subjects' data

	SAAM notation	Model 1	Model 2	Model 3	Model 4	Model 5
<i>Liver</i>						
Collection-injured platelets Percentage			9 4-16	10 5-16	10 5-16	10 5-16
Transit time (min)	$L(4,5)^{-1}$		27 14-40	29 20-38	28 14-53	24 18-30
Functional platelets Transit time (min)	$L(4,6)^{-1}$	0.39 0.18-0.57	0.40 0.34-0.47	0.33 0.19-0.50	0.25 0.19-0.29	0.29 0.20-0.33
Flow to liver (min^{-1})	$L(6,4)$	0.45 0.24-0.68	0.31 0.24-0.35	0.40 0.28-0.63	0.48 0.35-0.65	0.45 0.35-0.67
<i>Spleen</i>						
Collection-injured platelets Percentage				9 5-12		7 4-12
Transit time (min)	$L(6,1)^{-1}$			208 60-471		133 85-200
Functional platelets Transit time (min)	$L(6,2)^{-1}$	8.8 5.7-13.0	6.6 5.0-9.6	5.1 3.5-6.0	6.3 4.9-8.2	5.0 3.5-6.7
Flow to spleen (min^{-1})	$L(2,4)$	0.05 0.02-0.10	0.06 0.03-0.09	0.06 0.03-0.09	0.08 0.04-0.12	0.07 0.04-0.11
<i>Vascular endothelium (VET)</i>						
Collection-injured platelets Percentage					15 10-20	11 5-20
Transit time (min)	$L(4,17)^{-1}$				384 70-761	226 99-382
Flow to VET (min^{-1})	$L(17,16)$				0.14 0.02-0.23	0.14 0.06-0.24
<i>Equilibrium distribution</i>						
Spleen (%)		24 18-33	25 17-31	20 15-27	28 23-34	23 17-32
Liver (%)		11 8-14	10 8-12	10 8-11	9 7-10	10 8-12
Blood (%)		65 59-70	66 60-72	70 64-75	63 58-69	67 59-73

Table 5. Mean sequestration parameter results obtained from standard curve fitting techniques of blood sample data and multicompartmental modelling methods

	Standard curve fit	Compartmental modelling
Platelet production ($10^9/\text{l}$ per hour)	1.98 (1.68-2.55)	2.16 (1.49-3.31)
Mean platelet survival time (h)	227 (203-250)	205 (188-255)

Discussion

It is virtually impossible to reach uniqueness in biological modelling, because one can almost always find an alternative or more detailed model as a result of the presence of noise or inexactness of fit (Kuikka et al. 1991). In this study, although five models were entertained to simulate the kinetics of functional and collection-injured platelets, we showed that the increase in detail or complexity of the last two models did not give substantial additional information. Model 3 (Fig. 3) can be regarded as a working definition of the system studied.

Circulating radioactivity could not be simulated adequately with model 3 (Fig. 8). The observed activity in the circulation showed an initial reduction during the first 30 min after the reinjection of the labelled platelets (Fig. 10). It then increased during the next 7 h. This gradual increase was not well simulated. Models 4 and 5 were entertained to obtain a better simulation of the platelets in the circulation (Figs. 4, 5). A compartment that represented the interaction of platelets with the vascular endothelium was included. Although the platelets in the circulation were better simulated (Table 2; Figs. 9, 10), the overall fit for all the subjects did not improve (Table 3). When model 5 was applied it was found that 11% (5%–20%) of the platelets accumulated transiently in the endothelial compartment with a flow rate constant of 0.14 (0.06–0.24) per minute and a transit time of 226 (99–382) min (Table 4). We could not identify this compartment by image quantification. The diffuse nature of the vascular endothelium, resulting in a small signal that could not be distinguished from that of the overlying tissue, is the most probable explanation. The absence of an identifiable vascular endothelial compartment is possibly the reason why model 5 did not fit the data better than model 2 for all subjects (Table 3).

The distribution of the functional platelets in the circulation, liver and spleen is assessed by the flow rate constant and the transit time in the organs. This resulted in a 10% accumulation of functional platelets in the liver. We also found that about another 10% of the injected platelets were collection injured and pooled transiently in the liver with a transit time of 27 min (Table 4). About the same transit time and percentage of collection-injured platelets were estimated by models 2–5. The transit time of the collection-injured platelets in the liver was significantly longer than that of the functional platelets. A similar transient accumulation of platelets in the liver was reported after the administration of protamine sulphate (Heyns et al. 1980a; Pelikan et al. 1979; Jacques 1982).

The splenic transit time for functional platelets was approximately 8.8 minutes when model 1 was used and collection injured platelets were not taken into account (Table 4). A similar transit time was found by other investigators when a two-compartmental model was used (Table 6). The addition of compartments to model the collection-injured platelets (model 3) reduced the transit

time of the functional platelets in the spleen to 5.1 (3.5–6.0) min (Table 4). The flow rate constant for model 3 was increased compared with model 1. The changes in transit time and rate constant can be explained by the following. The two-compartmental model is based on the assumption that all the labelled platelets are accounted for by the platelets in the spleen and blood compartments, whereas the multicompartmental model took into consideration extra "sites" of platelet accumulation. The number of platelets actually present in the circulation, i.e. the blood compartment, at any time was therefore less. The results was that the calculated rate of flow of platelets to the spleen was greater to maintain the same splenic content (Table 4).

The long splenic transit time and small flow rate constant for functional platelets (Table 4) resulted in platelet accumulation out of proportion to the splenic plasma volume (Peters et al. 1985a; Kotzé et al. 1986). The long transit time of the functional platelets in the spleen could possibly be the result of the physical structure of the spleen, which slowed the platelets down in its pulp (Aster 1966; Peters et al. 1983). We found that 9% (5%–12%) of collection-injured platelets accumulated transiently in the spleen (model 3) with a transit time of 208 (60–471) min (Table 4).

We have shown in this study that the transit time of the collection-injured platelets was longer than that of functional platelets. This could be as a result of structural changes induced by activation or as a result of interaction with specific receptors that are exposed on the platelets during the labelling procedure. Another possible mechanism is that the electrostatic repulsion between the platelet and endothelium is disturbed (Hoover et al. 1978; Engelberg 1985). An important finding was that the injury to the platelets, received during the *in vitro* labelling process, was reversible.

A good fit for the sequestration phase was obtained when modelling the observed data of the blood and the organs. The mean platelet survival time obtained by the model and by standard curve fitting methods using the blood samples did not differ significantly. However, when determining the mean platelet survival time from the disappearance of platelets from the circulation and their simultaneous appearance in the spleen and liver, a more reliable determination is obtained. This technique could prove useful especially for patients with shortened mean platelet survival time where fewer datapoints are available from which to determine the mean platelet survival time. The sequestration multicompartmental model was designed to estimate the relative contribution of random and senescent sequestration to the accumulation of organ activity.

This compartmental model may prove clinically useful in studying the platelet kinetic data obtained from patients suffering from idiopathic thrombocytopenic purpura. These patients have very short mean platelet survival times, which makes the results obtained from conventional means of studying platelet kinetics unreli-

Table 6. Platelet kinetic parameters obtained by investigators who used a two-compartmental model

References	Flow to spleen (min^{-1})	Transit time in spleen (min)
ξ		
Peters et al. (1980, 1984); Peters and Lavender (1982)	0.045	8.1–10.3
Kotzé et al. (1986)	0.057 ± 0.024	9.01 ± 2.72
Wadenvik et al. (1987)	0.048 ± 0.019	9.7 ± 1.6

able. Peters et al. (1985b) also reported that in some of these patients in whom increased hepatic platelet destruction was demonstrated, the transit time of platelets through this organ was increased.

As in vitro tests are doubtful markers of platelet viability, the in vivo function of platelets was investigated by the analysis of the kinetics of ^{111}In -labelled platelets. In this study we succeeded in establishing a multicompartmental model to simulate platelet kinetics during their entire in vivo life span in normal subjects and established reference ranges for platelet kinetics. Multicompartmental modelling succeeded in quantifying collection-injured platelets. Entertaining similar models to simulate in vivo injured platelets in platelet disease may be clinically useful.

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MULTICOMPARTMENTAL COMPUTER ANALYSIS OF ^{111}In -TROPOLONE LABELLED BLOOD PLATELET KINETIC DATA IN NORMAL HUMANS

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INTRODUCTION

Scintillation camera imaging of the in vivo distribution of platelets labelled with ^{111}In has become a routine investigative procedure. Computer assisted image analysis further enables one to determine the radioactivity in an organ and to quantify the percentage of whole body radioactivity in that organ¹. The limitation of imaging is that, within an organ, there is no distinction between platelets that are functional from those that have been injured during collection, or have been sequestered^{1,2}. Since imaging cannot distinguish between these components of organ radioactivity, we used compartmental

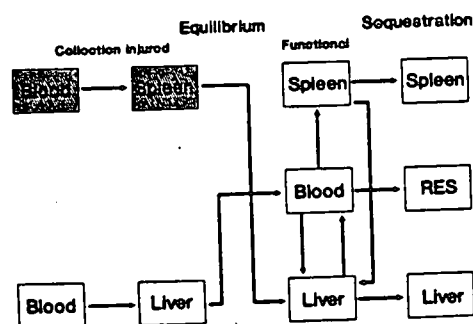


Figure 1. Compartmental model to simulate platelet movement in the body during both the equilibrium and sequestration phases. In Model 1, no compartment were included that simulated collection injured platelets. It was assumed that labelled platelets will equilibrate between the blood and the spleen and liver, and that labelled platelets from the spleen will enter the blood via the liver. Model 2 included a compartment to simulate collection injured platelets in the liver where they will accumulate transiently. Model 3 included compartments to simulate collection injured platelets in both the spleen and liver, and injured platelets in the spleen will enter the blood via the liver.

analysis (Figure 1) in order to attempt to quantify the contribution of each of the components to organ radioactivity. The SAAM program (simulation analysis and modelling) was used for this purpose.

METHODS AND RESULTS

Platelet kinetic data from 5 normal human subjects were used. Autologous platelets were labelled with ^{111}In -tropolone and reinjected². Standard methods were used to determine the mean platelet lifespan and in vivo distribution of the labelled platelets^{1,2}.

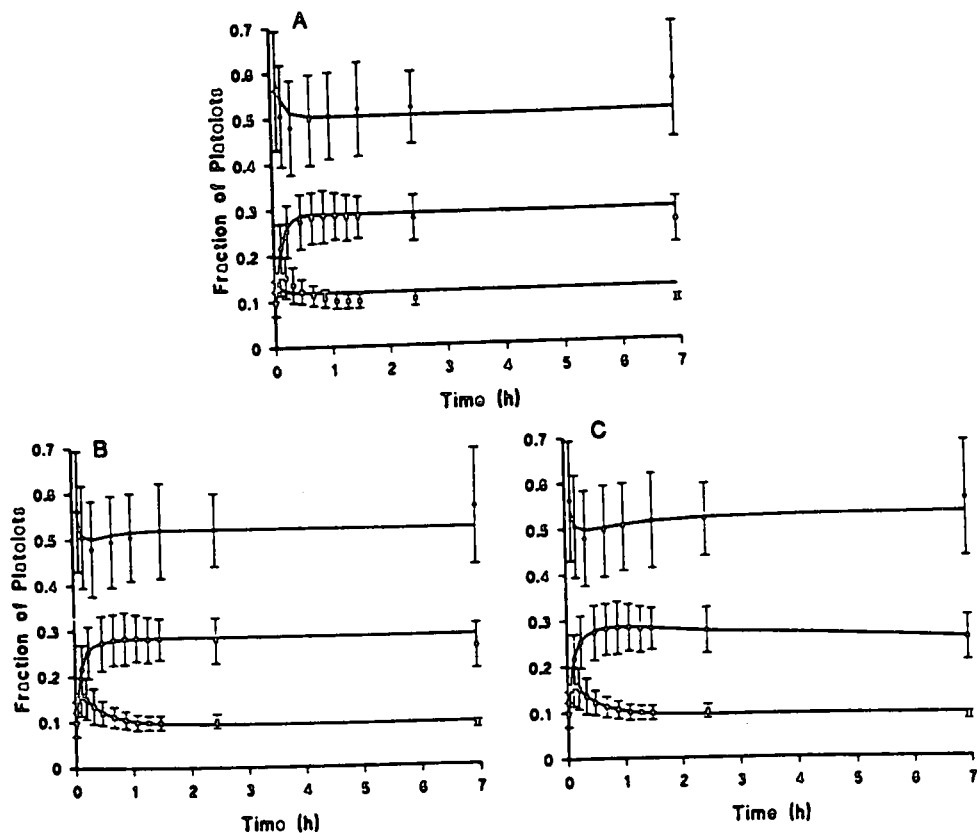


Figure 2. Simulation of the equilibrium phase with Models 1(A), 2(B), and 3 (C). The data points represent the mean experimental data (± 1 SEM) and the curves the simulated data.

When reinjected, labelled platelets redistribute between blood and organs until equilibrium between circulating and injected labelled platelets are reached. Some platelets accumulate transiently in the liver as a result of the collection injury (Figure 2). As labelled platelets age in the circulation, the senescent platelets are selectively sequestered by the macrophages of the monocyte-macrophage system, and thus disappear from the circulation (Figures 3). To simulate this, the platelet kinetic data were analysed in two phases. The first phase, the equilibrium phase, included the data that were measured

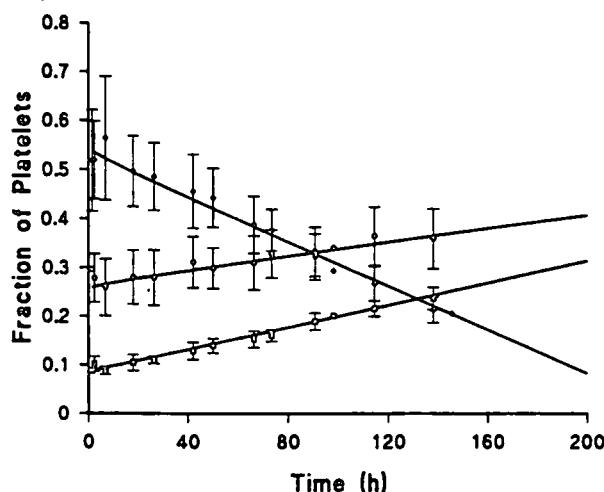


Figure 3. Simulation of the sequestration phase. The mean experimental data (± 1 SEM) is represented by the data points and the simulated data by the curves.

during the first 7 hours following reinjection of the labelled platelets (Figure 2). The second, the sequestration phase, included the data that were measured from 7 hours until the end of the platelet lifespan (Figure 3).

The first model (Model 1) that was used to simulate the equilibrium phase was rather simple. We assumed that platelets distribute from the blood to the spleen and liver, and that platelets re-enter the blood from the spleen via the liver (Figure 1). No compartment to simulate the movement of collection injured platelets was included. The fit of this model is given in Figure 2A. This model did not simulate the data well. In the second model (Model 2) a compartment to simulate the movement of collection injured platelets in the liver was added (Figure 1). Thus, collection injured platelet will accumulate transiently in the liver. The fit of this model is given in Figure 2B. Spleen and liver radioactivity, but not heart radioactivity, was simulated well. The third model (Model 3) included a compartment to simulate the movement of collection injured in both the spleen and the liver (Figure 1). Inclusion of this compartment improved simulation of heart radioactivity (Figure 2C). The constants obtained from the SSAM program with Model 3 gave the kinetics of labelled platelets through the spleen and liver. Accordingly, approximately 20% of the labelled platelets were collection injured. Of these, 10 % (range 5- 16%) accumulated transiently in the liver, and their transit time was 29 min (range 20-38 min). The normal transit time of platelets through the liver was 0.3 min. Nine per cent (range 5-12%) of the labelled platelets accumulated transiently in the spleen. Their transit time was 208 min (range 60-47 min), significantly longer than the 5 min of uninjured platelets.

Modelling of the sequestration phase was approximated by the Dornhorst function. We assumed that platelets were sequestered by a random and age dependent process. The fit of the sequestration model is given in Figure 3. The simulated data fitted the experimental data well. When the mean platelet lifespan was calculated from the compartmental model, the estimate compared favorably with that calculated using standard mathematical curve fitting techniques, i.e. 205 hours (range 188-255 hours) versus 227 hours (range 203-250 hours) respectively. The estimates of the in vivo distribution at equilibrium and the sites of sequestration of senescent platelets also compared favorable (Figure 3).

DISCUSSION

We have developed a compartmental model that can be used to quantify the movement of platelets in the body. The third model as shown in its entirety in Figure 1 allows one to determine the contribution of the different sources of radioactivity to total organ radioactivity, i.e. radioactivity from functional circulating platelets, senescent sequestered platelets and collection injured platelets. We were also able to show that collection injured platelets accumulate transiently in the spleen. An advantage of modelling of the platelet kinetic data may be that platelet survival can be more reliably determined by using the results of the disappearance of labelled platelets from the circulation and their simultaneous appearance in the spleen and liver.

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The sensitivity of tests to detect *in vivo* platelet activation induced by the removal of arterial endothelium of the baboon (*Papio ursinus*)

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Summary

The relative sensitivities for the various *in vivo* and *in vitro* tests for platelet activation are unknown. This was studied in a baboon model where limited and more substantial injury to the vascular endothelium was inflicted. The endothelium of a segment of the right carotid artery was removed with a balloon catheter on day 0 (limited de-endothelialisation), and that of the left carotid artery, abdominal aorta and left femoral artery on day 7 (substantial de-endothelialisation). Eight baboons (*Papio ursinus*) were used. Baseline tests for platelet activation (platelet volume, platelet density, platelet aggregate ratio, and platelet and plasma levels of platelet factor 4 [PF4] and β -thromboglobulin [β -TG]) were performed 7 days before de-endothelialisation and repeated on days 1, 9 and 16. The kinetics of indium-111-labelled platelets were measured after substantial de-endothelialisation. Sham operations were done on 3 animals exactly as in the test, except that the balloon injuries were not inflicted. No influence on the results of the platelet function tests was found. The only test capable of detecting limited injury to the endothelium was the measurement of plasma PF4. The mean platelet life-span (MPLS) shortened, mean platelet density decreased, the circulating platelet aggregate ratio decreased, and plasma levels of PF4 and β -TG increased ($P < 0,05$ in all instances) after the substantial endothelial injury. The mean platelet volume, intraplatelet PF4 and β -TG, and the *in vivo* distribution and sites of sequestration of labelled platelets were poor tests for *in vivo* platelet activation. Nine days after the substantial de-endothelialisation, the only remaining evidence of platelet activation was an increase in the number of circulating platelet aggregates. It is concluded that the most sensitive tests for *in vivo* platelet activation are the measurement of the circulating platelet aggregate ratio (sensitivity 0,63) and plasma β -TG (sensitivity 0,63), and the estimate of the MPLS (sensitivity 0,50).

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In clinical medicine platelet function tests are helpful for the diagnosis of platelet abnormalities as a possible cause of a bleeding diathesis, but they may also be used to determine whether the platelets have been activated *in vivo*. Provided that due attention is paid to the influence of various technical

factors,¹ it is usually relatively simple to diagnose qualitative platelet defects.

The detection of *in vivo* platelet activation is, however, more difficult. The usual *in vitro* tests of platelet function are not of much value in the evaluation of thrombo-embolism.² Attention has therefore recently focused on the direct measurement of the *in vivo* processes of platelet activation and consumption. The measurement of the mean platelet life-span (MPLS) is considered the most sensitive of these tests.³ However, to our knowledge, the relative sensitivities of the MPLS and the other tests for *in vivo* platelet activation have not been compared.

A study was undertaken to compare the relative sensitivities of a selection of readily available *in vivo* and *in vitro* tests of platelet function for the detection of limited and more extensive injury to the vascular endothelium. The effects of concomitant arteriosclerosis on platelet function tests were excluded by using baboons with no evidence of arterial disease for the experiments.

Materials and methods

Eight healthy, juvenile baboons (*Papio ursinus*), weighing 8 - 12 kg (mean $10,4 \pm 1,7$ kg), were used in a project approved by the Ethical Committee of the Provincial Administration and the University of the Orange Free State. They did not receive antiplatelet drugs before or during the study. Handling was accomplished through anaesthesia with intramuscular ketamine hydrochloride 10 mg/kg body mass.

Reference values for the MPLS, the *in vivo* distribution and the sites of sequestration of indium-111-labelled platelets had earlier been determined in our laboratory on a group of 8 baboons with similar physical characteristics.^{4,5} We employed the same standardised methods, reagents and internal and external quality control procedures in this study.

Experimental protocol

Tests for *in vivo* platelet activation were done on blood samples taken 7 days before vascular endothelium was removed (baseline values). The endothelium was then removed on two separate occasions with a balloon catheter. On day 0 only the endothelium of the right carotid artery was removed. Seven days later (day 7), a more substantial area involving the left carotid artery, the abdominal aorta and the femoral artery was denuded. We tested for platelet activation 1 day after de-endothelialisation of the right carotid artery (day 1), and 2 (day 9) and 9 (day 16) days after the more substantial de-endothelialisation. The MPLS, the *in vivo* distribution of platelets at equilibrium, and the sites of sequestration of platelets at the end of their life-span were determined after the substantial de-endothelialisation (starting on day 7).

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De-endothelialisation of arteries with a balloon catheter

Baboons were sedated with ketamine hydrochloride, intubated and anaesthetised with a mixture of halothane, O₂ and N₂O. The carotid artery or femoral artery was exposed to ensure the introduction of a balloon catheter with minimal injury to the vessel and the surrounding tissue. The catheter was advanced into the artery through a small cut-down. The balloon was then inflated with air and gently retracted. This procedure was repeated within 30 seconds. The arterial wall was sutured with 6-0 Prolene, and the skin wound closed with a silk suture. The arteries, as observed at autopsy on day 16, remained patent in all cases.

The catheter passed easily into the vessel lumen. After inflation with air, the vessel was visibly distended and there was palpable resistance when the catheter was retracted. The efficacy of the procedure to remove the endothelium was assessed by scanning electron microscopy in preliminary studies done on 8 baboons. In the final study, removal of endothelium was confirmed at the end of the investigation.

A 3 cm length of endothelium of the carotid artery was removed with a No. 6 balloon catheter inflated with 1.5 ml air. The endothelium of the abdominal aorta and femoral artery, extending from the site of entry in the femoral artery to the diaphragm, was removed with a similar catheter inflated with 2 ml of air.

Sham experiments without endothelial injury

Three baboons, similar in size to the experimental animals, were subjected to exactly the same procedures (anaesthetic, exposure of blood vessels) at the same time intervals but without the balloon injuries. The tests for *in vivo* platelet activation were done at the same time intervals as in the test protocol. The *in vivo* distribution of the labelled platelets and their final sites of sequestration were, however, not quantitated. The platelet concentrations of platelet factor 4 (PF4) and β -thromboglobulin (β -TG) were also not determined.

Platelet labelling and measurement of platelet kinetics

This was performed in 6 of the baboons. Blood was collected on day 7, within 30 minutes of de-endothelialisation. Autologous platelets were isolated^{6,7} and labelled with indium-111-tropolone.⁷ A platelet population, representing $94 \pm 16\%$ of that in the whole blood sample, was isolated with differential centrifugation and repeated washing of the red blood cell layer. Labelling efficiency was $78 \pm 12\%$. A total of $6.8 \pm 1.7 \times 10^9$ platelets, labelled with 20 ± 3 MBq ¹¹¹In, were re-injected within 4 hours of venesection. The labelled platelets were considered viable in view of their adequate *in vitro* aggregation response to adenosine diphosphate (ADP)⁸ and normal recovery in the circulation.⁴

The rate of disappearance of labelled platelets from the circulation was determined by measuring changes in the radioactivity in blood samples collected 15 minutes after re-injection of the labelled platelets, and then twice daily for 6 days. The MPLS was estimated by fitting a γ function⁹ to the data describing the removal of labelled platelets from day 1 to day 6. Recovery of labelled platelets in the circulation at equilibrium was calculated by back extrapolation of the platelet survival curve to zero time.⁴ The data of the test, sham and reference platelet survival curves were analysed in identical fashion.

Anterior and posterior images of the whole body were acquired daily for 7 days with a large-field-of-view scintillation

camera and an A² MDS data processing system. The *in vivo* distribution of the labelled platelets was quantified by the geometric mean method, and the distribution at equilibrium and the sites of sequestration of platelets at the end of MPLS estimated as described.^{5,6} Images (128 $\times$ 128 word mode) of the abdomen were taken for 30 minutes to detect the deposition of labelled platelets onto the de-endothelialised segment of abdominal aorta.

Tests for *in vivo* platelet activation

The mean volume of the platelets was measured according to the method of Corash *et al.*¹⁰ with an electronic particle counter interfaced with a multichannel analyser and logarithmic amplifier (Coulter Electronics, Hialeah, Fla, USA).

The density distribution of the platelets was determined on a non-linear Percoll (Pharmacia AB, Uppsala, Sweden) density gradient¹¹ calibrated with density marker beads. Percoll was made isosmotic with a buffered saline-glucose-citrate solution¹⁰ by adding 9 parts of Percoll to 1 part of a 10 $\times$ concentrated buffer-solution. Uniform mixtures of the isosmotic Percoll (4.5 ml) and buffer (5.5 ml) were poured into polycarbonate tubes. The density gradient was formed by centrifuging the tubes at 10 000 *g* for 30 minutes in a fixed angle rotor. Platelet-rich plasma (PRP), containing a fully representative platelet population ($96 \pm 6\%$ of those present in the blood), was prepared by repeated washing of platelets from the red cell layer.⁴ PRP 2 ml was gently layered onto the gradient. The platelets were separated by centrifuging at 400 *g* for 30 minutes in a swing-out rotor. The gradients were fractionated by carefully removing 1 ml aliquots from the top of the gradient.

Platelets in each aliquot were counted with the electronic particle counter, and the density of each aliquot determined by light refraction. The concentration of platelets in an aliquot was expressed as a fraction of the total number of platelets layered onto the gradient. A cumulative frequency of the platelets in the aliquots (representing the different densities) was calculated. The mean density of the platelet population was estimated at the 50th percentile.

Circulating platelet aggregates, expressed as an aggregate ratio, were determined according to Wu and Hoak.¹² Blood was collected into either formalin, which fixes aggregates, or ethylenediaminetetra-acetic acid (EDTA), which disperses them.

PF4 and β -TG. Blood (5 ml) was collected in precooled Thrombotect tubes (Abbott Laboratories, Chicago, Ill., USA).¹³ The tubes were centrifuged at 2 500 *g* for 20 minutes, the platelet-free plasma aspirated and frozen at -20°C until assayed with commercial kits (PF4 — Abbott Laboratories; β -TG — Radiochemical Centre, Amersham, UK). To measure intraplatelet PF4 and β -TG, platelets in PRP were lysed with Triton X-100, 1% final concentration. Cell debris was removed by centrifuging at 4 000 *g* for 30 minutes and PF4 and β -TG of the supernatant assayed. There is no evidence that these proteins show species-related antigenic differences.

Statistical analysis. Paired data were compared by Student's *t*-test if the distribution of data was normal or with the two-tailed Wilcoxon signed-rank test. The possible relationship between the MPLS and the results of the other tests for *in vivo* platelet activation was assessed with linear correlation analyses. Mean values are given with 1 SD.

The sensitivity of each test was assessed:¹⁴

$$\text{sensitivity} = \frac{\text{No. of positive tests}^*}{\text{No. of baboons studied}}$$

*A test was regarded as positive if the test result was outside 2 standard deviations of the mean baseline value.

Results

Sham experiments

The results of the sham experiments are given in Table I. Anaesthesia and the surgical procedures had no significant

effect on the *in vivo* and *in vitro* platelet function tests.

Baseline values

The peripheral blood platelet counts and the results of the tests for *in vivo* platelet activation are given in Table II.

TABLE I. EFFECT OF SHAM EXPERIMENTS, WITH NO BALLOON INJURY TO THE VASCULAR ENDOTHELIUM, ON TESTS OF PLATELET FUNCTION

Platelet function tests	Baseline	Anaesthesia and exposure	
		Carotid artery	Femoral artery
Platelet			
Count ($\times 10^9/l$)	346 $\pm$ 49	359 $\pm$ 46	382 $\pm$ 59
Volume (fl)	7.8 $\pm$ 0.3	8.1 $\pm$ 0.3	7.2 $\pm$ 0.8
Density (g/ml)	1.0446 $\pm$ 0.0014	1.0468 $\pm$ 0.0027	1.0470 $\pm$ 0.0009
Dense bodies	6.6 $\pm$ 1.0	6.6 $\pm$ 0.5	6.5 $\pm$ 0.5
Mean life-span (h)	135 $\pm$ 19	—	145 $\pm$ 11
Plasma			

TABLE II. RESULTS OF THE PLATELET FUNCTION TESTS AT BASELINE, AFTER LIMITED (DAY 1) AND SUBSTANTIAL (DAY 9) DE-ENDOTHELIALISATION OF THE ARTERY WALL, AND AFTER HEALING (DAY 16)

Platelet function test	Baboon								Mean $\pm$ 1 SD
	1	2	3	4	5	6	7	8	
Baseline									
Platelet count ($\times 10^9/l$)	446	525	447	561	644	425	452	530	504 $\pm$ 75
Platelet volume (fl)	4.64	5.40	5.25	4.08	4.25	5.37	5.74	5.49	5.03 $\pm$ 0.62
Platelet density (g/ml)	1.0489	1.0496	1.0476	1.0487	1.0470	1.0472	1.0472	1.0487	1.0481 $\pm$ 0.001
Platelet PF4 ($\mu g/10^9$)	2.55	3.82	0.57	4.16	3.99	1.75	2.60	1.87	2.60 $\pm$ 1.26
Platelet β -TG ($\mu g/10^9$)	2.18	3.54	2.89	2.53	6.82	1.07	2.14	1.83	2.85 $\pm$ 1.77
Platelet aggregate ratio	0.88	0.73	1.00	0.99	1.00	0.97	0.88	0.65	0.89 $\pm$ 0.13
Plasma PF4 (ng/ml)	5	7	15	10	21	12	29	26	16 $\pm$ 9
Plasma β -TG (ng/ml)	13	10	28	10	13	14	23	35	18 $\pm$ 9
β -TG: PF4 ratio	2.6	1.4	1.9	1.0	0.6	1.2	0.8	1.4	1.4 $\pm$ 0.6
Limited de-endothelialisation									
Platelet count ($\times 10^9/l$)	517	451	618	565	684	295	—	510	523 $\pm$ 75
Platelet volume (fl)	4.23	4.83	5.79	4.68	4.29	5.26	—	4.60	4.81 $\pm$ 0.55
Platelet density (g/ml)	1.0479	1.0486	1.0494	1.0447	1.0470	1.0472	—	1.0486	1.0479 $\pm$ 0.001
Platelet PF4 ($\mu g/10^9$)	2.85	2.40	4.09	1.52	2.30	3.12	—	7.24	3.36 $\pm$ 1.89
Platelet β -TG ($\mu g/10^9$)	2.74	1.65	4.77	6.06	2.34	3.47	—	6.32	3.91 $\pm$ 1.84
Platelet aggregate ratio	0.87	0.57	1.00	0.82	0.58	0.57	—	1.00	0.75 $\pm$ 0.22
Plasma PF4 (ng/ml)	18	33	18	74	65	13	—	41	37 $\pm$ 24*
Plasma β -TG (ng/ml)	24	25	17	74	56	15	—	32	35 $\pm$ 22
β -TG: PF4 ratio	1.3	0.8	0.9	1.0	0.9	1.2	—	0.8	1.0 $\pm$ 0.2
Substantial de-endothelialisation									
Platelet count ($\times 10^9/l$)	495	459	508	634	619	419	346	571	506 $\pm$ 99
Platelet volume (fl)	5.03	4.40	4.85	4.25	5.79	4.90	4.25	4.80	4.79 $\pm$ 0.51
Platelet density (g/ml)	1.0466	1.0467	1.0472	1.0452	1.0438	1.0465	1.0465	1.0460	1.0462 $\pm$ 0.001*
Platelet PF4 ($\mu g/10^9$)	4.94	4.53	4.51	4.69	1.74	3.03	3.25	2.58	3.66 $\pm$ 1.17
Platelet β -TG ($\mu g/10^9$)	4.36	3.85	4.54	6.59	2.82	2.34	2.89	1.97	3.65 $\pm$ 1.52
Platelet aggregate ratio	0.76	0.82	0.74	0.30	0.24	0.58	0.55	0.33	0.54 $\pm$ 0.23*
Plasma PF4 (ng/ml)	58	21	28	32	35	8	46	25	32 $\pm$ 15*
Plasma β -TG (ng/ml)	56	19	42	46	22	16	65	42	39 $\pm$ 18*
β -TG: PF4 ratio	1.0	0.9	1.5	1.4	0.6	2.0	1.4	1.7	1.3 $\pm$ 0.5
Effect of healing									
Platelet count ($\times 10^9/l$)	585	535	650	—	719	634	764	504	622 $\pm$ 88*
Platelet volume (fl)	5.54	4.64	4.29	—	4.25	4.81	4.81	4.44	4.68 $\pm$ 0.44
Platelet density (g/ml)	1.0490	1.0476	1.0475	—	1.0455	1.0472	1.0463	1.0462	1.0467 $\pm$ 0.001
Platelet PF4 ($\mu g/10^9$)	3.83	2.81	2.00	—	1.66	3.43	3.10	3.53	2.88 $\pm$ 0.79
Platelet β -TG ($\mu g/10^9$)	3.48	3.94	4.36	—	1.48	2.70	2.32	5.36	3.38 $\pm$ 1.32
Platelet aggregate ratio	0.58	0.64	0.584	—	0.52	0.55	0.80	0.73	0.63 $\pm$ 0.10*
Plasma PF4 (ng/ml)	8	76	19	—	10	11	54	44	32 $\pm$ 27
Plasma β -TG (ng/ml)	4	42	41	—	15	23	52	27	29 $\pm$ 27
β -TG: PF4 ratio	0.5	0.5	2.2	—	1.5	2.1	1.0	0.6	1.2 $\pm$ 0.7

*P < 0.05 versus baseline; Wilcoxon signed-rank test.

Changes induced by limited de-endothelialisation

The results are summarised in Table II. The mean blood platelet count did not change significantly from that at baseline ($P > 0,05$). Minimal endothelial damage increased the plasma concentration of PF4 significantly ($P < 0,05$). Evidence of *in vivo* platelet activation could not be detected by the other tests.

Changes induced by substantial de-endothelialisation

Tests for *in vivo* platelet activation. The mean platelet count, the mean platelet volume, the mean intraplatelet PF4 and β -TG and the plasma β -TG:PF4 ratio remained unchanged. Changes in the mean platelet density, the mean circulating platelet aggregate ratio, and the mean plasma concentrations of PF4 and β -TG did, however, provide evidence that the platelets were activated *in vivo* (Table II).

Platelet kinetics. The mean disappearance curves of the labelled platelets are given in Fig. 1. The results on platelet kinetics are summarised in Table III. The MPLS was significantly shorter than normal, and the mean platelet turnover increased significantly.

The *in vivo* distribution of the labelled platelets at equilibrium and their sites of sequestration at the end of platelet life-span, did not differ significantly from normal. Deposition of labelled platelets onto the abdominal aorta could not be detected by scintigraphy. Quantification of ^{111}In -deposition in this region was therefore not attempted.

Effect of healing of the injured vessel

The blood platelet count on day 16 was significantly higher than baseline ($P < 0,05$). Except for the increased numbers of circulating platelet aggregates, none of the other tests reflected residual platelet activation (Table II).

Sensitivity of the platelet function tests

The results are given in Table IV. The circulating platelet aggregate ratio, the plasma β -TG and MPLS were the most sensitive of these tests.

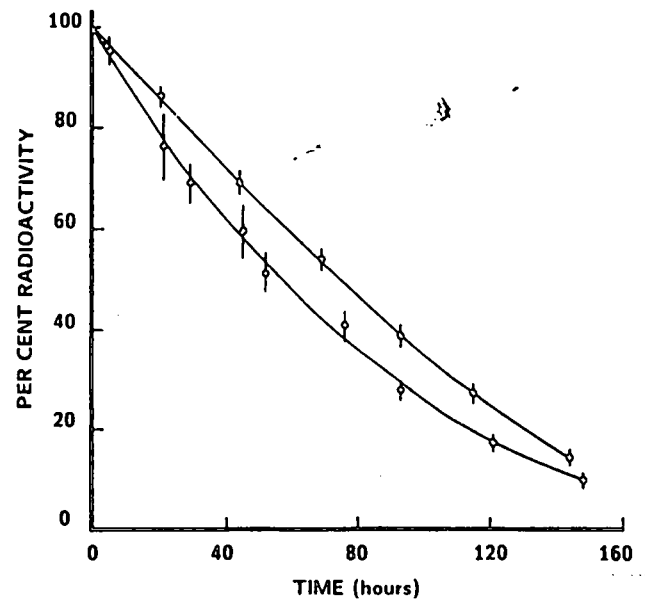


Fig. 1. The mean disappearance of ^{111}In -labelled platelets from the circulation. The top curve represents normal baboons. The bottom curve is the disappearance of labelled platelets after the more substantial injury. The values represent the mean % radioactivity ± 1 SEM of the radioactivity/ml injected, i.e. the recovery.

TABLE IV. SENSITIVITY OF THE PLATELET FUNCTION TESTS IN RESPONSE TO INJURY OF THE ARTERIAL ENDOTHELIUM

Platelet function test	Sensitivity of tests after		
	Limited injury	Substantial injury	7d healing
Platelet MPLS	—	0,50	—
Platelet volume	0,00	0,00	0,00
Platelet density	0,17	0,38	0,17
Platelet dense bodies	0,00	0,13	0,14
Platelet PF4	0,14	0,00	0,00
Platelet β -TG	0,00	0,13	0,00
Platelet aggregate ratio	0,57	0,63	0,57
Plasma PF4	0,43	0,38	0,43
Plasma β -TG	0,29	0,63	0,43

TABLE III. EFFECT OF DE-ENDOTHELIALISATION OF THE LEFT CAROTID ARTERY AND THE ABDOMINAL AORTA ON THE KINETICS OF ^{111}In -LABELLED PLATELETS

Baboon	Platelet			Distribution of labelled platelets*			
	Recovery (%)	MPLS (h)	Turnover ($\times 10^9/1/h$)	At equilibrium		At end of MPLS	
				Spleen	Liver	Spleen	Liver
1	67	132	3,4	16,5	17,1	22,6	33,2
2	94	113	4,3	16,4	16,2	21,6	19,6
3	90	87	5,1	13,5	17,0	19,6	36,9
6	99	111	3,1	17,4	19,1	22,4	26,8
7	71	148	2,8	19,2	15,6	23,6	17,9
8	71	130	3,3	18,4	19,9	22,0	25,9
Mean $\pm$ SD	82 $\pm$ 14	120 $\pm$ 21	3,7 $\pm$ 0,8	16,9 $\pm$ 2,0	17,5 $\pm$ 1,7	21,9 $\pm$ 1,4	30,0 $\pm$ 3,2
Reference	85 $\pm$ 9	148 $\pm$ 13	2,8 $\pm$ 0,9	16,0 $\pm$ 1,9	15,8 $\pm$ 2,9	23,3 $\pm$ 4,6	37,6 $\pm$ 6,0
P value†	0,56	0,02	0,03	0,30	0,20	0,46	0,59

* Organ radioactivity expressed as % of *in vivo* whole-body radioactivity.

† Student's *t*-test for unpaired data.

Linear correlation analyses

The results of the linear correlation analyses between MPLS and the other tests for *in vivo* platelet activation induced by the substantial injury to the vessel wall, were: platelet volume $r = -0,3768$; platelet density $r = -0,6922$; platelet PF4 $r = 0,3451$; platelet β -TG $r = -0,4217$; platelet aggregate ratio $r = -0,4267$; plasma PF4 $r = 0,5542$; plasma β -TG $r = 0,5842$; and β -TG:PF4 ratio $r = 0,1589$. None of these correlation coefficients were statistically significant ($P > 0,05$ in all instances).

Autopsy results in baboons

None of the animals had any macroscopic or microscopic evidence of atherosclerosis or systemic disease. As expected, the de-endothelialisation had induced hyperplasia of the intima of the arteries evident at the time of sacrifice.^{15,16}

Discussion

These experiments were performed to determine the relative sensitivities of *in vivo* and *in vitro* tests of platelet function in response to *in vivo* platelet activation. The latter was accomplished by exposing circulating platelets to a site or sites of injured vascular endothelium. A limited and a more substantial lesion was used to determine the sensitivity of the various tests. Healthy juvenile baboons, with no evidence of degenerative arterial disease, were considered a prerequisite for the experiment to exclude the added effects of concomitant arteriosclerosis on the platelet function tests.

In animals the removal of the vascular endothelium with a balloon catheter is followed by rapid accumulation of platelets on the exposed sub-endothelium. Once the surface is covered by a layer of platelets, few platelets continue to interact with the surface.^{15,16} This may explain why de-endothelialisation of the aorta does not shorten the MPLS in rabbits.¹⁵ This reaction may be species-dependent, because in man and non-human primates there is evidence that platelets are activated *in vivo* if the endothelium is damaged by homocysteine,¹⁷ cigarette smoking¹⁸ or hypercholesterolaemia.^{19,20}

De-endothelialisation of the artery of a healthy young primate was, in the light of the above, considered an appropriate model. The baboons had no evidence of arterial disease at autopsy, and the results of the baseline platelet function tests were also normal. The sham experiments demonstrated that the anaesthesia, surgical exposure of the arteries and the stress of handling had no effect on platelet function (Table I). It is therefore reasonable to ascribe the evidence of platelet activation that was observed to the interaction of the platelets with the damaged arterial wall. We assessed the influence of limited and more substantial endothelial damage on platelets and determined the relative sensitivities of a group of tests to diagnose *in vivo* platelet activation.

The baseline values of all the measurements were as expected and the animals showed no evidence of *in vivo* platelet activation. It should be noted that in this study the β -TG:PF4 ratio, contrary to the finding in another study,²¹ did not indicate platelet activation. In these animals the baseline β -TG:PF4 ratio approximated unity, whereas in humans the ratio is approximately 6,3.¹³ We have no ready explanation for this discrepancy. It is possible that the difference in the half-lives of these platelet-specific proteins in man and non-human primates may play a role. In man the half-life of β -TG is 100 minutes²² and in non-human primates 8,1 minutes;²³ that of PF4 is unmeasurably brief in man²² and in Rhesus monkeys there is a slow component with a half-life of 92 minutes.²³ Our finding can also probably not be ascribed to the use of an assay

for human β -TG and PF4 in our experiments with baboons; there is no species-related antigenic differences between β -TG and PF4 of humans and Rhesus monkeys.²³

The only test result that increased significantly after de-endothelialisation of a segment of one carotid artery was the mean plasma PF4 level. This increase was slight and individual results varied widely. It is therefore unlikely that the measurement of this platelet-specific protein will prove clinically useful for the diagnosis of *in vivo* platelet activation. Although the platelet aggregate ratio, indicative of circulating platelet aggregates, decreased in 5 of the 7 baboons, this change was not of statistical significance. The other tests for platelet activation clearly could not detect the effect of such a limited degree of endothelial injury on platelets. It should be noted that we did not measure the sensitivity of the MPLS because it was technically not feasible to perform this test on the same animal within 2 weeks.

More of the tests were able to detect de-endothelialisation of a substantial segment of the vascular system: the MPLS shortened, there was a decrease in the circulating platelet aggregate ratio, mean platelet density changed and plasma PF4 and β -TG levels increased.

Although this was seen in only 3 of the 6 animals, the MPLS shortened moderately, but significantly. Baboon 3 had a short platelet survival time of 87 hours. Removing the data from this animal does not influence the statistical comparison with the control group and therefore does not affect the conclusions. Mean platelet turnover also increased, compensating for the shortened platelet life-span, thus maintaining a normal blood platelet count. The decrease in the MPLS could not be linked to the visualisation of the deposition of platelets on the injured areas. If there was only slight deposition, the sensitivity of imaging will clearly be hampered by the relatively high concentration of radioactivity associated with platelets in the blood pool compared with those reacting with the lesion of the vessel wall.

It should be noted that this study was not performed to assess the effect of endothelial injury on the MPLS. It is well known that platelets will adhere to injured endothelium. We argue that it will be of more clinical value to know what the effect of an established, but not old, injury to the endothelium will be on the platelet life-span. Also, platelet kinetic measurements assume a steady state, therefore it is difficult to interpret the curve reflecting platelet life-span if labelled platelets are re-injected before the endothelial damage.

The interaction of platelets with the sub-endothelium had no influence on the sequestration pattern of the labelled platelets in the reticulo-endothelial system. This finding was not unexpected, since in patients with aortic aneurysms²⁴ or with arterial prostheses²⁵ labelled platelets are not permanently retained in the thrombogenic areas but are sequestered in the spleen, liver and bone marrow. The quantification of the organ distribution of labelled platelets at the end of their life-span is clearly not a useful test of *in vivo* platelet activation.

The most sensitive test for *in vivo* platelet activation was the determination of the circulating platelet aggregate ratio; this was low in 7 of the 8 baboons. One should, however, recognise that the test of Wu and Hoak¹² for platelet aggregates may reflect the propensity for platelets to aggregate *in vitro*.²⁶ The result of this test should therefore be interpreted to reflect hyperreactive platelets rather than a chronic state of micro-embolisation.

Plasma β -TG increased in all of the baboons; the mean increase was significant and the sensitivity of the test was high. However, the extent of the increase of the β -TG levels was slight and varied widely in individual animals. The finding that the plasma PF4 levels increased twofold is of some interest. It has been stated that the plasma concentration of PF4 should be low in absence of renal disease²¹ because the

half-life of PF4 in the circulation is very short.²³ According to this view, elevated PF4 in the plasma reflects *in vitro* platelet activation during blood sampling and manipulation.^{21,23} This may, however, not always be so; for instance, plasma PF4-levels may be elevated in patients with vascular disease.¹³ The results of our study strengthen the view that elevated PF4 levels may reflect *in vivo* platelet activation. The sensitivity of the measurement of PF4 for this purpose was, however, low.

The density of platelets is considered to be dependent on their α -granule content²⁷ and their densities decrease after the release reaction.²⁸ Such a test for an acquired storage pool deficit may therefore be a sensitive indicator of *in vivo* platelet activation. The validity of this approach was confirmed in this study in which it was found that the mean platelet density does decrease after *in vivo* platelet activation. This result is consistent with some, but not all, published reports. Some of these discrepancies may be related to species-differences. It is generally agreed that in the rabbit increased platelet consumption may be reflected by an increase in numbers of platelets of decreased density.²⁹ In humans, results are conflicting and it is not clear whether active vascular disease is associated with a decrease in mean platelet density²⁹ or not.³⁰ Clearly, further studies are needed to resolve this question.

We could not determine the cause of the decrease in platelet density induced by platelets reacting with a more substantial area of de-endothelialisation. Intraplatelet PF4 and β -TG were not altered by platelet activation. The concomitant increase in plasma PF4 and β -TG therefore suggests that these platelet-specific proteins are released mainly, or only, from those platelets that are rapidly removed from the circulation. Furthermore, our results do not support the view that the density of platelets are determined only by the β -TG and PF4 content of the α -granules.²⁷ A plausible explanation of the small decrease in platelet density may be that, because of the increased platelet turnover and shortened MPLS, the number of young platelets in the circulation increases.³¹

After the endothelium was allowed to heal for 16 days, almost no evidence of continuing *in vivo* platelet activation remained. Again, measurement of the circulating platelet aggregate ratio proved to be the most sensitive, and indeed the only, indicator of residual *in vivo* platelet activation.

It is evident from the results of this study that there are important discrepancies between the results of the various tests for *in vivo* platelet activation. It is clear that there is no ideal test to demonstrate such platelet activation. If the MPLS is accepted as the most specific and the most sensitive procedure,³ it is noteworthy that there is no relationship between the result of this 'gold standard' and those of other tests. The limitations of these tests as indicators of *in vivo* platelet activation become even more apparent if results in individual animals are noted. For instance, after substantial de-endothelialisation β -TG remained normal in 3 baboons and the platelet aggregate ratio only decreased in 4; there was no consistent pattern from animal to animal.

We acknowledge that these data cannot readily be extrapolated to those pertaining in humans. However, injury to the endothelium is basic to diseases affecting the vascular system and, as such, central to the process of *in vivo* platelet activation. It therefore seems reasonable to expect that results similar to those observed in this study are likely to be encountered when the process of *in vivo* platelet activation is measured in humans. It should therefore be noted that the results of this study indicate that the measurement of the circulating platelet aggregate ratio, the assay of plasma β -TG levels and the determination of the MPLS are the most sensitive indicators of *in vivo* platelet activation. However, because of notable discrepancies between the results in tests it would be prudent to use these tests in combination, and to interpret results with caution.

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Quantification of the thrombogenicity of Dacron aortic prostheses

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Summary

Eight patients with double-velour Dacron aorto-femoral grafts implanted for 2-60 months were studied. Indium-111-labelled platelets were found to have a shortened mean platelet lifespan (MPLS) of 138 ± 32 hours compared with the normal platelet lifespan of 224 ± 23 hours. Labelled platelets accumulated on all grafts, irrespective of graft age, to a maximum of $1.80 \pm 0.83\%$ of whole body radioactivity; thereafter graft radio-activity decreased. This suggests that platelets on the graft are in dynamic equilibrium with those in the circulation. Platelets may be lost from the graft by embolization or radioactivity may be released because of platelet lysis. Although MPLS may depend on the age and surface area of the graft, concomitant arterial disease probably also contributes to the shortened MPLS.

Dacron prostheses are commonly used in aortofemoral reconstruction.¹ These grafts, however, have one of the more thrombogenic surfaces.^{2,3} Mean platelet life span (MPLS) is significantly shortened soon after implantation of the graft and normalizes progressively during the following 12 months.⁴⁻⁸ Also, the deposition of platelets onto the graft decreases with graft age.^{4,7-9} These changes are generally interpreted as being due to a decreased thrombogenicity of the grafts. This is, however, not absolute and platelets may still be deposited onto the graft surface for as long as 120 months after implantation.^{10,11}

It is known that MPLS is shortened in patients with vascular disease.¹² There may be extensive atherosclerosis in

patients who need aortofemoral reconstruction. The interpretation that MPLS reflects only graft thrombogenicity may therefore not be valid. We have therefore re-evaluated the specificity of MPLS as an indicator of aortofemoral graft thrombogenicity. This was facilitated by using the new technology of quantitative imaging of indium-111-labelled platelets.

Patients and methods

Eight patients were studied. The relevant physical characteristics of the patients and the age and surface area of the double-velour Dacron prostheses are given in Table I. The patients did not receive antiplatelet drugs for at least 2 weeks before or during the study. The project was approved by the Ethical Committee of the Provincial Administration and the University of the Orange Free State.

Evaluation of arterial sufficiency

All patients had clinically severe aorto-iliac occlusive disease which was confirmed by aortography. Doppler pressures (tibialis posterior) were measured before implantation of the grafts and again at the time of the study (Table II).

Labelling and measurement of MPLS

Platelets were labelled with ¹¹¹In-oxine as previously described.¹³ Briefly, autologous platelets were harvested from 84 ml blood (collected in 16 ml acid citrate dextrose, NIH formula A) by differential centrifugation and repeated washing of the red cell layer. A total of $94 \pm 11\%$ of the platelets were

TABLE I. PATIENT AND PROSTHESIS CHARACTERISTICS AND THE INTERACTION OF THE LABELLED PLATELETS WITH THE GRAFT

Patient	Sex	Age (yrs)	Graft		MPLS (h)	Maximum graft radioactivity	
			Age (mo.)	Area (dm ²)		% administered*	%/dm ² †
1	M	47	60	2,38	165	2,07	0,87
2	F	68	50	2,77	116	1,00	0,36
3	M	59	48	2,38	135	1,95	0,82
4	M	57	38	2,11	103	2,25	1,07
5	M	57	37	1,81	114	3,26	1,80
6	M	54	25	2,76	135	2,00	0,72
7	F	69	9	0,33	130	0,63	1,88
8	M	63	2	1,15	202	1,23	1,07
Reference value					224 ± 24		

*Radioactivity expressed as a percentage of ¹¹¹In-radioactivity administered to the patient.†Radioactivity expressed as a percentage of ¹¹¹In-radioactivity administered to the patient and related to the surface area (dm²) of the graft.

TABLE II. DOPPLER PRESSURE (mmHg) OF THE POSTERIOR TIBIAL ARTERY

	Right leg	Left leg
Before implantation	117 ± 61	64 ± 45
After implantation	155 ± 40	133 ± 45

Values are given as a mean pressure ± 1 SD.

recovered from the blood, and $96 \times 10^9 \pm 37$ platelets labelled with 19 ± 4 MBq ¹¹¹In-oxine in a saline medium. Labelling efficiency was $75 \pm 13\%$. Platelet viability was assessed by their *in vitro* aggregation response to adenosine diphosphate (ADP), and the labelled platelets were not injected unless aggregates of 10 or more platelets were observed.

The MPLS was calculated by fitting a gamma function to the disappearance curve of the labelled platelets from the circulation.¹³ The curve was constructed by counting the radioactivity in blood samples collected at 5 and 90 minutes and again daily after the injection of the labelled platelets. Recovery of labelled platelets in the circulation at equilibrium was calculated by back extrapolation of the survival curve to time zero and by correcting for the blood volume.¹³ Platelet turnover was calculated by dividing the MPLS by the platelet count and correcting for the pooling of platelets in the spleen.

Quantification of graft-labelled platelets

The procedure is described in detail elsewhere.¹⁴ Briefly, anterior images of the abdomen were recorded at 30 and 90 minutes with a scintillation camera, and thereafter daily for 6 days after the labelled platelets were injected. The images were stored on a A²-MDS data processing system. The distance between graft and the anterior body surface was measured. The radioactivity deposited on the graft was quantified as follows: the count rate in the graft and in an adjacent area, reflecting background radioactivity, was determined with the data processing system by region of interest analysis. The count rate of the adjacent area was subtracted from that in the graft. Graft radioactivity was corrected for attenuation because of the tissues lying between the graft and the camera detector and for physical decay of the radio-isotope. Radioactivity in the graft was expressed as a percentage of the ¹¹¹In injected. A radioactivity/time curve reflecting the accumulation of ¹¹¹In-labelled platelets onto the graft was constructed.

Statistics and correlation analysis

A linear least squares analysis of MPLS as dependent variable and graft age, graft surface area and graft radioactivity as independent variables was done.

Results

Blood platelet kinetics

The mean peripheral blood platelet count was normal; $270 \pm 60 \times 10^9/l$. The recovery of labelled platelets in the circulation was also normal; $59 \pm 12\%$. The disappearance curve of labelled platelets from the circulation fitted an exponential function best (Fig. 1). The MPLS was 138 ± 32 hours (Table I), which was significantly shorter ($P < 0,01$) than that in normal humans; 224 ± 23 hours.¹⁵ Mean platelet turnover, $69 \pm 13 \times 10^9$ platelets/l/day, was approximately twice normal.

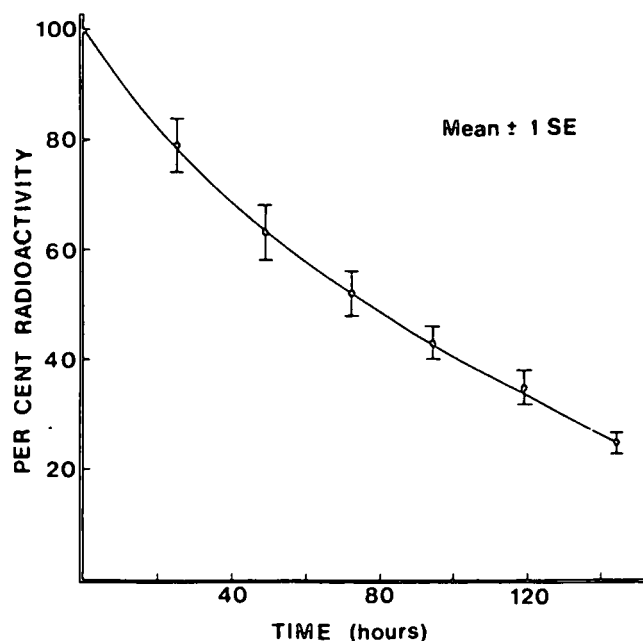


Fig. 1. Disappearance of ¹¹¹In-labelled platelets from the circulation. The values are given as a mean % of the count rate of the blood sample collected 5 minutes after the injection of the labelled platelets.

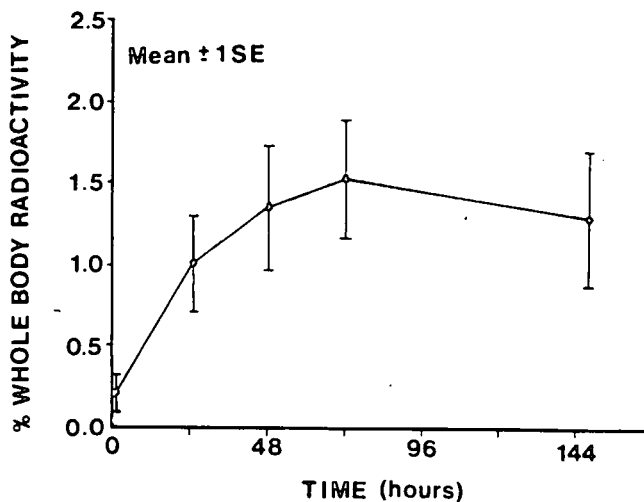


Fig. 2. A time/radioactivity curve illustrating the accumulation of ^{111}In -radioactivity on the graft. Graft radioactivity is expressed as a % of the ^{111}In administered to the patient.

Dynamics of graft ^{111}In -labelled platelets

All the grafts were thrombogenic, i.e. platelets accumulated on the graft surface, irrespective of the age of the graft. The dynamics of the deposition of labelled platelets onto the graft is illustrated as a time/radioactivity curve in Fig. 2. There was an initial rapid deposition of labelled platelets. The time to reach maximum radioactivity varied from patient to patient: in patients 3 and 6, maximum radioactivity on the graft was reached after approximately 25 hours; in patient 1 this was reached after approximately 145 hours, and in the remainder of the patients this took approximately 48 hours. After reaching a maximum, radio-activity was lost from the graft.

Correlation analysis

The relevant results are summarized in Table III. It is evident that MPLS was not related to the age, the surface area of the graft or the maximum number of labelled platelets deposited on the graft.

TABLE III. LINEAR REGRESSION ANALYSIS

Dependent variable	Independent variable	t-value for correlation	P
MPLS	Graft age	1,015	0,10
	Graft area	0,683	0,25
	Maximum activity	-0,746	0,10
	Maximum activity/area	-0,248	0,40

Discussion

There is direct and indirect evidence that platelets play a major role in the performance of arterial grafts, especially during the initial months after implantation.⁵⁻¹¹ Apparently the interaction between platelets and the graft diminishes with time. This is reflected by a progressive normalization of MPLS in serially studied patients, and a decrease in the thrombogenic index during the first 12 months after implanta-

tion.⁴⁻¹¹ This has been ascribed to endothelialization of the graft,⁵ which renders it less thrombogenic.

The results of the *in vivo* quantification of platelets labelled with ^{111}In -oxine by our method have been validated.¹⁶ In this study the labelled platelets were viable: the *in vitro* platelet aggregation response to ADP and the *in vivo* recovery in the circulation was normal. Furthermore, labelled platelets reacted with the thrombogenic surface indicating that the platelets retained their function.

In this study the disappearance of the labelled platelets from the circulation fitted an exponential function best. This indicates that platelets of all ages were randomly removed from the circulation. MPLS remained shortened and platelet turnover was almost doubled in 7 of the 8 patients. This is contrary to the results mentioned above, where a progressive normalization was observed in patients studied serially. The difference in results may be due to the fact that our group of patients were randomly selected. Patient 8, with the youngest graft (2 months) had a normal MPLS. There was no correlation between graft age and MPLS.

Nevertheless, it should be noted that there was a tendency for these two parameters to be positively related. This finding is somewhat surprising. In patients studied serially, it has been shown that MPLS does normalize and that the thrombogenicity of the graft decreases.⁵⁻¹¹ A likely explanation for the discrepancy is: (i) the number of patients studied is small; and (ii) the same patients were not studied serially. Another, and likely, explanation is that the MPLS may be related not only to the graft but may be interacting with other thrombogenic surfaces such as atherosclerotic lesions. That this view may be correct is supported by the finding that MPLS was not related to age or the surface area of the graft or to the maximum radioactivity deposited on the graft. The finding that the MPLS does not correlate with the surface area of the graft may be somewhat surprising at first sight. Indeed, in normal baboons the MPLS correlated inversely with the thrombogenic surface area.² Our findings could, however, be explained by the differences in the rest of the vascular tree: in normal baboons the thrombogenic graft is the major cause of platelet consumption, whereas in our patients the graft was only one of the factors decreasing MPLS.

The dynamics of the deposition of the labelled platelets onto the graft is of some interest. At maximum $1,80 \pm 0,83\%$ of the total injected labelled platelets were on the graft. The shape of the time/radioactivity curve reflecting the deposition of labelled platelets onto the graft (Fig. 2) may be interpreted as follows. After injection, the labelled platelets are incorporated into the graft. This is relatively rapid, but it takes some time (about 54 hours) to reach a dynamic equilibrium. Thereafter graft radioactivity decreases. This decrease is due to several factors. First, the deposition of platelets is not permanent and platelets on the graft are in equilibrium with those in the circulation. The immediate fate of the platelets released from the graft is unknown: they may continue to circulate, they may be rapidly sequestered by the reticulo-endothelial system or they may embolize or lyse. These different factors are at present being investigated.

The results in this study suggest that the determination of MPLS as an index of graft thrombogenicity is not entirely specific. The shortening in MPLS observed in patients with peripheral vascular disease which necessitates arterial grafting probably largely reflects diffuse vascular disease and is therefore not only determined by the graft surface. Clearly, platelet dynamics in patients with prostheses is complex, and may involve a variety of pathways mediated by the platelet and blood coagulation systems.

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Opsomming

Agt pasiënte, elk met 'n dubbel-velour Dacron aorto-femorale prostese, is bestudeer. Die prosteses was ingeplant vir tydperke wat tussen 2 en 60 maande gewissel het. Die gemiddelde leeftyd van ^{111}In -gemerkte bloedplaatjies, 138 ± 32 uur, was betekenisvol korter as die 224 ± 23 uur in normale mense. Gemerkte plaatjies het op die prostese-oppervlak geakkumuleer tot 'n maksimum van $1,80 \pm 0,83\%$ van die heelligaamaktiwiteit, waarna dit weer afgeneem het. Die plaatjies op die prostese was dus in 'n dinamiese ewewig met sirkulerende plaatjies. Die verlies van radioaktiwiteit kan of te wyte wees aan embolieë wat vanaf die prostese losgekom het, of aan radioaktiwiteit wat uit disintegreerende plaatjies vrygestel is. Alhoewel die verkorte plaatjieleeftyd deur die ouderdom en grootte van die prostese bepaal kan word, kan meegaande aterosklerose ook tot die verkorting van plaatjieleeftyd bydra.

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The influence of platelet-graft interaction on platelet survival in patients with aortobifemoral Dacron grafts

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Summary

In patients with arterial grafts, platelet consumption may be due either to platelet interaction with the graft, and/or concomitant platelet consumption in the rest of the arterial tree. This hypothesis was tested by quantifying the kinetics and platelet-graft interaction of indium-111-labelled platelets with double velour Dacron grafts in 13 patients with arterial insufficiency ascribed to atherosclerosis. Mean platelet lifespan (MPLS), 149 ± 46 hours, was significantly shorter ($P = 0,001$) than normal. Labelled platelets were transiently deposited onto the graft surfaces. Peak ^{111}In deposition on the grafts, $1,33 \pm 1,02\%$ of injected labelled platelets, was reached at 70 ± 33 hours. Thereafter the graft-platelet radioactivity decreased in parallel with platelet radioactivity in the circulation. There was no statistical correlation between MPLS and the factors known to be associated with graft platelet deposition: graft size; peak graft radioactivity; and the time to attain peak graft radioactivity. It is therefore concluded that in patients with arterial disease requiring graft implantation, the observed increased platelet consumption cannot only be ascribed to the interaction of platelets with the graft. Concomitant atherosclerosis is probably the important modifier of platelet consumption. The significant contribution of this factor to the shortening of the MPLS should be taken into account when assessing, by measuring platelet lifespan, the thrombogenicity of grafts.

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Patients with thrombo-embolic disorders have increased platelet utilisation. This may be ascribed to the interaction of platelets with a thrombogenic surface. Such a surface may be a prosthetic graft or an aneurysm.¹⁻¹⁰ The thrombogenicity, i.e. the ability to induce platelet deposition and/or increase platelet consumption of an aneurysm or graft may be indirectly demonstrated by a shortened mean platelet lifespan (MPLS),^{1-4,9,10} or quantitated by measuring the deposition of radiolabelled platelets onto the surface of the graft.⁵⁻¹⁰

However, patients with arterial disease of a magnitude that will necessitate grafting, usually have concomitant atherosclerosis. This, in its own right, may shorten the MPLS.¹¹⁻¹³ This notion is supported by compartmental analyses, which have indicated that in patients with grafts or aneurysms, atherosclerosis

has a cumulative effect on platelet utilisation.¹⁴ It therefore seems reasonable that shortening of the MPLS in such patients may reflect not only the thrombogenicity of a graft. It may thus be necessary to quantitatively measure platelet-graft interactions in conjunction with MPLS to reliably evaluate *in vivo* thrombus formation on prosthetic surfaces.

We have tested this hypothesis by studying the effect of Dacron grafts on platelet kinetics. By measuring the influence of graft age, graft size and extent of platelet accumulation on the graft on MPLS, we could assess whether other factors also modulated MPLS. None of these factors, usually thought to determine graft thrombogenicity, correlated with MPLS. MPLS can therefore not be regarded as a good indicator of thrombogenicity of the grafts.

Patients and methods

Thirteen patients, 10 men, who had a double-velour Dacron aortobifemoral prosthesis (USCI, Billerica, Mass., USA) implanted for arterial insufficiency secondary to atherosclerosis, were studied. All patients gave informed consent to participating in the project, which was approved by the Ethical Committee of the Provincial Administration and the University of the Orange Free State. The patients were aged 47 - 76 years, and their body mass varied between 53 kg and 90 kg. Their peripheral blood platelet counts, measured at the start of the study, were normal ($271 \pm 49 \times 10^9/l$) and did not change during the study. The patients were not on drugs known to affect platelet function for at least 2 weeks before and during the study.

Reference values for the kinetics of indium-111-labelled platelets were determined in our laboratory employing the same standardised methods, reagents and internal and external quality control procedures used in this study. The results, ascertained in 28 healthy volunteers, have been reported.¹⁵

Platelet labelling and determination of MPLS

Autologous platelets were isolated from whole blood and labelled with ^{111}In -oxine (Amersham International PLC, Amersham, UK), as described in detail elsewhere.^{15,16} Briefly, platelets were isolated from citrated blood by differential centrifugation and repeated washing of the red cell layer. The platelets were concentrated, resuspended in Plasmalyte B (Baxter Travenol Laboratories Inc., Deerfield, Ill., USA), and ^{111}In -oxine added. After incubating for 30 minutes at room temperature, the labelled platelets were concentrated and resuspended in platelet-poor plasma for re-injection. The viability of labelled platelets was assessed by their *in vitro* aggregation response to adenosine diphosphate.¹⁷ The labelled platelets were not injected unless aggregates consisting of 10 or more platelets were observed.

MPLS was determined from the rate of disappearance of labelled platelets from the circulation. The disappearance curves were constructed from the radioactivity in blood sam-

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ples collected 15 minutes and 90 minutes after re-injection of the labelled platelets, and then twice daily. MPLS was estimated by fitting a γ -function¹⁸ to the disappearance curves. Recovery of labelled platelets in the circulation at equilibrium was calculated from the radioactivity/ml injected and back extrapolation of the survival curve to time zero.^{15,16}

The turnover of the labelled platelets was calculated from the MPLS and platelet count, correcting for the splenic platelet pool, measured by the recovery of labelled platelets in the circulation.¹⁹

Image acquisition and quantification of deposited platelets

The deposition of labelled platelets onto the graft was imaged with a large-field-of-view scintillation camera and the scintigrams stored on, and analysed with an A² MDS data processing program. The fraction of injected labelled platelets deposited onto the graft was quantified as previously described.²⁰ Briefly, anterior images (128 x 128 word mode) of the abdomen were acquired for 30 minutes. The images were taken 15 minutes, 90 minutes and daily for 6 days after re-injecting the labelled platelets. The depth of the graft was determined according to the method of Links *et al.*²¹ and an attenuation factor was derived for the γ -photons at that depth.²² The accuracy of the method to determine the depth of a graft with ¹¹¹In was evaluated and validated in a human-like phantom. The range of depths tested in the phantom varied between 5 cm and 11 cm. The mean difference between the measured and actual depth of the radioactive source was $2,4 \pm 5,1\%$. In the patients the depths of the grafts varied between 5 cm and 9 cm.

The fraction of injected labelled platelets deposited onto the graft was measured by selecting a region of interest (ROI) of the graft and quantifying the radioactivity in the region. Selection of the ROI was done automatically with the standard-edge detection program of the image analysis computer program, using a 5% cut-off point. Earlier experiments where ROIs were selected with 5%, 10% and 15% cut-off points all gave similar counts; the 5% point used in this study was therefore considered satisfactory.

The size of the graft was related to the number of pixels in the ROI. Background radioactivity was measured in an area adjacent to the graft. Graft radioactivity was then appropriately corrected for background radioactivity, attenuation, blood radioactivity and physical decay of the radio-isotope, as described in detail elsewhere.^{9,20}

The injected radioactivity was determined by counting the injectate with the scintillation camera. Graft radioactivity was expressed as a percentage of that of the injectate. The time to reach peak graft radioactivity and the peak graft radioactivity was calculated by compartmental analysis.¹⁴ Changes in the radioactivity deposited on a graft at a given time were expressed as a percentage of peak graft radioactivity. A time/radioactivity curve was constructed.

Statistical analysis

The Shapiro-Wilk test was used to assess whether the data were normally distributed. The relationship between different parameters was tested by the Spearman rank correlation analysis. The difference between samples was compared by the *t*-test for different means and the 95% confidence intervals.

Results

Platelet labelling

A fully representative platelet population, consisting $93 \pm 7\%$ of those platelets in the whole-blood sample, were labelled with ¹¹¹In with an efficiency of $76 \pm 11\%$. A mean of $7,7 \pm 2,0 \times 10^9$ platelets were labelled with 19 ± 4 MBq ¹¹¹In. Contamination of the labelled platelet injectate with red cells was $0,7 \pm 0,9\%$ and with plasma proteins $3,2 \pm 1,8\%$. White blood cell contamination was less than 1/200 platelets.

The labelled platelets were viable: platelets aggregated *in vitro* in response to ADP and the recovery of labelled platelets in the circulation, $64 \pm 14\%$, was within the normal limits ($61 \pm 12\%$, $P = 0,68$) of this laboratory. The interaction of labelled platelets with the grafts could also be visualised (Fig. 1).

MPLS and platelet turnover

The platelet survival curves fitted an exponential function best, i.e. platelet consumption was random. The results are given in Table I. Ten patients had a shortened MPLS; patients 11, 12 and 13 with the most recently implanted grafts, had a MPLS within the mean ± 2 SD of that of the reference group. The MPLS of the patient group was significantly shortened to 149 ± 46 hours ($t = 6,897$; $df = 39$; $P = 0,001$). The mean shortening of their MPLS was 75 hours, with a 95% confidence interval (CI) from 53 hours to 97 hours.

TABLE I. GRAFT CHARACTERISTICS, PLATELET KINETICS AND THE DYNAMICS OF PLATELET-GRAFT INTERACTION

Patient	Graft		Platelet		Graft: peak	
	Age (mo.)	Pixels	Lifespan (h)	Turnover ($\times 10^9/l/d$)	Radioactivity (%)	Time to (h)
1	62,5	425	126	50	0,21	62
2	60,3	1122	143	55	1,78	58
3	49,7	1325	116	79	1,03	108
4	48,2	1186	135	56	1,32	73
5	38,6	1538	103	85	3,10	17
6	37,8	599	114	75	2,97	88
7	23,8	1264	135	77	2,20	72
8	28,2	309	162	63	0,32	35
9	9,2	425	106	101	1,00	89
10	8,4	183	130	84	0,23	97
11	3,3	1192	202	61	0,51	57
12	1,5	1478	228	49	2,09	134
13	1,0	1272	241	37	0,49	25

The number of pixels in the graft region of interest was used as an indication of the size of the graft.

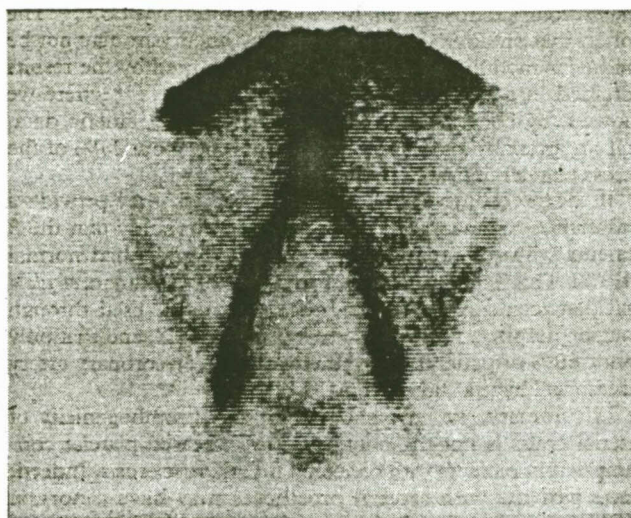


Fig. 1. Scintigram of a 60-month-old Dacron graft 96 hours after re-injection of ^{111}In -labelled platelets. Deposition of the labelled platelets onto the graft is more prominent at the anastomoses.

The patients maintained a normal blood platelet count in the face of a shortened MPLS, by an increase in platelet turnover; $70 \pm 16 \times 10^9$ platelets/l/day, i.e. approximately 1.6 times normal. The mean increase was 27×10^9 platelets/l/day with a 95% CI from 14 to 40×10^9 platelets/l/day. The *t*-statistic was 4,1301 with 39 df and an associated *P*-value of 0,002.

Kinetics of ^{111}In -labelled platelets with the graft

Platelets were deposited onto every graft, regardless of their age. The platelets tended to be especially laid down at the sites of the anastomoses of the graft with the vessel wall (Fig. 1).

The mean changes in graft ^{111}In radioactivity with time are illustrated in Fig. 2. Platelets rapidly accumulated on the graft and their deposition reached a peak between 17 hours and 134 hours (mean $70 \pm 33\text{h}$).

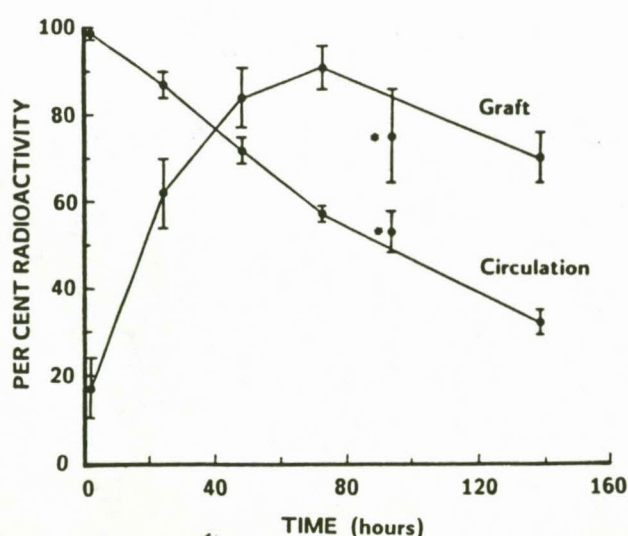


Fig. 2. Time-activity curves reflecting the changes in circulating radioactivity (% of the count rate of the 15-minute blood sample) and graft radioactivity (% of peak graft radioactivity) with time. Values are given as a mean $\pm$ 1 SEM (* only 5 of the 13 patients were studied 92 hours after re-injection of labelled platelets).

At equilibrium, $1,33 \pm 1,02\%$ of the total injected ^{111}In radioactivity resided in the graft (Table I). After reaching its maximum level, graft radioactivity decreased in parallel with ^{111}In platelet radioactivity in the blood (Fig. 2).

Correlations

The relationships between various parameters that may influence platelet lifespan and platelet-graft interactions were tested with linear correlation analyses. The results are summarised in Table II. MPLS correlated negatively with graft age. The MPLS did not correlate with graft size, peak graft radioactivity or the time to reach peak graft radioactivity. Graft age did not correlate with peak graft radioactivity or the time to reach peak graft radioactivity. The size of the graft correlated with peak graft radioactivity, but not the time taken to reach peak graft radioactivity. Time to reach peak graft radioactivity was unrelated to peak graft radioactivity.

TABLE II. SPEARMAN RANK CORRELATION ANALYSES

Dependent variable	Independent variable	r-value	P-value
MPLS	Graft		
	Age	-0,5117	0,08
	Size	0,1185	0,68
	Peak graft radioactivity	-0,2775	0,34
	Time to reach peak radioactivity	-0,1733	0,55
Graft age	Peak graft radioactivity	0,0989	0,73
	Time to reach peak radioactivity	-0,0495	0,86
Graft size	Peak radioactivity	0,6080	0,04
	Time to reach peak radioactivity	-0,0935	0,75
Peak graft radioactivity	Time to reach peak radioactivity	0,0495	0,86

The 3 patients with the most recently implanted grafts and a near normal MPLS (patients 11, 12 13) were excluded and the correlation between the different parameters again determined. The results are summarised in Table III. MPLS did not correlate with graft age and graft size. However, peak graft radioactivity correlated significantly with graft size.

TABLE III. SPEARMAN RANK CORRELATION ANALYSES EXCLUDING 3 PATIENTS WITH MOST RECENT GRAFTS

Dependent variable	Independent variable	r-value	P-value
MPLS	Graft		
	Age	-0,0790	0,81
	Size	0,2988	0,38
	Peak graft radioactivity	-0,2737	0,41
	Time to reach peak radioactivity	-0,2553	0,44
Graft age	Peak graft radioactivity	0,0424	0,90
	Time to reach peak radioactivity	-0,2970	0,37
Graft size	Peak radioactivity	0,7416	0,03
	Time to reach peak radioactivity	-0,1520	0,65
Peak graft radioactivity	Time to reach peak radioactivity	-0,2727	0,41

Discussion

The validity of the findings of this study depends primarily on the accuracy of the methods to quantitate ^{111}In deposition on the graft surface. First, attenuation of γ -photons is affected by the depth of a radioactive source in the body.²⁰ The depth of a graft must therefore be accurately measured. We verified our method, based on that of Links *et al.*,²¹ the mean difference between the measured and actual depth was 2.4%. Second, this method corrects for excess radioactivity contributed by blood within the graft and from adjacent areas. The radioactivity measured in the graft ROI therefore only reflects that of radioactivity deposited on the graft surface. Third, the relative size of the graft was expressed as the number of pixels constituting the graft image. Observer bias, inherent in the methodology, was minimised by using an automatic edge detection computer program. Our methods may therefore be considered to reflect reasonably accurately the quantitative kinetics of the interaction of the labelled platelets with the graft surface.

Our results confirm that the MPLS is shortened in patients with arterial grafts.^{1,4,9,14} This shortening in MPLS is associated with a compensatory increase in platelet turnover, thus maintaining a normal peripheral blood platelet count. It is generally assumed that in patients with grafts the increased platelet consumption reflects the deposition of platelets on the graft surface,^{1,11,23} and thrombo-embolism resulting from activated platelets reversibly interacting with the thrombogenic surface.⁹

We tested the hypothesis that this view is an oversimplification and that other factors may modulate platelet consumption in patients with arterial grafts. For instance, MPLS is shortened in patients with atherosclerosis^{1,11-13} and a fixed number of platelets are consumed to maintain vascular integrity.²⁴ Obviously, measuring only MPLS will not allow discrimination between these various factors or assessment of their relative contributions to the excess platelet consumption. However, quantitation of platelet deposition onto the graft/blood surface reflects more accurately the role of the graft in platelet utilisation.²⁵ We therefore argued that if the MPLS reflected only, or predominantly, the interaction of labelled platelets with the graft, there should be a correlation between platelet lifespan and the graft factors that determine thrombogenicity.

All the prostheses were thrombogenic, as shown by platelet deposition on the graft surface. We also quantitatively determined the other graft parameters that are related to thrombogenicity — graft age^{2,6,7} and graft surface area.²⁶ We also used our technique of compartmental analysis¹⁴ to determine peak graft radioactivity and time to reach peak radioactivity and assessed whether these could be related to platelet turnover. The grafts of all patients were of the same material; the known influence of this factor on platelet kinetics²⁷ could therefore be ignored in this study.

The main thrust of our findings is that MPLS does not correlate with any of those factors that relate to graft thrombogenicity, i.e. patients with a short MPLS did not necessarily have either a recently implanted and/or large graft, and/or high graft radioactivity, and/or rapid deposition of labelled platelets on the graft despite the tendency of the larger grafts to accumulate more labelled platelets.

It is noteworthy that MPLS accurately reflects platelet consumption on a thrombogenic surface in animal models.²⁶ However, this situation is not comparable with that in engrafted human patients. The major difference that has an important impact on platelet turnover is that the arterial tree of the young non-human primate is healthy, whereas patients that require arterial reconstructive surgery invariably have concomitant atherosclerosis of varying severity and degree. It is

known that atherosclerosis *per se* can shorten MPLS.¹¹⁻¹³ The notion that an arterial graft or an aortic aneurysm may not be the major modulator of the MPLS is supported by the results obtained in a small group of engrafted patients,¹⁴ where we showed, by compartmental analysis of platelet kinetic data, that an arterial prosthesis only accounts for about 17% of the excess platelet utilisation.

If the preceding arguments are accepted, they provide a reasonable explanation for the finding in our study that the 3 patients with the most recently implanted grafts had normal MPLS. This finding also confirms that of Goldman *et al.*^{3,7} that platelet deposition on a graft may be visualised through imaging despite a normal or near-normal MPLS, and that only about 60% of patients with atherosclerosis or coronary artery disease will have a shortened MPLS.^{12,13}

In summary, we have shown that the thrombogenicity of arterial grafts is not the only cause of increased platelet consumption in patients with concomitant atherosclerosis. Indeed, some patients with arterial prostheses may have a normal MPLS. It is therefore important always to take into account these other factors when adjudging the thrombogenicity of various graft materials or when assessing the efficacy of inhibitory drug in these circumstances.

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THE KINETICS AND SITES OF SEQUESTRATION OF PLATELETS IN ARTERIAL DISEASE

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ABSTRACT

The measurement of mean platelet life span (MPLS) is generally regarded as the best test for in vivo platelet activation. However, results with this measurement have been disappointing and conflicting in arterial disease. This may be ascribed to the inadequacy of Cr-51 as a platelet label. The introduction of In-111-oxine and the techniques of quantitative imaging permits a re-evaluation of platelet kinetics in arterial disease. We have done this in patients with aortic aneurysms and in a group of subjects with severe peripheral vascular disease. MPLS was shortened in all patients. There was accumulation of radioactivity in the aneurysms. This was however not permanent. After reaching equilibrium a plateau was reached, and radioactivity then decreased in parallel with that in the blood. This indicates that platelets deposited on the aneurysm wall are in exchange with those in the circulation. Also, at the end of their MPLS, the sites of sequestration of labelled platelets were similar to those of normal subjects. We then investigated the fate of those platelets that come off the aneurysm or are activated on the atheromatous surface of the vascular tree. These labelled platelets accumulate in the microcirculation of the feet. This could be demonstrated by comparing the rate of clearance of In-111-labelled platelets from the blood with the clearance from the feet. The latter was measured with anterior and posterior detectors and the geometric mean method for the correction for attenuation and geometry. There was clear evidence that there was a accumulation of activated platelets in the microcirculation of the feet. Again, this was transient and was reflected by a prolonged residence time in this area. We compared the shape of the curves reflecting the clearance of labelled platelets from the blood and the feet. It seems that the initial rate of clearance of the In-111-platelets from the blood is affected by the prolonged transit time of platelets in the microcirculation. This may an important determinant of the estimate of MPLS in these patients. The second part of the survival curve is near normal, suggesting that many of the platelets, and perhaps also those that have been activated on the diseased arterial wall, in these patients have a normal MPLS. This approach may be a more sensitive index of the in vivo activation of platelets in arterial disease. It may also provide a more rational method of measuring the influence of antiplatelet drugs on in vivo platelet activation and the interaction of platelets with a diseased vessel wall.

INTRODUCTION

In vitro methods have been in use for the diagnosis of in vivo platelet activation for almost two decades. These techniques have inherent limitations. It is not possible with in vitro tests, to assess all the factors involved in the complex interaction of platelets with one another, and platelets with the blood vessel wall and its components, especially since all of these may be variously affected in different diseases. A more promising approach, and at present probably the best, has been to assess in vivo platelet activation by determining the mean life span of platelets (MPLS) in the circulation.

The MPLS is generally estimated from analysis of the rate of clearance from the blood of a radiolabelled platelet population. The MPLS may be influenced by the "collection injury", the rate of mixing of the labelled cells with the exchangeable splenic platelet pool, and by various technical factors involved in the counting of radioactivity, blood sampling and elution of radioactivity from the platelets. Removal of platelets from the peripheral blood may be age-related or at random. If platelet survival is an age-dependent process, the platelet survival curve will be linear. On the other hand, if the removal of platelets from the circulation is due to their random destruction, the platelet survival curve will fit an exponential function best. These considerations are the basis for the measurement of MPLS to eg assess the consumption of platelets in a patient with disease of the vessel wall.

The physical properties of In-111 are advantageous for a platelet label. These were discussed in depth at this Conference. The major advantages are: it is possible to label platelets with high specific radioactivity, and the in vivo distribution of In-111-labelled platelets can be quantitatively and accurately imaged with a scintillation camera and computer-assisted image analysis.

The aim of this study was to apply this new technology to reassess the kinetics of the consumption of platelets in arterial disease. The ultimate purpose of such studies would be to develop methods to quantitate the adhesion, incorporation and consumption of In-111-labelled platelets into atherosclerotic plaques in different stages of evolution and to assess the efficacy of antiplatelet drugs in this situation.

To illustrate the advantages of In-111 as a platelet label and to provide the essential data on which the hypothesis that I would like to put forward is based, I would like to briefly reiterate the findings of our previous study of the platelet kinetics in patients with aortic aneurysms (1).

The MPLS was shortened in all but one of the patients; the mean was 132 ± 38 hours, and the platelet survival curves fitted an exponential function. The labelled platelets accumulated in the aneurysm; $4.7 \pm 3.6\%$ of the whole body radioactivity at the end of platelet survival. However, as is evident from fig 1, the radioactivity in the aneurysm increased with time and only reached

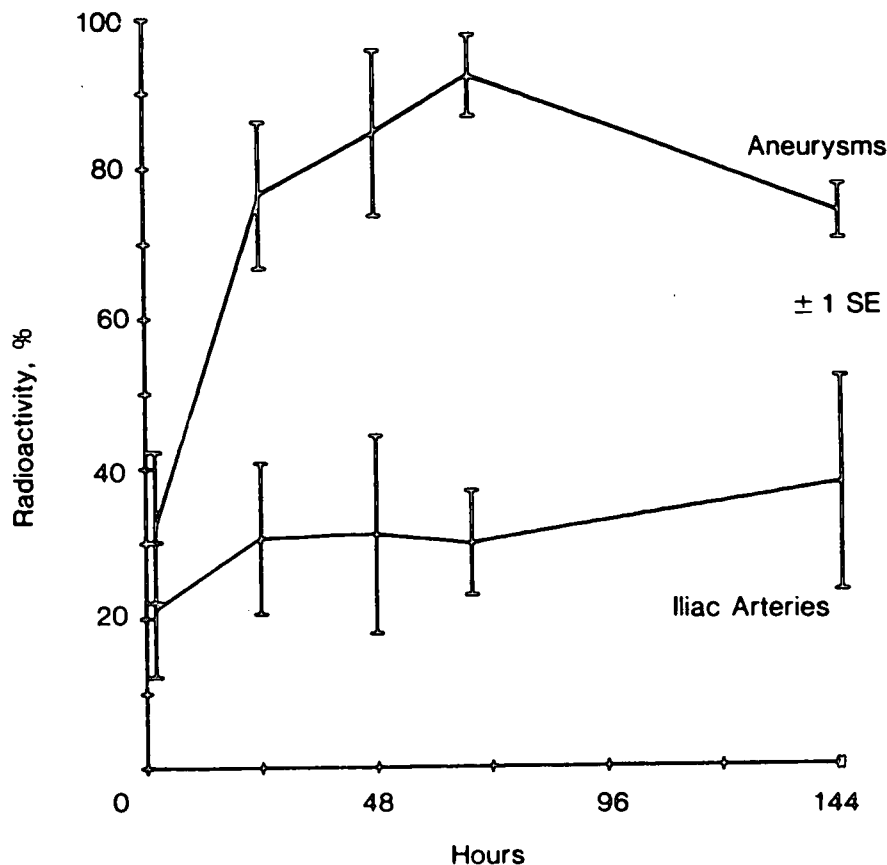


Fig 1: A time-activity curve of the rate of accumulation of In-111-platelets in patients with aortic aneurysms.

maximal activity at 48 to 72 hours after reinjection of the labelled platelets. Thereafter, radioactivity in the aneurysm decreased slowly, more or less in parallel with that of the blood. Interestingly, the final sites of sequestration of the labelled platelets did not differ significantly from that of normal subjects (Table 1): the deposition was confined to the reticuloendothelial system (RES) and relatively few platelets were deposited in the aneurysm.

We interpreted these findings as follows. The aneurysm wall is thrombogenic and the platelets adhere to, and aggregate on this surface. The deposition of platelets in the aneurysm accounts for the shortened MPLS. However, these platelets are not all deposited permanently and irreversibly; only their residence time at this site is increased. This is not surprising, because permanent sequestration of the platelets on the vessel wall would inevitably lead to rapid occlusion of the vessel. The residence time is about 60 hours. The platelets on the aneurysm wall and those in the circulation are apparently in

dynamic equilibrium. When platelets are eventually detached from the aneurysm wall, they are sequestered in the RES. The immediate fate of such detached platelets is unknown: they may be nonviable and be sequestered almost immediately, or, this interaction may only have an effect on platelet life-span or function.

TABLE I: PLATELET SEQUESTRATION: ANEURYSMS

Final In-111-radioactivity (% whole body)

	Aneurysm	Liver	Spleen	Lower limbs
Mean $\pm$ SD	5.2 $\pm$ 4.2	23.6 $\pm$ 8.4	30.8 $\pm$ 9.1	4.7 $\pm$ 4.2
Reference values	N/A	28.7 $\pm$ 8.3	35.6 $\pm$ 9.7	1.3 $\pm$ 1.0

We have previously shown that there is almost no accumulation of labelled platelets in the lower limbs of normal subjects (2). This was interpreted to indicate that relatively few platelets are used to support vascular integrity. Interestingly, in the patients with aortic aneurysms, there was a significant increase of the In-111-activity in the lower limbs.

These results are the basis for the following hypothesis. Platelets normally do not react with the intact vessel wall. In diseases of the arteries, such as aneurysms and probably also atherosclerosis, platelets are activated and will adhere to the exposed subendothelial structures of the damaged vessel. This interaction has several effects: the residence time of platelets in such a region is prolonged; the platelet-vessel wall interaction is however not irreversible, or permanent, and these platelets will enter the circulation after about 60 hours. This interaction of platelets with the diseased vessel wall is a random event, shortens platelet survival and will decrease the MPLS. Also, these activated platelets, either singly, or in the form of microaggregates, may be trapped in the microcirculation of the feet (or other organs distal to the lesion). This entrapment may theoretically be either permanent, owing to the sequestration of platelets in the RES of the bone marrow of the small bones of the foot, or transient, leaving the region after the microaggregates disintegrate. These two mechanisms may result in the accumulation of the In-111-radioactivity of the feet, but will have distinctive and different kinetic patterns.

These possibilities and their effects are illustrated in Fig 2.

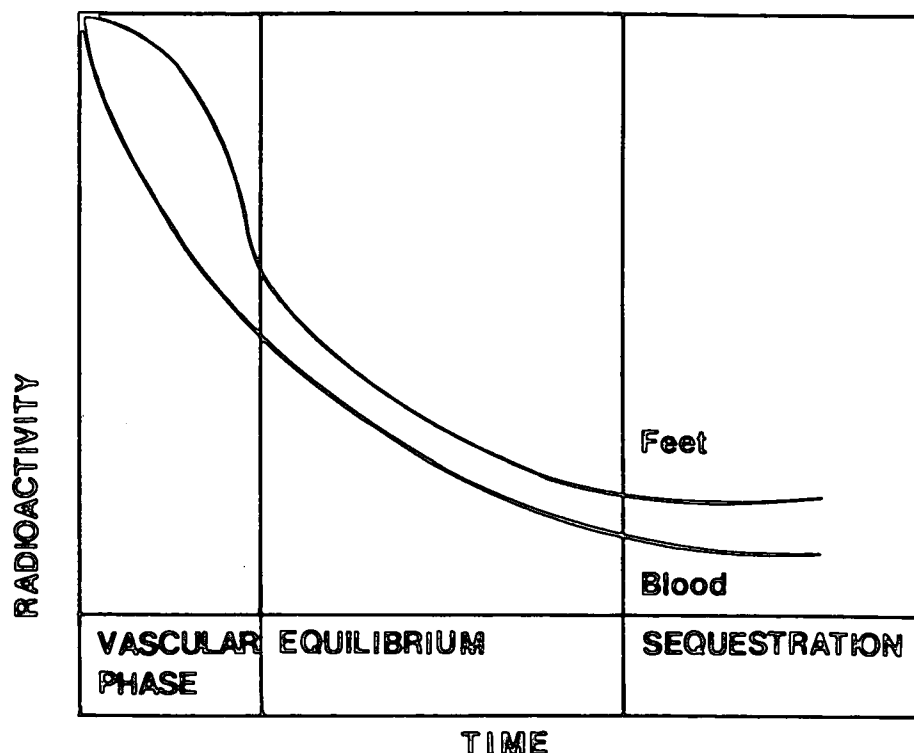


Fig 2: Theoretical patterns of accumulation of platelet microaggregates in the microvasculature of the feet. The lower curve is the shape of the blood platelet survival curve.

METHODS

This hypothesis was tested in patients with clinically evident peripheral arterial disease. Autologous platelets were labelled with In-111 (3), reinjected, and the MPLS estimated by fitting the data reflecting the rate of clearance of labelled platelets from the circulation, to a multiple hit model (4).

The accumulation of labelled platelets in the feet was serially measured by the geometric mean method. Radioactivity was measured with detectors placed anteriorly and posteriorly. The effects of attenuation and geometry were further minimised by marking the placement of the detectors, and by placing the feet into a constraining perspex box. This prevented movement of the feet and facilitated their accurate repositioning on subsequent days.

RESULTS

The results of time-radioactivity curves, reflecting the comparative rates of clearance of platelets from the blood and from the feet, are shown in Fig 3. The results in different patients could be compared by normalizing the data. It is evident that the mean clearance rates of the In-111 activity of the feet and blood differ. There is an initial accumulation of In-111 activity in the feet, it reaches a peak after 24 to 80 hours, and then decreases to parallel the activity of the blood.

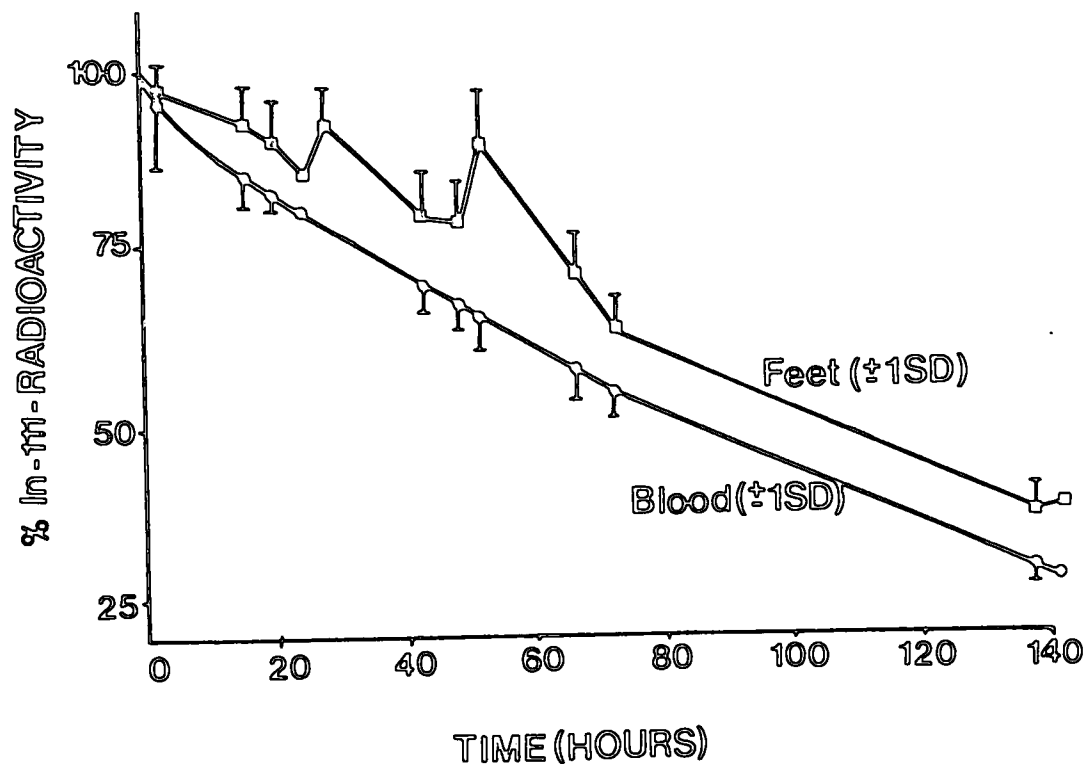


Fig 3: Comparison of the shapes of the rates of accumulation and clearance of In-111-platelets from the feet and the peripheral blood of six patients.

This phenomenon, illustrating the prolonged residence time of the platelets in the feet, is better seen in an individual patient (Fig 4). Note that the radioactivity counts in the foot varied from time to time. This may reflect true variations in the rates of clearance of In-111 (perhaps the platelet aggregates come off the vessel wall in showers and not as a constant stream), but may also be due to changes in geometry and attenuation associated with the positioning of the patient on different occasions.

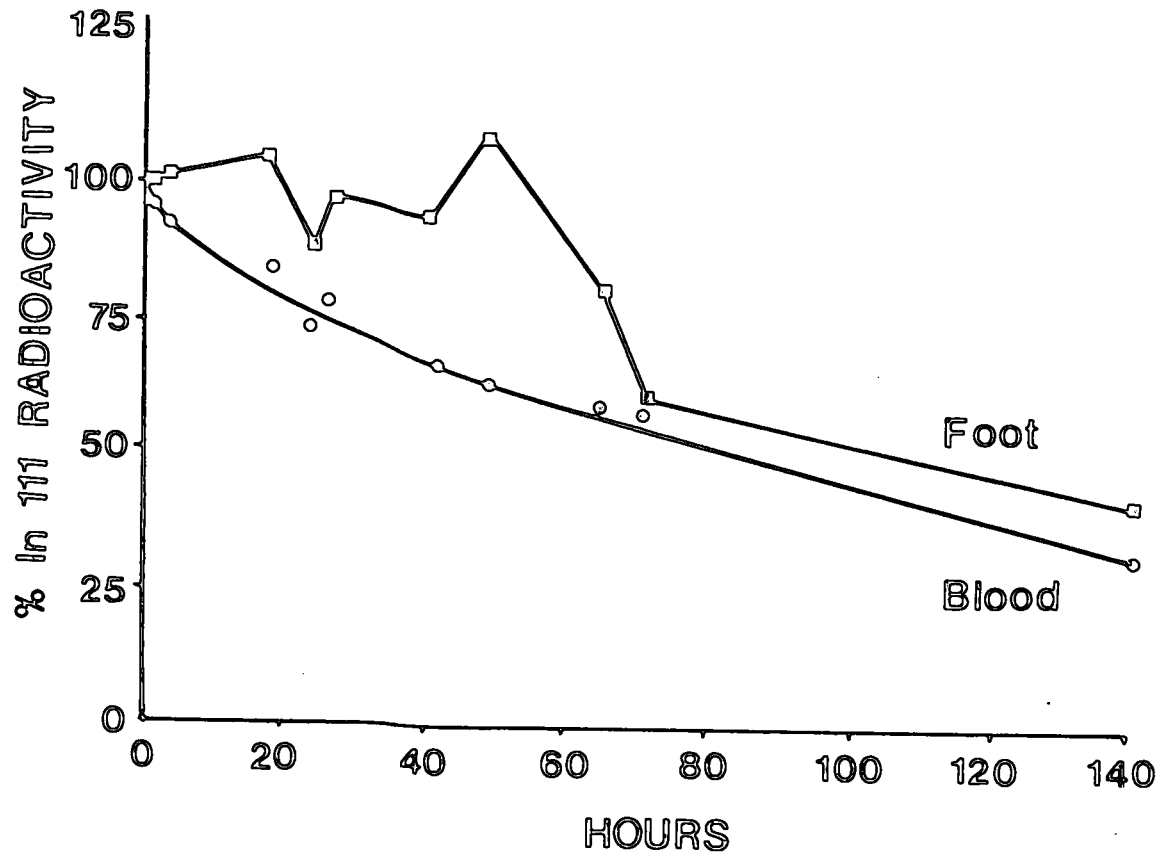


Fig 4: The rate of clearance of platelets from the foot and blood of a patient with peripheral vascular insufficiency. In this patient the extent of vascular insufficiency in the two limbs also differed: more In-111-activity accumulated in the more severely compromised limb (data not shown).

It may be concluded that in arterial disease, not complicated by thrombosis, the MPLS is shortened. This platelet-vessel wall interaction is reflected by an increase in the residence time of activated platelets or platelet aggregates in the microvasculature of the feet.

Although it may be overinterpreting the data at this stage of the study, the shape of the blood platelet survival curve may well be influenced by this platelet-vessel wall interaction. The platelet survival curve may be separated into two components. The early phase, which we name the "vascular phase", may be affected by this platelet-vessel wall interaction. It is noteworthy that the MPLS is largely dependent on the initial slope of the curve which reflects the clearance of platelets from the circulation. This part of the curve will therefore be especially susceptible to the transient accumulation of activated platelets on the vessel wall. The second part of the platelet survival curve, named the "equilibrium phase", is of a different kinetic order and starts

when the circulating platelets and those reacting with the vessel wall are in equilibrium. This part of the platelet survival curve may therefore more accurately reflect the "true" rate of clearance of platelets from the circulation, ie MPLS.

This possibility was tested by analyzing the two components of the platelet survival curves of the patients. The curves were divided into a "vascular phase", lasting from the time of injection of the In-111 platelets to 60 hours; and an "equilibrium phase" lasting from 60 hours to the end of the platelet survival time of the patient. The blood radioactivity data of the two phases were fitted to the multiple hit model and the respective estimates of the MPLS compared to that obtained when all the data points were included. The results are summarised in Table II.

TABLE II: ANALYSIS OF THE COMPONENTS OF THE PLATELET SURVIVAL CURVE

	ESTIMATE OF MPLS (HOURS)					
	ARTERIAL DISEASE			REFERENCE SUBJECTS		
	Vascular phase	Equili- brium	All Data	Vascular phase	Equili- brium	All Data
Mean	132	177	133	233	198	210
SD	11	47	35	75	19	16

It is evident that in normal subjects, the MPLS estimated if all data points are included, corresponds closely to that of the vascular as well as the equilibrium phases of the curve analyzed separately. This finding is not unexpected since normally the survival curve is essentially a straight line. In the patients the results were different. First, the estimate of MPLS with all data included, corresponded closely to that of the vascular phase. Second, the MPLS estimated from the data of the equilibrium phase, was considerably longer, although not quite normal.

This approach to the analysis and the interpretation of the platelet survival curve is of some interest. The first part of the curve is known to be "unstable". This may be due to several factors: the influence of the "collection injury"; and the varying time it takes the labelled platelets to equilibrate with those of the splenic platelet pool (2). The present study indicates that the interaction of platelets with the vascular pool may also play a significant role in this regard. It would seem that the size of this vascular pool depends on the extent of the arterial disease and reflects the in vivo activation of platelets.

The second phase of the curve is of a different kinetic order and operates only after equilibrium has been reached. This part of the curve may more closely reflect the "true" life span of platelets in the circulation. Our findings, and

it must be stressed that these should be considered as preliminary at this stage, suggests that this may be near-normal, even in the presence of severe arterial disease.

CONCLUSIONS

In this study, we have exploited the superior qualities of In-111 as a platelet label. We have demonstrated the interaction of platelets with the vessel wall in patients with either aortic aneurysms or peripheral vascular disease. This interaction is complex and not fully understood. It does however seem that the platelet interaction with the vessel wall is transient. This is reflected by a prolongation of the residence time of the platelets in the diseased vessel. After detachment from the vessel wall, the activated platelets, either singly or more likely as aggregates, are trapped in the microcirculation of the periphery. After leaving the vascular compartment, the platelets are destroyed (? immediately, or can they interact with the vessel wall again?) in the RES.

This interaction of platelets with the vessel wall may be used to demonstrate the presence of arterial disease in two ways. First, the residence time of the platelets in the microcirculation of the feet may reflect the extent of the disease, be it an aneurysm or severe atherosclerosis. Second, and this is mere speculation at this time, it may be possible to dissect the vascular and equilibrium phases of the platelet survival curve. The early part of this curve may reflect arterial disease, whereas the second part of the curve is perhaps more closely related to the true platelet survival time. These findings support the finding that reversibly aggregated platelets and some loss of their granule content, may have a normal survival time in the circulation (5).

These results indicate that it is worthwhile to re-examine platelet kinetics in arterial disease with In-111-labelled platelets. This technology has been instrumental in generating a fresh approach to the understanding of the interaction of platelets with the vessel wall. It will be interesting to apply this method to study the effect of antiplatelet drugs in patients with arterial disease or vascular prostheses.

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Analysis of Indium-111 Platelet Kinetics and Imaging in Patients with Aortic Grafts and Abdominal Aortic Aneurysms

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To quantitatively characterize processes of platelet thrombus formation *in vivo*, the kinetics and incorporation into thrombus of autologous In-111-labeled platelets were compared in six patients with aortic aneurysms and in seven patients with prosthetic aortic grafts. Although platelet survival was comparably shortened in both patient groups (mean, 5.8 days), the maximum radioactivity (percentage of whole body radioactivity) as determined by gamma camera imaging was higher in the aneurysms than in the grafts ($3.3\% \pm 1.6\%$ vs. $1.6\% \pm 1.1\%$, $p=0.05$). Maximum In-111 uptake was also attained more quickly in the aneurysm patients (2.3 ± 0.8 days vs. 3.5 ± 1.3 days; $p=0.07$). The experimental platelet kinetic and imaging data were subsequently evaluated by compartmental analysis to estimate both normal and disease-related components of platelet destruction. This analysis indicated that deposited platelet radioactivity had a longer residence time on grafts (2.9 ± 1.7 days vs. 1.4 ± 0.9 days, $p=0.07$) but accumulated at a faster rate in aneurysms ($5.0\% \pm 3.4\%$ per day vs. $1.4\% \pm 0.9\%$ per day, $p=0.02$). As determined by imaging, only a proportion of increased platelet destruction was specifically due to the aneurysms ($55\% \pm 38\%$) or grafts ($17\% \pm 11\%$, $p=0.03$). This result indicates additional components of platelet destruction unrelated to graft and aneurysm thrombus formation which, in some graft patients, may reflect a greater severity of vascular disease or other mechanisms causing a preferential shortening of platelet survival. Thus, the analytical approach described may be a useful one for discriminating components of *in vivo* platelet utilization including platelet removal due to normal hemostatic and senescent mechanisms, localized thrombus formation, and more generalized vascular disease. (Arteriosclerosis 10:1037-1044, November/December 1990)

In patients with thromboembolic disorders and cardiovascular devices, measurements of reduced platelet lifespan and of localized platelet deposition have proven clinically useful for assessing the severity of disease and the effectiveness of antithrombotic therapy.¹⁻⁹ Although these measurements are quantitative and objective, they are not readily interpreted in terms of rates of thrombus formation and dissolution. For example, platelet survival studies, which reflect all mechanisms of platelet utilization, cannot discriminate the interdependent processes of senescent platelet removal,^{1,6} normal platelet utilization for the maintenance of vascular integrity,¹⁰ and additional components of platelet destruction due to vascular disease. Similarly, measurements of localized platelet depo-

sition by scintillation camera imaging or other methods may have little relation to overall platelet turnover.

In this study, we evaluated patients with aortic aneurysms and prosthetic aortic grafts since both are actively thrombogenic as demonstrated by shortened platelet survival times,^{1,2,6} imaging of In-111 platelet deposition,³⁻⁸ and morphologic and clinical observations.^{11,12,13} In these patients, measurements of platelet lifespan have, in general, correlated poorly with measurements of In-111 platelet deposition.^{6,14} Thus, in aortic graft recipients, platelet survival tends to normalize over the first postoperative year, while platelet accumulation onto such grafts may continue at a reduced rate for periods of up to 10 years and perhaps indefinitely.^{5,7} In addition, in both patient groups, it has been demonstrated by dynamic gamma camera imaging that the accumulation of platelet radioactivity may be nonlinear with time, suggesting an exchange or equilibration between circulating and deposited platelet pools.^{6,14} At present, the quantitative interpretation of these various observations remains uncertain.

In the aneurysm and prostheses patients described in this report, the mechanisms of platelet removal (senescence, thrombosis, and hemostasis) were compared by a method of compartmental analysis of data from concurrent platelet kinetic and platelet imaging studies. It should be noted that it was not the objective of this study to

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compare by scintillation camera imaging the uptake of In-111 platelets by aortic grafts and aneurysms, a process that has been described for both patient groups.^{2-8,14} Rather, we employed well-documented methods for platelet kinetics and imaging to more clearly define the different components of platelet utilization in these disorders and to estimate the steady-state rates of platelet destruction due to normal physiologic mechanisms and direct interactions with the grafts, aneurysms, and other sites of vascular disease.

Methods

Patients

Thirteen patients gave informed consent to take part in the study, which was approved by the Ethical Committee of the Provincial Administration and the University of the Orange Free State. Six patients (five men, one woman) having abdominal aortic aneurysms of similar size and location were diagnosed according to clinical criteria.⁶ The diagnosis was confirmed by ultrasonic examination. Seven other patients (five men, two women) had double velour Dacron aortobifemoral grafts (U.S. Catheter, Inc., Billerica, MA). The period from graft implantation ranged from 2 to 60 months (mean, 32 ± 21 months). The mean age of the aneurysm patients was 60 ± 12 years (45 to 75 years), and that of the prosthesis patients was 59 ± 7 years (47 to 69 years). At the time of these studies, all grafts were patent as determined by Doppler scanning, and no patient was clinically symptomatic for peripheral ischemia or thromboembolism. Determination of platelet survival was also performed in six normal individuals ages 25 to 45 years. For a period of at least 2 weeks before each study, neither the patients nor the control subjects received any therapy that might have influenced platelet survival or function.

Platelet Labeling and Survival Measurements

Autologous platelets were isolated from whole blood under sterile conditions and were labeled with In-111-oxine as described.^{15,16} The labeled platelets were viable as documented by a normal aggregation response after exposure to adenosine 5'-diphosphate *in vitro*¹⁵ and by their normal recovery in the circulation after infusion (Tables 1 and 2).

The mean platelet lifespan was calculated by least-squares fitting the disappearance pattern of circulating In-111 platelet radioactivity to gamma functions.^{17,18} After the injection of labeled cells, the disappearance curve was generated by counting blood samples collected at 90 minutes and daily thereafter for 6 days. The recovery of labeled platelets in the circulation was calculated by estimating patient blood volumes and by back extrapolation of the disappearance curve to zero time.^{17,19}

Platelet Imaging

In-111 images were made with a Searle large-field-of-view scintillation camera and were analyzed with a Medical Data Systems A² image processing system. In patients with aortic aneurysms; the percentage of total injected radioactivity associated with the aneurysm was

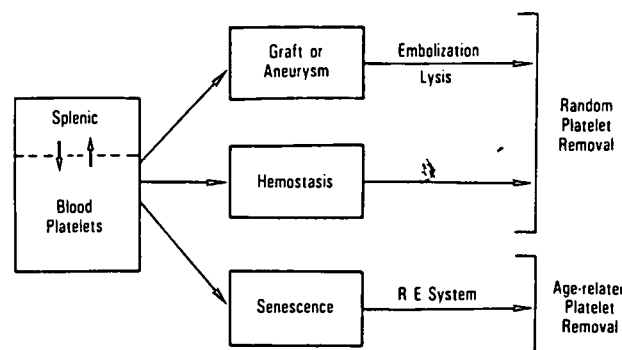


Figure 1. Schematic representation of pathways of platelet destruction. Circulating platelets, in equilibrium with the splenic platelet pool, are removed by hemostatic, senescent, or disease-related mechanisms.

determined by using the geometrical mean method. This method corrects for the attenuation of gamma photons by intervening tissues.^{15,20,21} In brief, anterior and posterior images of the whole body were acquired daily; the count rates in the aneurysm and whole body were determined by region-of-interest analysis and were corrected for background radioactivity. Aneurysm radioactivity (counts/pixel) was corrected for sequestered bone marrow and circulating blood radioactivities by subtracting the count rate (corrected for background radioactivity) in an adjacent region positioned over an iliac artery. Whole body and aneurysm radioactivities were then corrected for In-111 decay (half-life, 2.8 days).

In patients with Dacron aortic prostheses, the deposition of In-111 platelets was measured as described elsewhere.²¹ The total amount of radioactivity injected was determined by counting the injectate with the scintillation camera. After administration of the labeled cells, 30-minute anterior images of the prostheses were acquired daily. The count rates in the prosthesis and in an adjacent area were determined by region-of-interest analysis. Prosthesis radioactivity was corrected for sequestered bone marrow and blood radioactivities by subtraction of the count rate in the adjacent area from that of the prosthesis. The attenuation of gamma photons by intervening tissue was measured by determining the depth of the prostheses and deriving an attenuation factor.²¹ Prosthesis depth ranged from 5 to 9 cm. The accuracy of this method of depth determination was confirmed in a human-like phantom with an In-111 source at depths ranging from 5 to 11 cm. The accuracy of determination of depth was $2.4\% \pm 5.1\%$. The prosthesis count rate was then corrected for tissue attenuation and radioisotope decay and was expressed as a percentage of the amount of radioactivity injected. Since little In-111-oxine radioactivity is lost from the whole body over time,^{15,16} the methods used to quantify the amount of radioactivity associated with both the aneurysms (fraction of whole body In-111) and arterial grafts (fraction of total In-111 dose) were essentially equivalent and in both cases reflected only deposited (noncirculating) radioactivity.

Analysis of Platelet Kinetic and Imaging Data

The important pathways of platelet removal considered in this study are shown in Figure 1. In this scheme, circulating platelets are in dynamic equilibrium with the splenic platelet pool, which comprises about one-third of the total body platelets.^{10,15,16} Platelets are removed from the circulation by normal physiologic mechanisms (senescence and hemostasis). Thus, based on an observed platelet lifespan of 9 to 10 days and a predominantly linear platelet disappearance pattern in normal individuals, it is accepted that approximately 10% of the circulating platelet pool will be removed daily by senescent mechanisms (reticulo-endothelial system) after a finite lifespan *in vivo*.^{10,17,22} In addition, there appears to be a modest, fixed, hemostatic platelet requirement for the maintenance of vascular integrity¹⁰ (Figure 1). In patients, platelets are also lost from the circulation after direct interactions with thrombus associated with an arterial graft or aneurysm or at other sites of vascular disease.

In the present analysis, platelet utilization by both normal hemostatic and disease-related mechanisms were considered to be random processes, so that with increasing severity of disease, the extent of age-related platelet removal (senescence) would be reduced relative to the random component. The relative proportion of platelets destroyed by random vs. senescent mechanisms was calculated according to an application of the Mills-Dornhorst model:

$$\frac{N(t)}{N(0)} = \frac{e^{-kt} - e^{-kT}}{1 - e^{-kT}} \quad (1)$$

This model, which was originally developed to describe the survival of red cells,^{23,24} applies equally well to any element having a finite lifespan but which is also subject to risk of destruction by external hazards.^{17,18} In this formula, the number of labeled platelets circulating at time *t* after infusion, *N(t)*, is determined by a maximum platelet lifespan (*T*), which will be reduced depending upon the rate of random platelet destruction (*k*) due to extrinsic mechanisms.^{23,24} Since the initial slope of the platelet survival curve, *N(t)/N(0)*, intercepts the time axis at the mean platelet lifespan,^{23,25} it follows that:

$$\tau = \frac{1 - e^{-kT}}{k} \quad (2)$$

where τ is the estimated mean platelet lifespan (days), and *k* is the rate of random platelet removal (fraction of circulating platelets per day). Equation 2 is the well-known expression of Mills-Dornhorst,^{23,24} which has been widely used for the interpretation of platelet survival data.^{10,17,18,25}

The application of Equations 1 and 2 to platelet survival curves obtained in normal subjects and in patients with decreased platelet production has suggested a finite platelet lifespan, *T*, averaging approximately 10.5 days.^{10,17,22} Therefore, this value was used in the subsequent analysis. Similarly, a fixed hemostatic requirement of approximately 4700 platelets per microliter of whole blood per day has been determined.¹⁰

With these approximations, both the normal and disease-related components of platelet utilization were calculated in the following manner. The fractional rate of random platelet destruction due to normal physiologic mechanisms (fraction per day) was determined by dividing the fixed platelet requirement (4700/ μ l/day) by the circulating platelet count (platelets per microliter) for each patient and normal subject.¹⁰ Given the mean platelet survival time (τ) as determined in all individuals studied, the rate constant for random platelet removal (*k*) was calculated directly from Equation 2 with *T*=10.5 days. It should be noted that when platelet lifespan is reduced significantly, as in the present studies, calculations of platelet destruction rates are relatively independent of assumed values of *T* within normal limits as well as possible variations in maximum lifespan of individual platelets in different subjects (i.e., when platelet survival is short, few cells reach their maximum possible lifespan). The fractional rate of senescent platelet removal was calculated by subtracting the rate of random platelet destruction, *kP* (platelets per microliter per day), from the overall turnover rate of circulating platelets, *P/τ*, and dividing by the platelet count, *P*. The fractional rate of senescent platelet removal was therefore simply given as: $1/\tau - k$. The fraction of platelets removed by random (vs. senescent) mechanisms throughout their lifespan was calculated by dividing the rate of random platelet removal (*kP*) by the overall turnover rate of circulating platelets (*P/τ*), or equivalently, as *kτ*. By subtracting the fractional rate of platelet removal due to normal hemostatic processes (4700/*P*) from the fractional rate due to all random mechanisms (*k*), the component due to vascular disease and other processes could be calculated. Of this value, the fraction of platelets removed specifically through interactions with the aortic grafts or aneurysms was determined by analysis of the In-111 imaging data as described below.

Since the graft and aneurysm patients were clinically stable, an approximate steady-state was presumed; thus, over the course of the dynamic imaging studies, an equilibrium existed between the rate of platelet thrombus accumulation and the rate of removal from thrombus of deposited platelet material. In the present instance, this assumption would appear reasonable since: 1) platelet counts were stable during the study period; 2) In-111 platelet radioactivity was, in fact, lost from both grafts and aneurysms after the clearance of labeled platelets from the circulation (see the Results section); and 3) In-111 platelet radioactivity deposited in aneurysms is largely confined to a surface layer in contact with the flowing blood.⁶ Under these conditions, the accumulation of platelet radioactivity by grafts and aneurysms after infusion of labeled cells may be described mathematically as:

$$\frac{dI(t)}{dt} = K \frac{N(t)}{N(0)} - \frac{I(t)}{\theta} \quad (3)$$

where *I(t)* is the fraction of injected In-111 platelet radioactivity deposited at time *t*, *K* is the fraction of circulating platelets daily incorporated into thrombus (day^{-1}), θ is the average residence time or period that deposited platelet radioactivity is retained locally before removal by emboliza-

Table 1. Platelet Utilization in Patients with Aortic Aneurysms

Patient	Platelet			Peak radioactivity		Average residence time (days)	Platelet destruction rates (% per day)					% Aneurysm	% Random
	Count/l ($\times 10^{-9}$)	Recovery (%)	Lifespan (days)	% Whole body	Time (days)		Total random	Hemostasis	Aneurysm	Other	Senescence		
1	198	78	6.29	2.0	2.2	1.1	10.8	2.4	3.2	5.2	5.1	38	68
2	303	79	4.21	3.1	3.0	2.3	21.2	1.6	2.9	16.7	2.6	15	89
3	285	57	4.67	3.7	3.4	2.8	18.3	1.7	2.9	13.7	3.1	17	85
4	230	84	8.46	1.0	1.6	0.6	4.3	2.0	2.2	0.1	7.5	96	36
5	236	58	4.96	5.3	1.7	0.8	16.7	2.0	9.7	5.0	3.5	66	83
6	205	59	6.13	4.4	1.6	0.6	11.4	2.3	9.1	0.0	4.9	100	70
Mean	243	69	5.79	3.3	2.3	1.4	13.8	2.0	5.0	6.8	4.5	55	72
1 SD	43	12	1.54	1.6	0.8	0.9	6.1	0.3	3.4	7.0	1.8	38	19
Normals	260	74	8.95	0	0	0	1.9	1.9	0	0	9.4	0	17
1 SD	43	17	0.75				0.3	0.3			1.0		3

Theoretically derived parameters included the average residence time for aneurysm-associated platelets (θ) and rates of platelet destruction due to senescence and random mechanisms (hemostasis, aneurysm, other disease). % Aneurysm (column 13) is the percentage of pathological platelet destruction (aneurysm plus other) that can be attributed directly to the aneurysm. % Random (column 14) is the fraction of platelets destroyed by all random (vs. senescent) mechanisms throughout their lifespan. The calculations are described in the Methods section.

tion or lysis, and $N(t)/N(0)$ is the fraction of injected platelets still circulating at time t as given by Equation 1. Or, in brief, Equation 3 states that the rate of change of deposited radioactivity at time t , $dI(t)/dt$, is equal to the deposition rate, $K \cdot N(t)/N(0)$, minus the removal rate, $I(t)/\theta$, of platelet-associated Indium-111. The general solution to Equation 3 is obtained by direct integration as:

$$I(t) = \frac{\theta K}{(1 - k\theta)(1 - e^{-kT})} \cdot [e^{-kt} - e^{-t/\theta} - (1 - k\theta)e^{-kT} + (1 - k\theta)e^{-(t/\theta + kT)}] \quad (4)$$

Equation 4 gives deposited Indium-111 activity, $I(t)$, as a function of time and four parameters (k , K , T , θ) and was fitted to the dynamic imaging data obtained in the patients with grafts and aneurysms to determine values for the parameters, K and θ . In the curve fitting procedure, the value of T was assumed to be equal to 10.5 days, k was determined a priori from Equation 2, and the parameters, K and θ , were determined for each patient by least-squares regression analysis. The apparent maximum value of platelet uptake and the time required to reach this maximum were also calculated for each patient from the best-fit equation. By subtracting the components due to normal hemostasis and thrombosis associated with the grafts or aneurysms from the total rate of random platelet destruction, an additional component of platelet removal (presumably due to other disease processes) was calculated.

Curve Fitting and Statistical Analyses

Curve fitting of prosthesis and aneurysm time/activity data to Equation 4 was performed on a microcomputer (IBM PC-AT) according to the nonlinear least-squares iteration procedure of Snedecor and Cochran.²⁶ Comparisons were made by using Student's t test (two-tailed) for unpaired sample groups. The data are expressed as the mean values ± 1 SD.

Results

Platelet Survival Studies

The mean values for platelet count and platelet recovery were similar (Tables 1 and 2) and not different from the control values obtained in six normal subjects ($p > 0.1$). The mean platelet lifespans were also equivalent, averaging 5.79 ± 1.54 days in the aneurysm patients (Table 1) and 5.75 ± 1.46 days in the group with aortic prostheses (Table 2). The composite platelet disappearance patterns obtained in the patients and normal subjects are shown in Figure 2. In the aneurysm and graft patient groups, the averaged best-fit platelet survival curves derived from gamma function modeling were

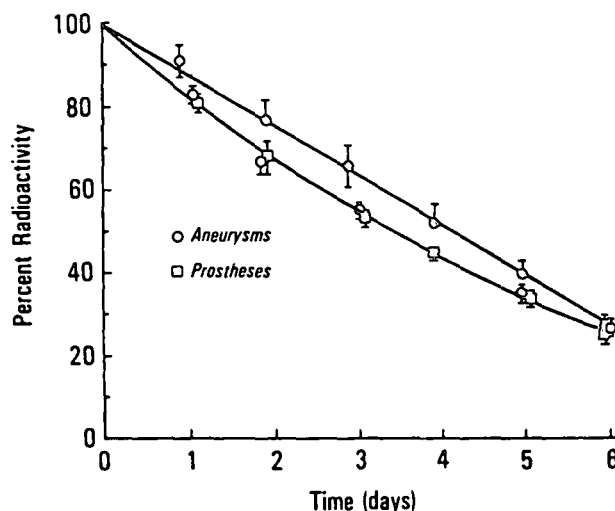


Figure 2. Platelet survival curves in patients and normal subjects. In-111-platelet disappearance patterns were equivalent in patients with aortic aneurysms ($\circ$) and aortic Dacron grafts ($\square$), but platelet clearance was increased as compared to normal control subjects ($\circ$). For both patient groups, mean platelet lifespans were reduced significantly ($p < 0.01$) vs. control values (see Results).

Table 2. Platelet Utilization in Patients with Aortic Prostheses

Patient	Platelet			Peak Radioactivity		Average residence time (days)	Platelet destruction rates (% per day)					% Prosthesis	% Random
	Count/l ($\times 10^{-9}$)	Recovery (%)	Lifespan (days)	% Whole body	Time (days)		Total random	Hemostasis	Prosthesis	Other	Senescence		
1	252	69	6.88	0.8	5.1	5.0	8.7	1.9	0.4	6.4	5.8	40	60
2	250	39	4.29	2.2	4.2	4.7	20.7	1.9	1.3	17.4	2.7	7	89
3	335	70	5.63	2.0	3.0	1.9	13.4	1.4	1.9	10.1	4.3	16	76
4	206	52	4.75	3.7	3.6	3.0	17.8	2.3	2.7	12.8	3.3	18	85
5	239	56	4.83	1.0	1.6	0.7	17.4	2.0	1.9	13.5	3.4	12	84
6	379	75	5.42	0.6	2.1	1.1	14.4	1.2	0.8	12.4	4.9	6	78
7	289	52	8.46	1.1	4.8	3.8	4.3	1.6	0.6	2.1	7.5	21	36
Mean	279	59	5.75	1.6	3.5	2.9	13.8	1.8	1.4	10.7	4.4	17	73
1 SD	60	13	1.46	1.1	1.3	1.7	5.7	0.4	0.8	5.0	1.7	11	19
Normals	260	74	8.95	0	0	0	1.9	1.9	0	0	9.4	0	17
1 SD	43	17	0.75				0.3	0.3			1.0		3

Theoretically derived parameters included the average residence time for prosthesis-associated platelets (θ) and rates of platelet destruction due to senescence and random mechanisms (hemostasis, prosthesis, other disease). % Prosthesis (column 13) is the percentage of pathological platelet destruction (prosthesis plus other) that can be attributed directly to the prosthesis. % Random (column 14) is the fraction of platelets destroyed by all random (vs. senescent) mechanisms throughout their lifespan. The calculations are described in the Methods section.

virtually coincident. In both cases, platelet survival was reduced significantly ($p < 0.01$) compared to normal control values, which averaged 8.95 ± 0.75 days.

Since platelet survival was comparably shortened in both patient groups, the calculated rates of total random platelet destruction (excluding senescent platelet removal) were also equivalent, and averaged $13.8\% \pm 6.1\%$ per day in the aneurysm group and $13.8\% \pm 5.7\%$ per day in the prostheses patients. These rates were significantly greater ($p < 0.001$) than the control values of $1.9\% \pm 0.3\%$ per day, which in the normal individuals reflected only hemostatic platelet utilization (Tables 1 and 2). Similarly, senescent platelet removal averaged $4.5\% \pm 1.8\%$ per day in the aneurysm patients and $4.4\% \pm 1.7\%$ per day in the graft recipients ($p > 0.5$). These rates were significantly reduced as compared to control values ($9.4\% \pm 1.0\%$ per day, $p < 0.001$). Also, the fraction of platelets that were removed by random mechanisms throughout their lifespan (% random, Tables 1 and 2) was elevated in both the aneurysm patients ($72\% \pm 19\%$) and graft patients ($73\% \pm 19\%$) relative to the control group values ($17\% \pm 3\%$, $p < 0.001$). The platelet requirement to support hemostasis and/or other normal physiologic mechanisms averaged 1.8% to 2.0% per day in both the patient groups and normal subjects.

Platelet Imaging Studies

Despite the similarities in platelet kinetics between the aneurysm and graft patients, dynamic imaging demonstrated markedly dissimilar patterns of In-111 platelet accumulation onto the abnormal endovascular surfaces (Figure 3). The maximum accumulation of platelet radioactivity and the time required to reach this peak value after injection of the labeled cells were calculated directly from Equation 4 following least-squares fitting to the time-radioactivity data for each patient. The aneurysms accumulated platelets more rapidly, with the peak uptake of radioactivity observed at 2.3 ± 0.8 days compared to

3.5 ± 1.3 days in the graft patients ($p = 0.07$). Maximum accumulation of radioactivity was also greater in the aneurysms, averaging $3.3\% \pm 1.6\%$ of total radioactivity (vs. $1.6\% \pm 1.1\%$ on the vascular grafts, $p = 0.05$).

These differences between patient groups, which were demonstrated experimentally by dynamic imaging (Figure 3), were also reflected in the parameters derived from the analysis (Equation 4). Thus, the average residence time (θ) for aneurysm-associated radioactivity was 1.4 ± 0.9 days, vs. an average residence time of 2.9 ± 1.7 days for graft-associated In-111 radioactivity ($p = 0.07$). The rate constant (K) for platelet deposition in aneurysms ("Aneurysm," Table 1) averaged $5.0\% \pm 3.4\%$ of circulating platelets per day and was significantly greater ($p = 0.02$) than the rate of platelet deposition onto the aortic prostheses, $1.4\% \pm 0.8\%$ per day ("Prosthesis,"

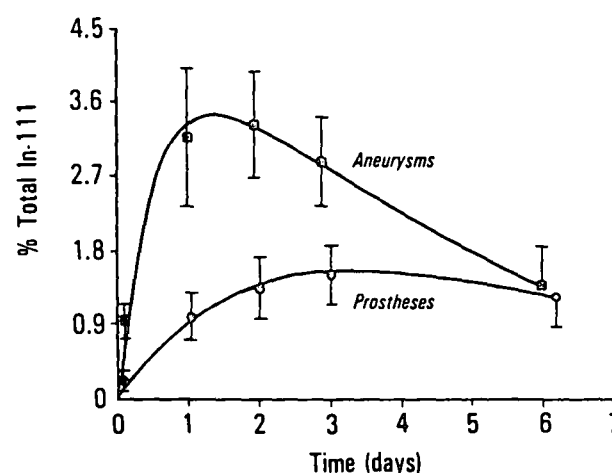


Figure 3. In-111-platelet accumulation onto aortic aneurysms (upper curve) and Dacron aortic grafts (lower curve), expressed as a percentage of total injected radioactivity for images taken serially over a 6-day period. Solid lines are the best least-squares fit to the data according to Equation 4.

Table 2). The component of random platelet destruction not due to the grafts, aneurysms, or hemostasis (termed "Other," Tables 1 and 2) averaged 6.8% per day in the aneurysm patients and 10.7% per day in the graft patients.

The rate of platelet destruction by the aneurysms expressed as a percentage of the total rate of platelet removal due to abnormal mechanisms (aneurysm plus other) averaged $55\pm38\%$ (Table 1). Despite the variability in this measurement between patients (range, 15% to 100%), which presumably reflects differences in aneurysm age, thrombogenicity, and the relative contribution of nonaneurysm components of platelet removal, platelet destruction by the aneurysms was clearly a major factor contributing to shortened platelet survival in most patients. However, in the seven prosthesis patients, the extent of abnormal platelet destruction that could be attributed directly to the vascular grafts (% Prosthesis, Table 2) averaged only $17\pm11\%$ (range, 6% to 40%). The difference between the aneurysm and graft patients in this regard was statistically significant ($p=0.03$).

Discussion

While both arterial grafts and aneurysms are thrombogenic, these vascular abnormalities differ in terms of their pathologic mechanisms of thrombus formation. Important variables may include the effects of altered blood flow patterns, the presence of synthetic (Dacron) vs. biologic (aneurysm) nonendothelialized surfaces and differences in the relative importance of platelet-mediated vs. coagulation-mediated pathways of platelet recruitment.^{1,4,8,27,28} Thus, aneurysms may contain large visible thrombi, while grafts generally accumulate thinner layers of platelets and compact fibrin.^{1,12,13}

In the present study, the mean lifespan of circulating platelets was significantly and comparably shortened, both in patients with aortic aneurysms and in patients with Dacron aortic prostheses, although the patterns of In-111 platelet deposition, as determined from gamma camera imaging, were markedly different in the two patient groups. The aneurysms accumulated approximately twice as much injected In-111 platelet radioactivity as the vascular prostheses, and the maximum incorporation of isotope was observed at an earlier time in the aneurysm patients. Thus, measurements of platelet survival alone were clearly unable to predict the rate or extent of platelet incorporation by the grafts or aneurysms, suggesting additional and perhaps unrecognized components of platelet destruction in these disorders. These experimental observations are generally consistent with previous clinical studies in patients with aortic grafts^{2,5,7,8,14} and aneurysms.^{3,6} It should be noted that the control subjects for platelet survival studies were of a younger average age than the patients, and it is conceivable that the normal platelet lifespan may decrease modestly with advancing age. Nonetheless, platelet survivals in both patient groups were considerably reduced as compared to the generally accepted normal value of 9 to 10 days.^{1,10,16-18,22}

The analytical approach used to estimate the rates of platelet removal by different normal and pathological mechanisms allowed a broader interpretation of these findings. The results were well explained quantitatively on the basis that the aneurysms caused more destruction of circulating platelet than the grafts ($5.4\pm3.0\%$ per day vs. $1.4\pm0.8\%$ per day), with platelets deposited in aneurysms having a shorter residence time than platelets associated with the Dacron prostheses (1.4 ± 0.9 days vs. 2.9 ± 1.7 days). Since rates of thrombus formation and dissolution are equivalent under steady-state conditions, the observations that aneurysms accumulated platelets more quickly and to a greater extent suggests that they are typically more thromboembolic/thrombolytic, as well as more thrombogenic, than vascular prostheses. This rapid turnover of aneurysm (vs. graft) thrombi could reflect a more active thrombin-mediated deposition process and locally enhanced fibrinolysis and/or the formation of thrombus, which is inherently less stable toward fluid shear forces or more susceptible to degradation by thrombolytic mechanisms. This concept is also consistent with clinical observations that aneurysms may produce a chronic consumption coagulopathy,²⁸ while arterial grafts selectively remove platelets without increased turnover of fibrinogen and plasminogen or elevation of plasma levels of fibrinogen/fibrin degradation products.^{1,2} Our results are also consistent with the increased frequency of clinical embolic phenomena observed in aneurysm patients¹³ and support the view that aneurysm segment reconstruction by vascular grafting is therapeutically warranted.

Graft-related platelet deposition accounted for a relatively minor proportion of increased platelet turnover above normal physiologic levels ($17\pm11\%$) as compared to the aneurysm patients ($55\pm38\%$). This may explain, in part, the poor correlation found between platelet lifespan and maximum graft platelet deposition and may be consistent with observations of reduced platelet lifespan in preoperative atherosclerotic patients.⁹ The additional components of platelet removal could be related to unrecognized (by In-111 imaging) sites of vascular disease, particularly in the graft recipients. While there is no reason to speculate that graft patients have more unrecognized preoperative atherosclerosis than aneurysm patients, in the present study the graft patients were evaluated an average of 32 months postoperatively. Over this interval, significant progression of disease at sites removed from the grafts seems likely, at least in some patients. Alternatively, this result could reflect a reduced intrinsic lifespan of individual platelets, which continued to circulate, perhaps as a consequence of reversible or sublethal platelet interactions. We consider this explanation less likely since it has been shown in rabbits that platelets degranulated *in vitro* survive normally *in vivo*^{29,30} and that in primates with vascular prostheses, platelet survival and function are normal and independent of platelet aging in the circulation after platelet cross-transfusion into normal recipients or after removal of the thrombogenic stimulus.^{31,32} The analytical approach used here is consistent with either possibility since it demonstrates only that there is an additional

component of platelet removal (i.e., a shortening in platelet survival to a degree greater than expected) occurring through mechanisms that remain to be defined.

Despite the heterogeneity of each patient population, the two groups differed most significantly with regard to the theoretically predicted rates of platelet deposition onto grafts vs. aneurysms ($p=0.02$) and were less well differentiated on the basis of experimental measurements such as the mean platelet survival time ($p>0.5$) or the maximum incorporation of radioisotope ($p=0.05$). This approach may, therefore, have a greater sensitivity for distinguishing thromboembolic disorders and for assessing the effects of therapy. In addition, information on platelet deposition rates and platelet thrombus turnover may be important in certain settings for the proper selection of radioisotopes or for the design of thrombus-directed probes (e.g., labeled monoclonal antibodies) to be used for thrombus detection. Moreover, this analytical method may also be useful for the interpretation of clinical assessments of peripheral thromboembolism or ischemia or for correlating laboratory measurements of ongoing thrombosis such as plasma levels of platelet specific proteins and other markers of active coagulation and fibrinolysis.

Finally, a plateau and subsequent decrease in graft or aneurysm radioactivity was observed in each patient studied, demonstrating both active platelet uptake and the reduction of platelet-containing thrombus, presumably by microembolization and/or lytic mechanisms. This phenomenon has generally not been demonstrated in previous reports, perhaps since imaging was not performed at later time points^{3,4,5,7,8} or, because the presentation of imaging data simply normalized with respect to reference areas or circulating blood radioactivity does not fully exploit the information contained in the dynamic curves.^{4,5,7,8} Thus, while measurements such as mean platelet lifespan and maximum incorporation of isotope have clearly proven useful for thrombus detection, we believe that the additional determination of both normal and pathological components of platelet destruction will provide a more thorough characterization of platelet mechanisms in thromboembolic disease.

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Index Terms: aortic aneurysms • vascular grafts • Indium-111 platelet kinetics • Indium-111 platelet imaging

Cardiovascular Topics

Changes in platelet function in atherosclerotic vervet monkeys after long-term dietary enrichment with n-3 and n-6 essential fatty acids

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Summary

Plaque formation is a complication of atherosclerosis that predisposes to thrombotic myocardial and cerebral infarction. Platelet activation, vascular stenosis and anomalous vasoconstriction are contributory factors. Hypothetically, dietary supplementation with essential fatty acids could modify the pathogenesis of atherosclerosis by inhibiting platelet function, by promoting synthesis of vasoactive eicosanoids, and by altering the composition of plasma lipids. To test this theory primates (vervet monkeys) with confirmed atherosclerosis were supplemented for 20 months so that matched individuals received 2% of their dietary energy from either n-3 or n-6 essential fatty acids added to an atherogenic diet. In matched subsets of each essential fatty acid treatment, the atherogenic diet was changed to a therapeutic diet known to regress some components of atherosclerosis.

Mean platelet lifespan, the plasma beta-thromboglobulin/platelet factor 4 ratio (BTG:PF4), the circulating platelet aggregate ratio, the *in vitro* aggregation response of platelets to ADP and collagen, and thromboxane A₂ production by platelets that have been stimulated with collagen were measured before and after treatments. Platelet function was not significantly inhibited by n-3 essential fatty acid supplementation of either diet. N-6 essential fatty acid supplementation lengthened the mean platelet lifespan significantly by 33 ± 23 hours and 28 ± 22 hours in animals receiving the atherogenic diet and therapeutic diet respectively. This lengthening is either a result of less severe atherosclerosis in these groups or of inhibition of the interaction of platelets with atherosclerotic lesions.

Atherogenesis is a complex process that involves accumulation of lipoprotein in the arterial wall and attachment of monocytes and T-lymphocytes to adhesive proteins expressed on the surface of injured endothelial cells. The monocytes and lymphocytes migrate into the subendothelium where the monocytes become macrophages that accumulate lipid to change into foam cells. Together with the accompanying lymphocytes, the foam cells form the fatty streak. Continued cell influx and proliferation lead to more advanced lesions, distinguished by their fibrous character, and ultimately to the fibrous plaque. In humans, monkeys and rabbits the affected arteries contain sites with retracted endothelial cells which provide foci for platelet interactions leading to the formation of mural thrombi. Thus,

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TABLE I THE DAILY ENERGY AND CHOLESTEROL SUPPLIED BY DIET, THE RATIO OF POLYUNSATURATED TO SATURATED FATTY ACIDS (P/S RATIO) AND DOSES OF THE OIL SUPPLEMENTS. VALUES ARE GIVEN AS A MEAN $\pm$ 1 SD

	AD/SO N = 8	AD/FO N = 9	TD/SO N = 10	TD/FO N = 8	Reference N = 10
Total energy (kcal)	318 $\pm$ 7	312 $\pm$ 18	363 $\pm$ 10	366 $\pm$ 13	366 $\pm$ 8
Energy from lipids (%)	35 $\pm$ 2	34 $\pm$ 4	17 $\pm$ 2	17 $\pm$ 3	17 $\pm$ 2
Energy from carbohydrate (%)	49 $\pm$ 1	50 $\pm$ 3	69 $\pm$ 2	69 $\pm$ 2	69 $\pm$ 2
Energy from protein (%)	16 $\pm$ 1	16 $\pm$ 1	14 $\pm$ 1	14 $\pm$ 1	14 $\pm$ 1
Cholesterol (μ g/kcal)	310 $\pm$ 39	326 $\pm$ 37	50 $\pm$ 10	56 $\pm$ 9	53 $\pm$ 14
Cholesterol (% total food)	0.050	0.056	0.007	0.008	0.008
P/S ratio	0.37 $\pm$ 0.05	0.28 $\pm$ 0.06	1.53 $\pm$ 0.46	1.56 $\pm$ 0.01	1.48 $\pm$ 0.30
N-3 supplementation (mg)	0	254 $\pm$ 11	0	254 $\pm$ 11	0
N-6 supplementation (mg)	260 $\pm$ 8	0	260 $\pm$ 8	0	0

AD = atherogenic diet, TD = therapeutic diet, FO = fish oil, SO = sunflower oil.

atherosclerosis is a principal contributor to the pathogenesis of myocardial and cerebral infarction, and gangrene and loss of function in the extremities.

It has been confirmed that the feeding of diets rich in saturated fat to non-human primates promotes atherogenesis, while decreasing dietary saturated fat or supplementing the diet with n-3 fatty acids, or both, may halt the progress of the lesions or even cause them to regress.^{2,3} In addition, it has been shown in humans that aggressive treatment with lipid-lowering drugs regresses coronary atherosclerosis.^{4,7} The fatty acids may influence the metabolism of lipoproteins or other plasma proteins, fluidity of membranes, and the functions of platelets, endothelial cells, monocytes and macrophages.^{2,21} The effect of supplementing diets with n-3 fatty acids derived from marine fish oil is, however, not always anti-atherogenic in non-human primates^{2,3} or man.^{22,24}

Researchers have shown, in short-term studies (< 30 days) of the effects of marine fish oil enrichments of atherogenic diets in humans, rats and rabbits, that it might be possible to prolong the bleeding time, decrease the tendency of platelets to aggregate, inhibit platelet adhesion, reduce formation of arterial thrombi in carotid arteries after endarterectomy, alter monocyte-neutrophil function and reduce the rate of restenosis after coronary angioplasty.^{8,21} The explanation may be the competition between eicosapentaenoic acid and arachidonic acid as potential substrates of cyclo-oxygenase. Whereas the predominant product of arachidonic acid formed by platelets, thromboxane A₂, is a potent vasoconstrictor and platelet agonist, the corresponding product of eicosapentaenoic acid, thromboxane A₃, is biologically virtually inert.²⁵ The effects of n-3 fatty acids on platelet function are, however, variable,^{8,21} and either increasing the dose or prolonging the course may inhibit platelet function further.⁸

In this study we investigated the effect that a long-term dose of fish oil, which was clinically safe in primates, has on platelet function. The dose of Atlantic pilchard (*Sardinops ocellata*) oil provided 2% of dietary energy. Other potential modifiers of platelet function imposed in addition to the fish oil were long-term intake of an atherogenic diet and dietary change from this to a therapeutic diet known to regress some components of atherosclerosis.^{2,3} The treatments were replicated with equivalent doses of sun-

flower (*Heliantes annuus*) oil n-6 polyunsaturates instead of fish oil n-3 polyunsaturates (Table I).³

Material and methods

Study design

All the primates used were mature adult vervet monkeys (African green monkeys) (*Cercopithecus aethiops*). To quantitate aortic and peripheral atherosclerosis they were necropsied at the end of the study and the results published.³ Altogether 18 male and 17 female vervets were fed an atherogenic diet for at least 15 months (range 15 - 71 months) before the start of the study. Thereafter individual animals allocated to the dietary treatments were matched for exposure to the atherogenic diet, sex, body weight and plasma low-density lipoprotein cholesterol. Seventeen remained on the atherogenic diet and were subdivided into a group that was given sunflower oil (N = 8) and a group given Atlantic pilchard fish oil (N = 9). The diet of the remaining 18 was changed to a therapeutic diet with sunflower oil (N = 10) and fish oil (N = 8) enrichment. The sunflower oil was given as a control to balance the oil intake among groups. The composition and preparation of the therapeutic and atherogenic diets (Table I) are described elsewhere.²³ Briefly, in the atherogenic diet intake of saturated fat, red meat, refined carbohydrate and salt were increased, whereas in the therapeutic diet, in which the content and saturation of fat were reduced, cholesterol was minimal, little red meat was given, no salt was added and the amounts of vegetables and unrefined carbohydrate were increased. No extra cholesterol was added to either diet. N-3 polyunsaturated fatty acids in the sunflower oil balanced the dose of n-3 polyunsaturated fatty acids in the fish oil.

A reference group of 10 female vervets was also included in the study. They were fed a therapeutic diet for 6 months before the study started and remained on this diet until the end of the study. Before this, their diet from weaning had been high in carbohydrate and low in fat. This group was monitored to identify possible effects of non-treatment-related environmental influences, such as stress, on platelet function during the study.

Laboratory tests

The baseline tests were done 1 week before dietary manipulation was started and repeated after 20 months. All measurements were taken over a period of 7 days. Blood was collected without stasis from the femoral vein. The monkeys were anaesthetised with ketamine hydrochloride (5 mg/kg body mass) to allow handling.

Markers reflecting diets. Total cholesterol levels in plasma low-density lipoproteins and the platelet membrane phosphatidylcholine fatty acid composition were determined as previously described.^{3,4} Eicosapentaenoic acid and linoleic acid were markers of fish oil and sunflower oil, respectively. The fatty acid content was assessed by gas liquid chromatography. Briefly, platelet-rich plasma was prepared by differential centrifugation, the platelets washed free of plasma and then resuspended in Plasmalyte B (Baxter Travenol). Platelet lipids were extracted with a chloroform/methanol (2:1) extraction mixture and phosphatidylcholine was isolated by thin-layer chromatography. The phosphatidylcholine fatty acids were transmethylated with a sulphuric acid/methanol mixture, and after extraction by *n*-hexane, the fatty acids were analysed by gas liquid chromatography. The individual fatty acids were each expressed as a percentage of the total fatty acid content in the platelet membrane phosphatidylcholine.

Platelet function tests. Mean platelet lifespan of ¹¹¹In-labelled platelets was measured.²⁶ A sample of 8.5 ml of blood was collected in 1.5 ml ACD-A (Baxter Travenol). Platelets were harvested from the blood by differential centrifugation, pelleted by centrifugation and resuspended in 0.2 ml plasma. Tropolone and ¹¹¹In-chloride were added to the platelets and incubated for 3 minutes. Plasma was added, the platelets were concentrated and finally resuspended in platelet-poor plasma before reinjection. Labelled platelets were not injected unless aggregates consisting of 10 or more platelets formed in response to adenosine diphosphate (ADP), which was added *in vitro*.²⁶ Blood, 2 ml collected in EDTA, was collected 10 minutes after the labelled platelets were injected and again after approximately 18, 28, 45, 68, 75 and 93 hours. The radioactivity in the blood samples was determined with a well-type scintillation counter (Packard, Packard Instrument Company, Downers Grove, IL). Radioactivity/time curves were constructed and MPLS calculated by the fitting of a gamma function to the disappearance curve of labelled platelets. The recovery of labelled platelets in the circulation was calculated by back extrapolation of the disappearance curve of labelled platelets from the circulation to time zero to assess platelet viability.²⁶ The recovery and mean platelet lifespan were also determined in 6 young (< 1 year old), normal monkeys fed a therapeutic diet, and not subjected to dietary manipulations.

Plasma platelet factor 4 (Abbott Laboratories, North Chicago, USA) and β -thromboglobulin (Amersham International plc, Buckinghamshire, UK) were assayed with commercially available radio-immune assay kits.²⁷ Blood was collected in precooled syringes containing EDTA, 2-chloro-adenosine and procaine-HCl (9:1, Abbott Laboratories, Chicago, USA) to prevent platelet activation and thus *in vitro* release of the platelet-specific proteins.

The circulating platelet aggregate ratio was measured by the modified method of Wu and Hoak.²⁸ The ratio of the platelet count in platelet-rich plasma of a blood sample collected in EDTA/phosphate-buffered saline/formalin to that in the platelet-rich plasma of a blood sample collected in EDTA/phosphate-buffered saline was calculated.

The aggregation response of platelets in platelet-rich plasma (platelet count = $150 \pm 20 \times 10^9/l$) was measured turbidimetrically with a Monitor VI plus aggregometer (Helena Laboratories, Texas) as described.²⁹ ADP (Sigma) and collagen (Hormon-Chemie, Germany) were used as aggregating agents. Final cuvette concentrations of ADP were 25, 15, 5 and 2.5 $\mu\text{mol/ml}$ and that of collagen was 20 $\mu\text{g/ml}$. The percentage change in light transmission 4 minutes after addition of the agonist was used as an indication of the aggregation response.

Thromboxane A₂ synthesis by platelets was measured.^{29,30} Samples were collected exactly 4 minutes after addition of 20 $\mu\text{g/ml}$ (final concentration) collagen to platelet-rich plasma. A sample of 50 μl of the platelet suspension was withdrawn into 950 μl of indomethacin ($1 \times 10^{-4}\text{M}$ in phosphate-buffered saline, pH 7.4) in a cold tube. After holding on ice for 5 minutes, the sample was frozen at -80°C until assayed for thromboxane B₂ with a commercial radio-immune assay kit (Amersham International plc, Buckinghamshire, UK).

Statistical analyses

As the data were normally distributed, Student's *t*-test for paired data was used to assess the significance of difference in changes between baseline and final measurements. The mean difference and 95% confidence interval (CI) were also calculated to identify the magnitude and direction of the observed differences.

Results

Changes in plasma LDL cholesterol and lipoprotein profiles reflected dietary manipulation (atherogenesis versus therapeutic), but not the oil enrichment.¹ Compliance with oil enrichment was confirmed by the platelet membrane phosphatidylcholine content of arachidonic and eicosapentaenoic acids (Table II). Fish oil added to both atherogenic and therapeutic diet enriched the platelet membrane with eicosapentaenoic acid ($P = 0.001$), most probably by replacing arachidonic acid that had decreased significantly ($P = 0.001$). Changing the diet from atherogenic to therapeutic, and supplementing the latter with sunflower oil, decreased the platelet membrane content of arachidonic acid ($P = 0.021$), without a simultaneous increase in either eicosapentaenoic acid or linoleic acid. The reference group showed no changes in the plasma cholesterol in low-density lipoproteins and in the platelet membrane contents of arachidonic, eicosapentaenoic or linoleic acid.

The results of the baseline platelet function tests and the mean changes measured at the end of the study are summarised in Table III. The 95% CIs are included as an indication of the magnitude of changes brought about by the dietary treatments and to provide an indication of the precision of the mean change.

TABLE II. THE MEAN $\pm$ 1 SD OF THE TOTAL PLASMA CHOLESTEROL IN LOW-DENSITY LIPOPROTEIN AND THE PLATELET MEMBRANE PHOSPHATIDYLCHOLINE COMPOSITION OF ARACHIDONIC ACID, EICOSAPENTAENOIC ACID AND LINOLEIC ACID

	AD/SO N = 8	AD/FO N = 9	TD/SO N = 10	TD/FO N = 8	Reference N = 10
Cholesterol in low-density lipoproteins (mg/dl)					
Baseline	216 $\pm$ 134	228 $\pm$ 129	220 $\pm$ 129	245 $\pm$ 149	61 $\pm$ 18
Final	227 $\pm$ 103	238 $\pm$ 135	43 $\pm$ 19	46 $\pm$ 20	86 $\pm$ 26
Arachidonic acid (percentage of total fatty acid content)					
Baseline	23 $\pm$ 4	23 $\pm$ 3	22 $\pm$ 4	22 $\pm$ 2	18 $\pm$ 1
Final	22 $\pm$ 1	18 $\pm$ 1	19 $\pm$ 2	14 $\pm$ 1	19 $\pm$ 1
Eicosapentaenoic acid (percentage of total fatty acid content)					
Baseline	0.5 $\pm$ 0.2	0.6 $\pm$ 0.2	0.6 $\pm$ 0.2	0.5 $\pm$ 0.1	0.3 $\pm$ 0.1
Final	0.7 $\pm$ 0.1	4.5 $\pm$ 0.7	0.5 $\pm$ 0.2	4.4 $\pm$ 0.1	0.3 $\pm$ 0.1
Linoleic acid (percentage of total fatty acid content)					
Baseline	1.2 $\pm$ 0.2	1.5 $\pm$ 0.3	1.4 $\pm$ 0.2	1.1 $\pm$ 0.2	1.5 $\pm$ 0.4
Final	1.1 $\pm$ 0.1	0.8 $\pm$ 0.1	1.2 $\pm$ 0.3	0.7 $\pm$ 0.1	1.4 $\pm$ 0.3

AD = atherogenic diet, TD = therapeutic diet, FO = fish oil, SO = sunflower oil

In all the animals the recovery of ^{111}In -labelled platelets at baseline ($68 \pm 13\%$) and at the end of the study ($72 \pm 14\%$) was not different from the $72 \pm 7\%$ measured in the 6 young, normal vervets (< 1 year old) fed a carbohydrate diet low in fat and not subjected to the dietary manipulations. The mean platelet lifespan before the start of dietary treatment (Table III) was markedly shorter than the 136 ± 11 hours determined in the 6 young vervets mentioned above. Fish oil enrichment of both the atherogenic and therapeutic diets did not significantly change the mean platelet lifespan, although the 95% CI indicates erratic responses. Enrichment of both diets with sunflower oil significantly lengthened the mean platelet lifespan, and the 95% CI suggested that more benefit may have accrued from enrichment of the atherogenic diet. The mean platelet lifespan lengthened slightly, but significantly, in the reference group.

Diet or oil enrichment, or both, did not change the BTG:FP4 ratio significantly, nor did it influence the circulating platelet aggregate ratio (Table III). The latter measurement was increased significantly ($P = 0.021$) in the group receiving a therapeutic diet with fish oil enrichment, indicating that there were fewer circulating platelet aggregates.

The production of thromboxane A_2 by platelets, measured as thromboxane B_2 , in response to collagen stimulation is also summarised in Table III. Fish oil enrichment of the atherogenic and therapeutic diets decreased thromboxane A_2 production. Changing of the diet from atherogenic to therapeutic, as well as supplementation of the latter with fish oil, also decreased production.

The *in vitro* aggregation responses of platelets to ADP and collagen are summarised in Table IV. An atherogenic diet enriched with fish oil significantly increased the aggregation response to 15 and 2.5 μmol ADP. Although aggregation with ADP was not significantly changed by the other treatments, the 95% CI strongly suggested an increase in aggregation of platelets in response to ADP, especially with

fish oil. Dietary enrichment with oil had no significant effect on collagen-induced platelet aggregation.

Discussion

At necropsy, varying degrees of aortic, peripheral and coronary atherosclerosis were confirmed.¹ This means that platelet function was measured during progressive atherosclerosis when the atherogenic diet was continued. Changing from the atherogenic to the therapeutic diet regressed components of atherosclerosis such as intimal lipid-laden macrophages and stainable lipid within 20 months. Components of advanced atherosclerosis such as cholesterol crystals, fibrosis and calcification did not regress within the 20 months. Lack of regression of these chronic components is logical, given the long progression of this disease.

The influence of dietary manipulation and enrichment of the diet with fish oil and sunflower oil on atherosclerosis is discussed in detail elsewhere.¹ Briefly, there was no evidence that the addition of fish oil to the atherogenic diet was anti-atherogenic, nor did the fish oil enhance regression when combined with a therapeutic diet. In addition, specialised lipophages (foam cells) derived from monocytes increased in both males and females when the atherogenic diet was enriched with fish oil, but not with sunflower oil. Changing the diet to a therapeutic diet and supplementing it with sunflower oil, but not fish oil, regressed some components of atherosclerosis.¹ Our negative results on atherosclerosis, where 2% of the energy was derived from fish oil, differed from those of a study where African green monkeys were given 22% of calories as menhaden fish oil.¹ However, when menhaden oil supplied 22% of dietary energy the demand for lipid-phase anti-oxidation was unphysiological and induced vitamin E deficiency. Deaths followed and

TABLE III. MEAN PLATELET LIFESPAN, THE BETA-THROMBOGLOBULIN/PLATELET FACTOR 4 RATIO, THE CIRCULATING PLATELET AGGREGATE RATIO AND THROMBOXANE A₂ PRODUCTION BY PLATELETS AT THE START OF THE STUDY, THE INCREASES OR DECREASES MEASURED AT THE END OF THE STUDY, AND THE P-STATISTICS AND THE 95% CIs OF THE DIFFERENCES (MEAN $\pm$ 1 SD)

	No. of monkeys	Baseline value	Mean change	P-value	95% CI
Mean platelet lifespan (hrs)					
Reference	10	59 $\pm$ 18	16 $\pm$ 13	0.030	3 to 29
AD/SO	8	62 $\pm$ 25	33 $\pm$ 23	0.003	18 to 48
AD/FO	9	70 $\pm$ 23	8 $\pm$ 34	0.536	-14 to 30
TD/SO	10	60 $\pm$ 21	28 $\pm$ 22	0.003	15 to 41
TD/FO	8	80 $\pm$ 24	15 $\pm$ 39	0.320	-11 to 41
β-thromboglobulin: platelet factor 4 ratio					
Reference	10	1.9 $\pm$ 0.7	-0.1 $\pm$ 0.7	0.349	-0.6 to 0.4
AD/SO	8	3.0 $\pm$ 2.2	-1.0 $\pm$ 2.8	0.384	-3.0 to 1.0
AD/FO	9	1.5 $\pm$ 0.9	-0.6 $\pm$ 1.3	0.227	-0.2 to 1.4
TD/SO	10	2.1 $\pm$ 1.2	0.0 $\pm$ 0.9	0.944	-0.5 to 0.5
TD/FO	8	2.2 $\pm$ 1.6	-0.3 $\pm$ 2.0	0.694	-1.6 to 1.0
Circulating platelet aggregate ratio					
Reference	10	0.82 $\pm$ 0.07	0.02 $\pm$ 0.12	0.340	-0.05 to 0.09
AD/SO	8	0.83 $\pm$ 0.08	-0.01 $\pm$ 0.06	0.752	-0.05 to 0.03
AD/FO	9	0.87 $\pm$ 0.09	-0.01 $\pm$ 0.18	0.913	-0.12 to 0.10
TD/SO	10	0.83 $\pm$ 0.13	-0.04 $\pm$ 0.12	0.357	-0.11 to 0.03
TD/FO	8	0.84 $\pm$ 0.09	0.09 $\pm$ 0.12	0.012	0.01 to 0.17
Thromboxane A₂ synthesis (pg/9x10⁷ platelets)					
Reference	10	14.8 $\pm$ 4.2	6.0 $\pm$ 8.6	0.029	1.0 to 11.0
AD/SO	8	15.5 $\pm$ 9.4	-2.6 $\pm$ 8.9	0.436	-8.6 to 3.4
AD/FO	9	28.2 $\pm$ 11.9	-13.5 $\pm$ 8.9	0.001	-19.0 to -8.0
TD/SO	10	26.3 $\pm$ 9.3	-11.2 $\pm$ 12.7	0.020	-18.6 to -3.8
TD/FO	8	23.5 $\pm$ 11.5	-12.9 $\pm$ 8.9	0.003	-18.9 to -6.9

AD = atherogenic diet; TD = therapeutic diet; SO = sunflower oil; FO = fish oil.

TABLE IV. THE MEAN *IN VITRO* AGGREGATION RESPONSE $\pm$ 1 SD OF PLATELETS AT THE START OF THE STUDY, THE MEAN CHANGES $\pm$ 1 SD AT THE END OF THE STUDY, AND THE P-STATISTICS AND 95% CIs OF THE DIFFERENCES. IN THE CASE OF ADP-INDUCED AGGREGATION, THE PERCENTAGE CHANGE IN LIGHT TRANSMISSION WAS NORMALISED TO THAT MEASURED WITH 25 μ MOL ADP

Group	No. of monkeys	Baseline value	Mean change	P-value	95% CI
ADP (15 μmol)					
Reference	10	83 $\pm$ 14	13 $\pm$ 22	0.050	0 to 25
AD/SO	8	87 $\pm$ 14	5 $\pm$ 14	0.336	-7 to 17
AD/FO	9	82 $\pm$ 15	19 $\pm$ 22	0.028	2 to 36
TD/SO	10	77 $\pm$ 22	9 $\pm$ 20	0.197	-6 to 24
TD/FO	8	79 $\pm$ 15	10 $\pm$ 19	0.157	-6 to 26
ADP (5 μmol)					
Reference	10	78 $\pm$ 22	0 $\pm$ 12	0.900	-9 to 9
AD/SO	8	80 $\pm$ 19	3 $\pm$ 17	0.549	-12 to 18
AD/FO	9	73 $\pm$ 19	13 $\pm$ 25	0.138	-6 to 32
TD/SO	10	64 $\pm$ 28	10 $\pm$ 23	0.199	-6 to 26
TD/FO	8	67 $\pm$ 22	19 $\pm$ 27	0.086	-5 to 43
ADP (2.5 μmol)					
Reference	10	6 $\pm$ 4	10 $\pm$ 15	0.027	2 to 19
AD/SO	8	20 $\pm$ 17	5 $\pm$ 28	0.603	-19 to 29
AD/FO	9	16 $\pm$ 9	16 $\pm$ 17	0.021	3 to 29
TD/SO	10	22 $\pm$ 13	-7 $\pm$ 17	0.223	-19 to 5
TD/FO	8	15 $\pm$ 10	2 $\pm$ 12	0.712	-8 to 12
Collagen (20 μg/ml)					
Reference	10	60 $\pm$ 16	10 $\pm$ 18	0.056	-11 to 31
AD/SO	8	66 $\pm$ 16	4 $\pm$ 16	0.507	-10 to 18
AD/FO	9	72 $\pm$ 19	2 $\pm$ 17	0.821	-15 to 19
TD/SO	10	63 $\pm$ 20	12 $\pm$ 19	0.128	-2 to 26
TD/FO	8	56 $\pm$ 23	16 $\pm$ 32	0.211	-12 to 44

AD = atherogenic diet; TD = therapeutic diet; SO = sunflower oil; FO = fish oil.

vitamin E supplementation was necessary to reverse the trend.³

The results of several short-term studies of eicosapentaenoic acid enrichment of westernised diets have suggested that the anti-atherogenic effects of eicosapentaenoic acid may be that it inhibits platelet function.^{4,21} Our results of long-term enrichment of a proven atherogenic diet with eicosapentaenoic acid do not support this hypothesis. We found no evidence that changes in the composition of platelet membranes of arachidonic acid and eicosapentaenoic acid had antiplatelet effects and were therefore anti-atherogenic. None of the indices of platelet function that were measured showed that platelet function was inhibited. Although short-term studies have indicated that platelet function can be inhibited by fish oil,^{4,18} we found no such effect after long-term enrichment. It is therefore likely that the observed short-term beneficial changes may have been temporary, and that the platelets adapted to decreased availability of arachidonic acid. Support for the latter hypothesis comes from a study in which platelet adherence to endothelial cells was measured before and during fish oil enrichment of a high-cholesterol diet. Platelet adherence was maximally inhibited 1 week after enrichment started. Inhibition of adherence decreased during the next 4 weeks of enrichment to about half of that measured at 1 week.²¹ In addition, habitual fish consumption (about 33 g/day) for 26 years also had no measurable effect on platelet function when it was compared with that measured in a matched group of people selected from the same population whose diet included about 2 g of fish per day.³¹

Measurement of the mean platelet lifespan is one of the more sensitive indices of *in vivo* platelet function. A shortening indicates random removal of platelets from the circulation as a result of increased utilisation secondary to platelet activation by atherosclerotic plaques or endothelial cell injury.³²⁻³⁴ The mean platelet lifespan was shortened before the diets started (Table III), suggesting the presence of atherosclerosis, which was confirmed at necropsy.³ Fish oil enrichment had no measurable effect on mean platelet lifespan. Although the changes were not significant, lifespan was lengthened by approximately 11% in the vervets that remained on the atherogenic diet and by approximately 19% after the diet was changed to the therapeutic one (Table III).

It is important to note that the mean platelet lifespan in vervets receiving the atherogenic diet lengthened significantly by approximately 53% as a result of the addition of sunflower oil. For vervets receiving the therapeutic diet, the lifespan lengthened significantly by approximately 47% (Table III). These results should be interpreted in relation to the significant lengthening of approximately 27% in the reference group receiving only the therapeutic diet. This supports regression of atherosclerosis with the therapeutic diet, and it appears that supplementation of both diets with sunflower oil resulted in an improved benefit. The vervets receiving sunflower oil did have slightly less atherosclerosis at end of the study compared with those that received fish oil. The increase in foam cells, derived from monocytes, was also less in the atherogenic diet group receiving sunflower oil.³ The relatively small difference in the degree of atherosclerosis between the sunflower oil and fish oil enrichment groups was probably not sufficient to explain

fully the difference in the effect of oil enrichment on mean platelet lifespan. There are some indications that linoleic acid may influence platelet function directly. Firstly, a diet deficient in linoleic acid increases thrombin-induced platelet aggregation.³⁵ Secondly, in response to indwelling catheters in rats, the mean platelet lifespan was shortened by 28% when the diet was enriched with safflower oil, whereas enrichment with milk fat shortened mean platelet lifespan by 41%. The safflower oil contained approximately 70% linoleic acid and milk fat roughly 3%.¹¹

Fish oil added to both the atherogenic and therapeutic diets decreased arachidonic acid and increased eicosapentaenoic acid in platelet membrane phosphatidylcholine. Interestingly, changing from a diet rich in fat (atherogenic) to a diet with a low fat content (therapeutic) and supplementing it with sunflower oil, also decreased platelet membrane arachidonic acid slightly. This decrease was, however, not accompanied by a simultaneous increase in either eicosapentaenoic acid or linoleic acid (Table II). The decrease in membrane arachidonic acid was reflected in the decrease in thromboxane A₂ formation by the platelets (Table III), which confirmed previously published results.^{8,10,17}

In contrast to other studies,^{17,20} we could not show that fish oil enrichment was related to reduced platelet aggregation. Long-term enrichment of the atherogenic diet with fish oil actually tended to increase aggregation, suggesting that the platelets may have become more reactive to stimulation by ADP (Table IV). The increased aggregation response and the finding that mean platelet lifespan was not markedly changed as a result of fish oil supplementation, supports more recent findings that enrichment of atherogenic diets with fish oil is not always advantageous.^{4,21, 24}

We could not associate decreased thromboxane A₂ formation with the reduced platelet aggregation response; this is not consistent with results obtained when diets were supplemented with fish oil in the short term.^{17,19} It is possible that the amount of eicosapentaenoic acid consumed (30 mg/kg body weight/day) was not sufficient to effect changes in the platelet membrane eicosapentaenoic acid/arachidonic acid ratio that will inhibit platelet function.^{8,18} In the other studies the dose of eicosapentaenoic acid was considerably higher.^{8,11,17,20} We do not believe that the changes in platelet membrane eicosapentaenoic acid/arachidonic acid ratio were too small. In this study, the eicosapentaenoic acid/arachidonic acid ratio in the platelet membrane phosphatidylcholine increased to between 0.25 and 0.31 after 20 months of enrichment with fish oil (Table II). In studies reporting inhibition of platelet aggregation, the ratios increased to 0.10¹⁷ and 0.22,²⁰ whereas in studies reporting no inhibition final ratios were 0.76⁸ and 0.09.¹¹ The results are so variable that interpretation is difficult. The platelet membrane may have contained enough arachidonic acid to produce thromboxane A₂ in sufficient amounts to support aggregation induced by ADP and collagen. It is known that the association between inhibition of thromboxane A₂ formation and thromboxane-dependent platelet aggregation is not linear, and that a 10% residual capacity to form thromboxane A₂ is sufficient to sustain platelet aggregation.³⁶

It is reasonable to assume that determination of the mean platelet lifespan was best suited to indicate the *in vivo* platelet activation that resulted from atherosclerosis. The

changes in the mean platelet lifespan measured at the end of the study also reflected the differences seen in atherosclerosis, especially in the indices of less advanced atherosclerosis such as lesion-free areas and the presence of foam cells in aortas.³ Thus, active atherogenesis at the start of the study was reflected in a shortened mean platelet lifespan. The lengthening in mean platelet lifespan after supplementation of the diets with sunflower oil strongly suggests that atherosclerosis regressed. The finding that fish oil supplementation had no effect on the platelet lifespan may indicate that atherogenesis was arrested. The changes in the lifespan are supported by the morphological observations in the same primates.³ The *in vitro* aggregation response of the platelets, the plasma BTG:PF₂ ratio, and the circulating platelet aggregates were apparently less sensitive in this model.

Conclusions

There was no evidence that long-term manipulation of the diet of vervet monkeys with established atherosclerosis with a low dosage of fish oil inhibited platelet function. Diet enrichment with sunflower oil, on the other hand, lengthened the mean platelet lifespan significantly. This may be the result of its beneficial effects on atherosclerosis,³ or its inhibition of the interaction of platelets with the atherosclerotic lesions,^{11,35} or both. In conclusion, no benefit in respect of platelet function was achieved by enriching the diet of vervet monkeys with a low dosage of Atlantic pilchard fish oil for 20 months. Enriching the diets with sunflower oil may have advantages, which should be investigated.

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Thromboembolic potential of synthetic vascular grafts in baboons

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We have compared in baboons the capacity of two types of synthetic vascular grafts to accumulate thrombus, activate circulating platelets, and generate occlusive platelet microemboli. Grafts were incorporated into femoral arterial-arterial shunts placed unilaterally in 10 baboons; the unoperated contralateral limbs served as controls. The accumulation of indium 111 (¹¹¹In)-labeled platelets onto the grafts (expanded polytetrafluoroethylene [ePTFE] or knitted Dacron, 4 mm inner diameter) and the appearance of ¹¹¹In radioactivity in distal microcirculatory beds (calf and foot) were quantified by dynamic scintillation camera imaging. After 1 hour total platelet deposition per graft was higher with Dacron ($49.0 \pm 8.0 \times 10^9$ platelets) than with ePTFE ($3.7 \pm 0.6 \times 10^9$ platelets, $p < 0.01$). Platelet counts decreased and β -thromboglobulin levels increased with Dacron graft placement but were unaffected by ePTFE graft placement ($p < 0.05$ and $p < 0.01$, respectively). Emboli shed from Dacron grafts were detected as multifocal, irregular, and changing deposits in the calves and feet. Indium 111 platelet activity in the feet distal to the Dacron grafts increased $81.1\% \pm 21.4\%$ from baseline values over 1 hour, whereas the activities in the feet distal to the ePTFE grafts were unchanged ($p < 0.05$). The increase in ¹¹¹In-platelet radioactivity above the control limb values (excess radioactivity) was higher for the Dacron graft group than for the ePTFE group in both the feet ($139.6\% \pm 46.9\%$ vs $6.2\% \pm 2.6\%$, $p < 0.05$) and the calves ($86.7\% \pm 21.7\%$ vs $7.3\% \pm 3.6\%$, $p < 0.05$). After graft removal the peripheral ¹¹¹In-platelet activity returned to normal over 24 hours. We conclude that knitted Dacron is more thrombogenic than ePTFE and that its use may be associated with peripheral thromboembolization. The present model may be useful for evaluating graft-related thromboembolic phenomena and the effects of mediating variables. (J VASC SURG 1989;10:75-82.)

Thrombotic and thromboembolic events complicate the use of prosthetic vascular grafts for arterial reconstruction.¹⁻³ The central role of blood platelets in graft-induced thromboembolism has been shown by studies documenting (1) selective platelet consumption and the release of platelet-specific proteins after the placement of synthetic arterial conduits⁴⁻⁶ and (2) reduced thrombus formation and improved

graft patency after inhibition of platelet reactions by antiplatelet agents or by endothelialization of the graft luminal surface.⁶⁻⁹

The eventual disruption of an enlarging platelet mass under high-shear arterial flow may result in the embolization of platelet thrombus to distal circulatory beds.^{1,4} Whereas this potentially serious complication has been observed in association with prosthetic grafts both clinically and experimentally, the determinants of embolic processes remain unclear.^{4,10-13} Unfortunately, no methods have been described that are capable of monitoring real-time embolic events associated with in vivo implants.¹⁴⁻¹⁶ Consequently, previous studies of thromboembolism in this setting have generally been qualitative or anecdotal.^{11,12,17} In the present article we document a quantitative method for evaluating platelet interactions with vascular prostheses, the subsequent embolization of platelet-containing thrombus to distal extremities, and the importance of factors influencing these sequelae of synthetic vascular graft placement.

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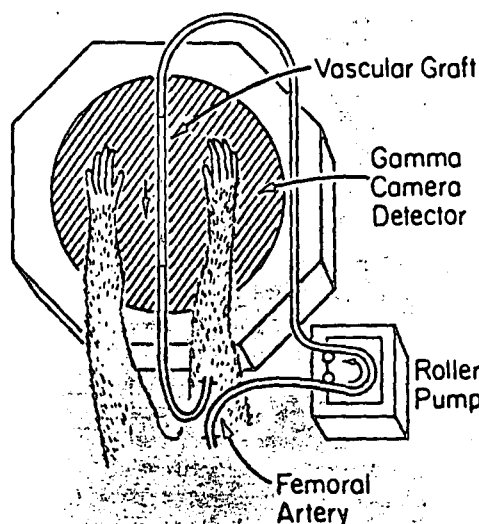


Fig. 1. The superficial femoral artery was isolated and cannulated proximally and distally with a silicone rubber arterial-arterial shunt. The graft preparation was placed in line distal to a roller pump by means of Teflon connectors. Platelet accumulation on the vascular graft and in the lower extremities was quantified by scintillation camera imaging of platelets labeled with ^{111}In -oxine.

MATERIAL AND METHODS

Graft preparation

Reinforced expanded polytetrafluoroethylene (ePTFE) vascular grafts (4 mm inner diameter [I.D.], 30 μm internodal distance) were supplied by W.L. Gore & Associates Inc., Elkton, Md., and knitted Dacron (4 mm I.D., uncrimped, external velour) vascular grafts were supplied by C.R. Bard, Inc., Billerica, Mass. All grafts were cut to a length of 40 cm, were placed inside Teflon tubing to prevent blood leakage, and were connected end to end to 4.0 mm I.D. silicone rubber tubing. As described in detail previously,¹⁸ this method produces a flow channel that is smooth in its transition from the silicone rubber to the graft, without imperfections caused by the coupling procedure.

Surgical procedure

In vivo studies were performed with 10 normal male baboons (*Papio anubis*), weighing 11.6 ± 2.0 kg, that had been observed to be free of disease for at least 6 weeks before use. All experiments were performed in compliance with the "Principles of Laboratory Animal Care" and the "Guide for Care and Use of Laboratory Animals" (NIH Publication No. 80-23, revised 1985). While they were under general anesthesia induced with 1% halothane, each animal

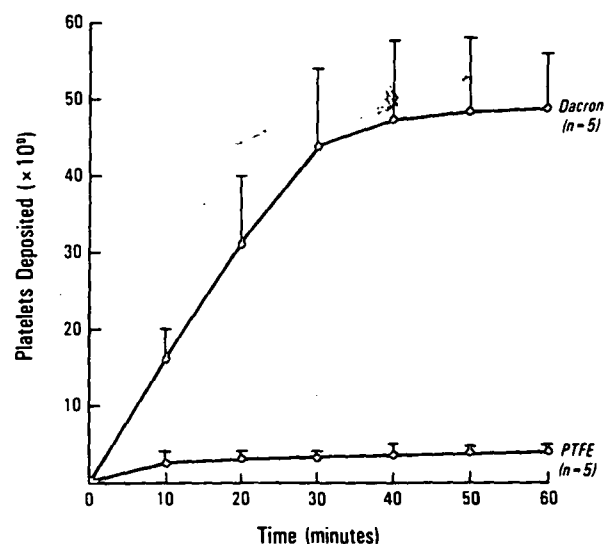


Fig. 2. Platelet deposition was significantly higher on the Dacron grafts than on the ePTFE grafts ($p < 0.01$). The mean platelet counts in the two groups were similar.

underwent the placement of a shunt between proximal and distal segments of the superficial femoral artery. The vessel was isolated after a femoral incision was made, heparin sulfate (100 units/kg) was administered, and two transverse arteriotomies were performed. Each arteriotomy was cannulated by means of a 13- to 15-gauge Teflon vessel tip (Lifemed, Vernitron Corp., Compton, Calif.) connected to silicone rubber tubing (3.0 mm I.D.), thus providing arterial inflow and outflow for the shunt. Each cannula was securely fixed by use of 3-0 silk ties that were placed around the vessel and the cannula. The silicone rubber cannulas were connected and flow was established. The wound was closed with interrupted sutures and the animal was positioned for imaging. This surgical approach was chosen since the vascular anatomy of the lower extremity in the baboon is similar to that in humans.¹⁹

Platelet imaging

Autologous baboon blood platelets were labeled with ^{111}In -oxine according to a previously reported protocol¹⁸ and were injected before each surgical procedure. Images of the grafts, feet, and calves were acquired with a Picker DC 4/11 Dyna scintillation camera (Picker Corp., Northford, Conn.). Data storage and image analysis were performed by means of a Medical Data System A³ computer (Medtron, Ann Arbor, Mich.) interfaced with the camera. A medium-energy collimator was used with good resolution by imaging both the upper and lower ene

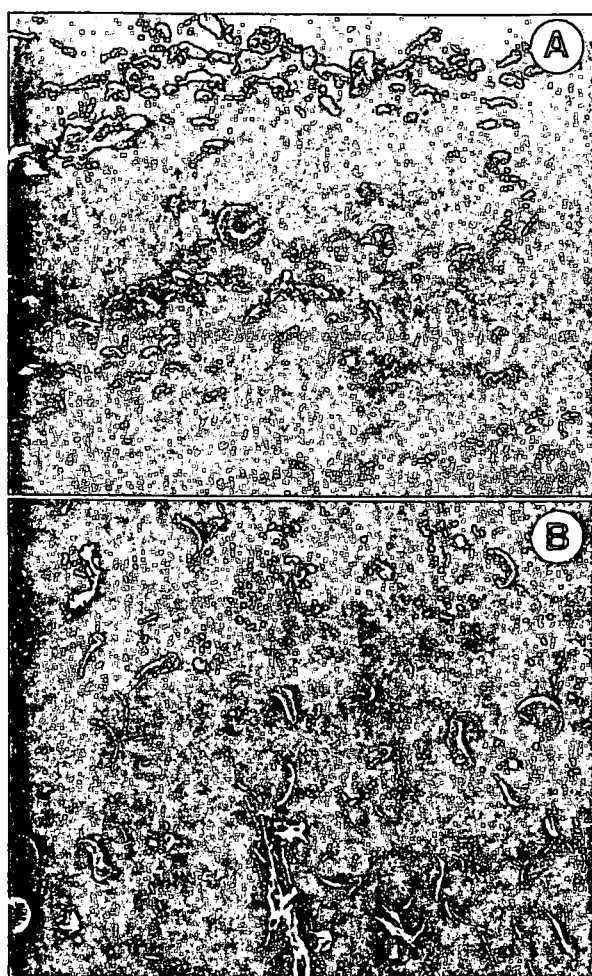


Fig. 3. A, Platelets were adherent to the ridges present on the surface of the ePTFE and underlying graft material was visible. B, Dacron grafts exhibited platelet-rich thrombus with fibrin strand formation that completely obscured the underlying knitted graft material.

peaks of ^{111}In with a 20% energy window. Images were also acquired of a 5 ml sample of whole blood (blood standard). These methods have been described previously.¹⁸

Total graft platelet deposition (labeled plus unlabeled cells) was calculated by dividing the deposited graft radioactivity by the platelet radioactivity contained in 1 ml of whole blood and multiplying by the platelet concentration per milliliter of whole blood.¹⁸ Platelet counts were performed on whole blood by means of an electronic platelet counter (J.T. Baker Model 810, Allentown, Pa.).

Indium 111-labeled platelet activity in each limb was determined by measuring the counts per minute in standardized regions of interest (ROI) for each foot and calf. The percent excess ^{111}In -platelet de-

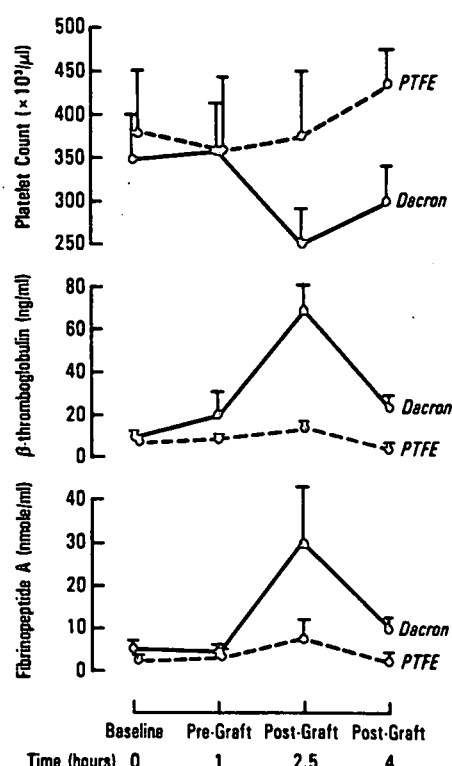


Fig. 4. The placement of Dacron grafts was associated with decreased platelet counts and increased levels of BTG and FPA as compared to ePTFE.

position in the experimental extremity (ipsilateral to the graft) as compared to the control extremity (contralateral to graft) was then calculated at each imaging time point by means of the following equation:

$$\frac{\text{cpm in ROI of experimental limb} - \text{cpm in ROI of control limb}}{\text{cpm in ROI of control limb}} \times 100$$

The excess (noncirculating) radioactivity in the experimental limb was thus expressed as a percentage of the control limb circulating blood activity.

Experimental protocol

Each animal underwent surgery in one limb; the contralateral limb served as a control. After the shunt was in place, scintillation camera imaging of baseline (time 0) labeled platelet activity in the calves and feet was performed. Blood was drawn from the shunt for measurements of platelet count, β -thromboglobulin (BTG), fibrinopeptide A (FPA), fibrinogen, hematocrit, and white blood cell count. The arterial-arterial shunt was then connected to a roller pump (Masterflex, Cole Parmer, Chicago, Ill.) and operated at a flow rate of 80 ml/min for



Fig. 5. After graft placement, platelet thrombus embolized from the Dacron grafts to the calves and feet. The contralateral control limbs were free of embolic material. This anterior image of the feet was taken after placement of a Dacron graft in the right femoral artery shunt for 1 hour. Emboli appeared as multifocal, irregular, and changing deposits in the right foot.

1 hour. Pregraft (time 1 hour) images were obtained of the calves and feet, and blood samples were drawn. Heparin sulfate (50 units/kg) was administered, and the graft preparation was interconnected between segments of the shunt tubing distal to the pump (Fig. 1). Vascular grafts that were placed unilaterally in 10 animals included five knitted Dacron vascular grafts and five ePTFE grafts. After placement, the graft and the feet were imaged continuously for 1 hour. The graft and the arterial-arterial shunt were then removed (referred to herein as *postgraft* period), the superficial femoral artery was ligated, and postgraft images of the calves and feet were obtained. Additional blood samples for biochemical assays were also taken at this time. Subsequent imaging of the calves and feet was performed at 30-minute intervals for an additional 1½ hours (2½ to 4 hours from time 0). Blood samples were taken at 2½ and 4 hours. Final images of the calves and feet were taken 24 hours after the procedure. None of the animals experienced clinical evidence of limb ischemia, an observation

consistent with previous studies that involved ligation of the baboon superficial femoral artery.^{7,10,18}

Morphologic characteristics

Immediately after they were removed, graft preparations were perfused with lactated Ringer's solution (Travenol Laboratories, Deerfield, Ill.) at 37° for 5 minutes. Perfusion was continued with 2.5% glutaraldehyde in lactated Ringer's solution at 37° for an additional 5 minutes. Samples were then stored at 4° C until they were processed for scanning electron microscopy, which was performed as described previously.¹⁸

Laboratory assays

Serial measurements of platelet count, BTPA, fibrinogen, hematocrit, and white blood cell count were performed. Plasma levels of BTG were determined by means of a commercially available radioimmunoassay (Amersham, Arlington Heights, Ill.).^{18,20} Blood for FPA was drawn

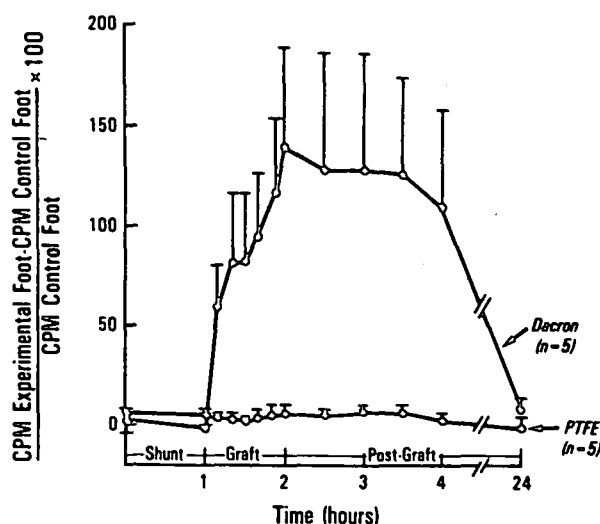


Fig. 6. Excess ^{111}In -platelet deposition in the experimental feet as compared to the contralateral control feet. There was a significant increase in percent excess of ^{111}In -platelet activity in the experimental limbs in which Dacron grafts were placed but no change with ePTFE placement. Indium 111 platelet radioactivities in the feet decreased after the graft was removed and returned to baseline within 24 hours.

2 ml samples into precooled syringes containing 20 ng PPACK (D-phenylalanyl-L-prolyl-L-arginyl chloromethylketone, a thrombin inhibitor), 20 units heparin sulfate, and a final concentration of 10 mmol/L ethylenediamine tetraacetic acid. Samples were centrifuged at 45,000 g for 20 minutes at 4°C. The measurement of FPA levels was performed by means of a radioimmunoassay (IMCO Corporation, Stockholm, Sweden).²⁰ Fibrinogen was measured as total thrombin-clottable protein.^{10,21} The mean fibrinogen level was 316 ± 43 mg/dl and remained stable in all animals during the study. White blood cell counts and hematocrits were performed by use of an electronic cell counter (J.T. Baker Model 150, Allentown, Pa.). The mean white blood cell count was $9.1 \pm 1.5 \times 10^3/\mu\text{l}$ and the mean hematocrit was $33.2\% \pm 0.5\%$. These remained unchanged over the course of the experiment.

Data analysis

Data were analyzed by means of CLINFO computer programs provided through the Division of Research Resources, National Institutes of Health. Comparisons between two groups were performed by use of the two-tailed Student t test.²² Unless otherwise stated, values are given as the mean $\pm$ 1 SEM.

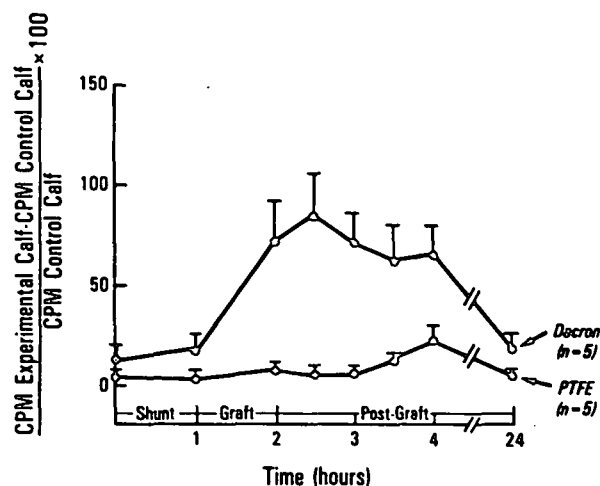


Fig. 7. Embolization to the calves also occurred in association with Dacron graft placement, and its course was similar to that seen in the feet.

RESULTS

Indium 111-platelet deposition was significantly higher on the Dacron grafts ($49.0 \pm 8.0 \times 10^9$ platelets per graft) than on the ePTFE grafts ($3.7 \pm 0.6 \times 10^9$ platelets per graft, $p < 0.01$) after 60 minutes of exposure to flowing blood (Fig. 2). Platelet deposition curves plateaued within 40 minutes of graft placement. The mean platelet count was $347 \pm 49 \times 10^3/\mu\text{l}$ in the Dacron graft group and $379 \pm 81 \times 10^3/\mu\text{l}$ in the ePTFE graft group. Gross evaluation of the graft specimens showed that the ePTFE surface had accumulated minimal thrombus, whereas the Dacron was covered with an evenly distributed layer of thrombus. Scanning electron microscopy of the ePTFE luminal surface showed platelets adherent to the graft mesh (Fig. 3, A). A marked accumulation of platelet-rich thrombus was present on the Dacron surface, which totally obscured the underlying graft substrate (Fig. 3, B).

Systemic blood alterations as determined from the in vitro biochemical assays are summarized in Fig. 4. Platelet counts decreased $27.7\% \pm 5.1\%$ with the placement of Dacron grafts and $1.3\% \pm 2.0\%$ with ePTFE graft placement ($p < 0.05$). Beta thromboglobulin increased after the placement of Dacron grafts (68.8 ± 11.9 ng/ml) and was higher than that observed with ePTFE grafts (12.8 ± 3.0 ng/ml, $p < 0.01$). Fibrinopeptide A levels were 29.9 ± 14.8 nmol/ml after the exposure of Dacron to flowing blood and 7.5 ± 4.2 nmol/ml with ePTFE ($p = 0.154$). By 4 hours the BTG and FPA

levels had returned to near normal values (Fig. 4). There were no significant changes in fibrinogen levels, white blood cell counts, or hematocrits during these studies in either the Dacron or the ePTFE groups.

When ^{111}In -platelet activities in the feet were expressed as a percentage of baseline (time 0) values, pregraft (time 1 hour) values for the 10 unoperated control feet ($95.6\% \pm 2.6\%$ of baseline) were similar to those in the operated experimental limbs ($98.0\% \pm 6.0\%$ for the Dacron graft group and $98.9\% \pm 2.0\%$ for the ePTFE graft group), indicating that there was no significant increase in peripheral ^{111}In -platelet activity as a result of the surgical procedure and placement of the extracorporeal circuit. Embolization of platelet thrombus to the feet began immediately after the exposure of Dacron grafts, but was not observed with ePTFE grafts or in the control feet (Fig. 5). Thus after 60 minutes of exposure to flowing blood, ^{111}In -platelet activity in the feet distal to the Dacron grafts increased $81.1\% \pm 21.4\%$ above baseline values but was unchanged in the ePTFE graft group ($p < 0.05$).

The measurements of excess ^{111}In -platelet activity in the feet of the experimental extremities as compared to the control limbs are given in Fig. 6. Subtracting the relative activity of the control limbs showed that the Dacron and ePTFE graft groups were similar before graft placement; excess ^{111}In -platelet activity in the experimental feet was $3.3\% \pm 3.0\%$ for the Dacron graft group and $4.9\% \pm 2.2\%$ in the ePTFE graft group at baseline (time 0). One hour after the graft was placed, the feet distal to the Dacron grafts showed a $139.6\% \pm 46.9\%$ excess of ^{111}In -activity as compared to a $6.2\% \pm 2.6\%$ increase for the ePTFE graft group ($p < 0.05$). Activity decreased in the postgraft period, and after 24 hours it was similar in the control and experimental limbs (Fig. 6), which indicated that the embolized material had been nearly eliminated.

Embolization of platelet thrombus to the calves also occurred after the placement of Dacron grafts (Fig. 7). Excess ^{111}In -platelet activity in the calves distal to the Dacron grafts was $86.7\% \pm 21.7\%$ as compared with a $7.3\% \pm 3.6\%$ increase in the ePTFE group ($p < 0.05$). Indium 111-platelet activity continued to increase for a short time after the Dacron graft was removed (2 to 2½ hours), decreased steadily during the remainder of the postgraft period, and by 24 hours was similar to that observed in the ePTFE graft group ($19.6\% \pm 9.6\%$ vs $5.4\% \pm 3.2\%$, $p = 0.219$). There was no significant

accumulation of ^{111}In -platelet activity in the control or ePTFE calves during the study.

The distribution of platelet emboli in the limbs distal to the Dacron grafts was variable. The feet were the most common targets for embolization; all five feet in the group with Dacron grafts sustained increases in ^{111}In -platelet deposition. Levels in two feet increased to over twice the baseline values, whereas the other feet sustained moderate increases. When the location of platelet thrombus deposited in the feet was evaluated by subdividing each foot in the Dacron graft group into three equal regions of interest, no significant differences in platelet accumulation were found in the forefoot, midfoot, and heel. Two calves showed no appreciable increase in platelet deposition, whereas the others sustained appreciable increases from baseline levels. The limb with the largest increase in platelet deposition in the foot also showed almost no change in the calf. Thus whereas platelet microembolization was a consistent finding in all cases, the absolute numbers of platelets retained in the calf or foot exhibited considerable variability among animals.

Finally, the total number of platelets (labeled plus unlabeled cells) deposited in the limbs after placement of the Dacron graft was evaluated by subtracting the number of platelets present at baseline (time 0) from the number present at graft removal (time 2 hours). There were $5.5 \pm 2.5 \times 10^9$ platelets deposited per limb (calf plus foot), a value equal to $11.8\% \pm 4.5\%$ of the total number of platelets deposited on the Dacron grafts.

DISCUSSION

The present studies document in a nonhuman primate model the effects of two different vascular graft materials on platelet destruction, systemic blood alterations consistent with platelet thrombus formation, and the generation of peripheral arterial thromboemboli. No significant changes in these measurements were induced by the surgical procedure or the experimental design, which included the placement of an extracorporeal circuit and roller pump. After grafts were placed, platelet deposition was significantly higher on Dacron grafts than on ePTFE grafts, as previously observed.^{23,24} The Dacron grafts also caused a significant decrease in circulating platelet counts, with corresponding increases in plasma levels of BTG and FPA. In contrast, no significant changes in these measurements were associated with exposure to the ePTFE graft. The placement of Dacron grafts, but not ePTFE grafts, resulted in the

embolization of platelet thrombus to distal micro-circulatory beds, which caused a significant increase in peripheral ^{111}In -platelet activity, as compared to both baseline images in the same limb and concurrent images obtained in the contralateral control limb. Approximately 10% of the platelets accumulated by the Dacron grafts embolized distally, and the foot was the most common and consistent site of embolization. After the Dacron graft was removed after 1 hour of blood exposure, there was a steady decrease in ^{111}In -platelet activity in the distal extremities, with most of the embolized platelet thrombus undergoing lysis and removal within 24 hours.

This model was designed to directly evaluate the thromboembolic consequences of graft placement and the role of graft material properties. Grafts were incorporated into a hemodynamically smooth, extracorporeal shunt system, thus avoiding the effects of vessel injury and possible differences in surgical technique. An occlusive roller pump was used to maintain constant blood flow during the procedure, thereby eliminating possible effects of hemodynamic variables. Under these conditions platelet deposition onto the Dacron grafts was comparable to values obtained in a previous study performed without mechanical blood pumping.¹⁸ In addition, blood pumping alone was not associated with direct activation of platelets (Fig. 4) or measurable damage to other formed elements. Heparin was given in standard anticoagulating amounts during surgery and again with graft placement, to prevent clot formation caused by stasis in the shunt system. Unoperated, contralateral limbs served as controls for comparison to the limbs in which the grafts were placed. Dacron and ePTFE grafts were evaluated because they are widely used for clinical arterial reconstruction.

Graft material is a major factor influencing platelet deposition on vascular prostheses.^{2-4,23,24} Dacron has generally been considered to be more thrombogenic than ePTFE, and this observation was confirmed in the present study. Increased rates of platelet destruction have also been reported to vary markedly depending on material type.^{4,6} The significant decrease in platelet count and increase in plasma levels of BTG that occurred in the hour after the placement of Dacron grafts further documents the important role of platelets in this process. Although levels of FPA were modestly increased, fibrinogen levels did not change appreciably as a result of graft placement, which suggests that thrombus formation in this arterial flow model is regulated primarily by platelets rather than by coagulation-dependent mechanisms.

Whereas thromboembolic complications have been associated with the use of cardiovascular devices, peripheral arterial thromboembolization has been difficult to evaluate quantitatively.^{4,11,12,25,26} Previous reports have documented qualitatively the presence of platelet microemboli in the renal parenchyma after placement of proximal tubular prostheses,^{4,25,26} and this process appeared to be a function of the physicochemical properties of the prosthetic materials evaluated. In the present study embolic material was detected within minutes of Dacron graft placement and before maximum saturation of the graft surface with platelets occurred. However, factors other than graft type may also play a role, since the amount of embolic material did not correlate closely with the total number of platelets collected by a given graft, the platelet count, fibrinogen level, or hematocrit. The embolic material was also rich in blood platelets as documented by scintillation camera ^{111}In -platelet imaging (Fig. 5), evaluation of graft surfaces by scanning electron microscopy (Fig. 3), and morphologic evaluations of renal microemboli shed from prosthetic surfaces in an earlier study in baboons.^{4,10} In healthy baboons without peripheral arterial disease, these thromboemboli were almost completely removed within 24 hours by endogenous fibrinolytic mechanisms. However, in patients with severe atherosclerotic peripheral arterial disease, and perhaps a diminished capacity for thrombolysis, these embolic processes may contribute to irreversible arterial occlusion, which is clinically significant.^{27,28}

Previous reports have indicated that embolization in the early period after graft placement may be primarily atheroembolic in nature. Whereas our observations suggest that platelet microembolism may also occur in humans after synthetic graft placement, the contribution of this process to the occasional but serious clinical syndromes of peripheral limb ischemia remains to be defined. Although late morbidity caused by the embolization of mural thrombus accumulated by synthetic grafts has been documented qualitatively by the retrieval of surgical specimens, a more complete understanding of the mechanisms responsible for this process has been hampered by the inability to monitor real-time embolic events *in vivo*. Therefore the present model involving ^{111}In -platelet imaging of microcirculatory beds distal to prosthetic grafts may prove useful for evaluating quantitatively the extent of occlusive microembolization *in vivo* with respect to graft-related variables and therapeutic interventions.

In summary, graft-induced platelet destruction

with subsequent embolization of platelet thrombus was observed in a baboon model after placement for 1 hour of Dacron grafts, but not after placement of ePTFE grafts. This mechanism may play a role in arterial occlusive disease or ischemic syndromes occasionally associated with the use of prosthetic vascular grafts.

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Platelet Interactions with Dacron Vascular Grafts

A Model of Acute Thrombosis in Baboons

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We have developed a model of acute Dacron graft thrombosis in baboons in order to assess platelet alterations secondary to arterial thrombus formation. In this model, thrombus formation was initiated by Dacron vascular grafts inserted as extension segments into chronic arteriovenous Silastic shunts. Following platelet labeling with ^{111}In -oxine, platelet deposition was measured for 1 hour following blood contact under arterial flow conditions using a scintillation camera. Graft platelet activity rapidly increased 40- to 50-fold, plateauing by 1 hour. All grafts produced equivalent reductions in circulating platelet count and blood ^{111}In -platelet radioactivity, demonstrating that the labeled cells were functionally equivalent to the total platelet population. After graft placement, the remaining platelets survived normally. Acute platelet deposition was equivalent on grafts placed 1, 24, 48, and 72 hours following injection of the labeled cells, indicating that a variable delay between platelet labeling and graft imaging was without detectable consequence. Platelet destruction by the graft produced a tenfold increase in plasma levels of platelet factor 4 (PF4) and β -thromboglobulin (BTG) but did not modify either the α -granule (PF4, BTG) or dense granule (ADP, ATP) contents of circulating platelets.

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Animal models of acute arterial thrombosis have had limited usefulness because the assessment of vascular occlusion has usually involved the use of experimental animals of questionable relevance to humans with observations at a single point in time after an initiating vascular insult.¹⁻³ Some of the uncontrolled variables that compromise the quantitative aspects of such approaches include: the type and extent of the initial vascular injury, loss of thrombus by embolization, loss of thrombus by thrombolysis, hemodynamic characteristics of the model, and the timing and type of observations at sacrifice.

To quantify continuously the overall rate of acute arterial thrombus formation in vivo in a manner that is free of uncontrolled variables, we used a primate model of vascular graft thrombosis. This model was developed specifically to produce an acute, significant, and reproducible thrombotic challenge such that the relative importance of in vivo variables and therapies could be determined. In these studies, we first wished to develop an efficient method for the labeling of baboon blood platelets with ^{111}In -oxine without altering platelet survival or function. Subsequently, we determined the effects of the repeated placement of Dacron vascular grafts on the following variables: graft platelet deposition, platelet survival, platelet count, platelet α -granule contents, and platelet adenine nucleotides (ADP, ATP).

The choice of the baboon for these studies was based on the baboon's hemostatic similarity to humans, together with its size, logistical ease of frequent blood sampling, and its long-term acceptance of chronically patent A-V cannulas.^{4,5} Although it is difficult to establish equivalent thrombogenicity among humans and other species, available evidence suggests that the baboon is a good animal for simulating arterial thrombotic processes in humans.

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We have studied the hemostatic mechanism in the baboon and found it to resemble closely that of humans in a number of respects, including concentrations of coagulation factors and plasminogen, bleeding time, platelet count, platelet volume, platelet morphology, platelet aggregation and release in vitro, and plasma levels of platelet factor 4, beta thromboglobulin, and fibrinopeptide A using human radioimmune assays^{6,7} (unpublished observations).

Methods

Animals

We studied 14 normal male baboons (*Papio anubis*) weighing 8 to 14 kg who had been dewormed and observed to be disease-free for at least 6 weeks prior to use. Circulating platelet concentrations averaged $380,000 \pm 91,000$ platelets/ μl (± 1 sd), and white cell counts averaged $8300/\mu\text{l} \pm 4100$. All animals had a chronic arteriovenous shunt surgically implanted between the femoral artery and vein.⁷ Ketamine hydrochloride (10 mg/kg intramuscularly) was given as a preanesthetic agent, and the operation was performed under general 1% halothane anesthesia. All procedures were in accordance with institutional guidelines. The permanent shunt system consisted of two 25-cm lengths of Silastic tubing, 3.0 mm i.d., (Dow Corning Corporation, Midland, Michigan) connected to 13- to 15-gauge Teflon vessel tips (Lifemed, Vernitron Corporation, Compton, California). In addition, the two Silastic lengths were fixed with Dacron sewing cuffs (Dupont, E.I. de Nemours and Company, Wilmington, Delaware) at skin exit sites. The cannulas were sterilized by autoclaving before surgical placement. Blood flow was established by connecting the two Silastic shunt segments with a 1-cm length of blunt-edge Teflon tubing (2.8 mm i.d.). The permanent Teflon-Silastic shunt did not detectably shorten platelet survival or produce measurable platelet activation (see Results). In all experiments, Dacron vascular grafts were subsequently interposed between the segments of the permanent Silastic A-V shunt.

Vascular Grafts

Uncrimped knitted Dacron grafts (Sauvage external velour, mean porosity 2000 to 2200 ml/H₂O/min at 120 mm Hg) were a gift from Roger Snyder, U.S. Catheter, Incorporated, Billerica, Massachusetts. All grafts were 10 cm in length, 4.0 mm i.d. Prior to evaluation in the arteriovenous shunt system, the grafts were rendered impervious to blood leakage in the following manner. First, a 4.0-mm Teflon rod (that had been thoroughly cleaned using a mild soap solution, then ethanol, and finally by rinsing with sterilized distilled water) was inserted through the graft. The graft was then externally wrapped with a 5 × 10 cm sheet of Parafilm (American Can Company, New York, New York) and placed inside a 10-cm length of 6.3-mm i.d.

"heat shrink" Teflon tubing (Small Parts Incorporated, Miami, Florida). The Teflon tubing containing the graft segment was gently heated over a low Bunsen flame until shrinkage to about 5.3 mm had occurred, resulting in a compression of the Parafilm onto the external fabric interstices without modification of the luminal graft surface as documented by scanning electron microscopy (data not shown). Silicone rubber tubing, 10 cm × 4.0 mm i.d., was transferred onto the Teflon rod and connected to both ends of the graft segment with Silastic Medical Adhesive (Type A, Dow Corning Incorporated). When the polymer had cured for 24 hours, the Teflon rod was carefully pulled from the tubing lumen (Figure 1). This procedure produced impervious grafts rigidly constrained to a linear geometry, and having an inner diameter of precise dimensions (4.0 mm). The resulting flow channel was smooth in its transition from the Silastic to the graft without imperfections due to the coupling procedure.

The 4.0-mm i.d. Silastic tubing segments proximal and distal to the Dacron graft were connected to the 3.0-mm i.d. Silastic tubing comprising the chronic arteriovenous shunt with 2-cm long tapered Teflon connectors (Small Parts, Incorporated).

Mean blood flow rates through vascular graft segments incorporated into the A-V shunt system were measured continuously using a Doppler ultrasonic flowmeter (L and M Electronics, Daly City, California) and "C" shell cuff type transducer probe which fitted snugly around the Silastic tubing comprising the chronic shunt. The flowmeter and probe were calibrated in vitro using whole baboon blood. In all studies initial blood flow rates ranged from 170 to 220 ml/min.

Following 60 minutes exposure to flowing blood, vascular graft segments were removed and perfused with 2.5% glutaraldehyde in 0.1 M phosphate buffered saline, pH 7.4. All grafts were perfused at a rate of 100 ml/min for 5 minutes. Graft segments were subsequently dehydrated in increasing concentrations of ethanol, critical point dried, and sputter-coated with approximately 35 nm of gold-palladium for scanning electron microscopy.

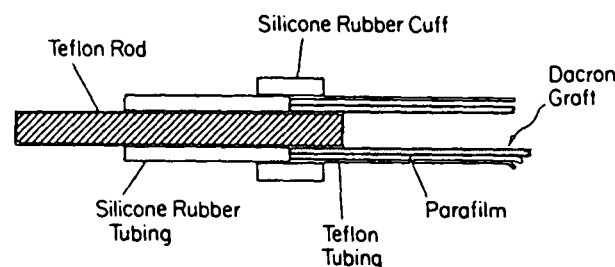


Figure 1. Dacron vascular graft connected to silicone rubber tubing. After removal of the Teflon rod, a smooth flow channel is achieved.

Laboratory Procedures

Autologous baboon blood platelets were labeled with ^{111}In -oxine according to the following protocol. Whole blood (100 ml) was collected directly into plastic bags (TA-3, Fenwal Labs, Deerfield, Illinois) containing 20 ml acid-citrate-dextrose anticoagulant (NIH formula A). The blood was centrifuged in the bag at 300 g for 10 minutes. The supernatant platelet-rich plasma (PRP) was transferred to a second bag and pH adjusted to 6.5 by the addition of 0.15 M citric acid (0.1 ml/10 ml PRP). The red blood cell fraction was returned to the donor animal. The platelets were formed into a pellet by centrifugation of the PRP at 1300 g for 15 minutes. The supernatant platelet-poor plasma (PPP) was completely decanted and discarded. To remove residual plasma proteins, the bag containing the platelet pellet was carefully washed once by overlaying with 30 ml of Ringer's citrate dextrose (RCD; pH 6.5) which was then decanted and discarded. The pellet was then gently resuspended in 5.0 ml RCD, and incubated for 30 minutes with 500 to 700 μCi ^{111}In -oxine (Amersham Corporation, Arlington Heights, Illinois). Contaminating red cells were removed by a final slow centrifugation at 200 g for 5 minutes. Labeling efficiency was determined by diluting 200 μl of the labeled-platelet concentrate with 5.0 ml RCD, and comparing the activity in 0.5 ml of the diluted platelet suspension with the activity in 0.5 ml of cell-free supernatant following centrifugation at 3000 g for 30 minutes. In the 14 studies involving platelet labeling and subsequent graft imaging, labeling efficiency was $87\% \pm 6\%$. A measured volume of labeled platelet suspension containing approximately 13% non-platelet-bound isotope was then injected directly into the recipient animals following the preparation of a 100 μl standard. Additional washing procedures to remove nonplatelet-bound isotope were deemed undesirable since they may produce *in vitro* cell damage. Moreover, in all studies with the scintillation camera, blood pool activities from all sources (platelet and nonplatelet) were a small fraction of total deposited platelet activity and were subtracted using appropriate computer-assisted image analysis routines as described below.

To determine whether the centrifugation procedures resulted in the isolation and labeling of platelets which were representative of the parent platelet population, density separation was performed on platelets harvested from citrated whole blood (10 ml), diluted with a half volume of Ca^{2+} -free modified Tyrodes-ACD buffer, pH 6.5 containing EDTA (0.5 mM). Following centrifugation at 200 g for 10 minutes, the upper supernatant layer of PRP was removed without disturbing the interface, and the remaining cells diluted to the original volume with Tyrodes-ACD buffer, remixed and centrifuged as before. The second supernatant fraction was harvested and pooled with the initial harvest. This procedure was performed a total of four times; over 94% of the

total platelet population was recovered with minimal leukocyte contamination ($<0.01\%$).

Percoll (Pharmacia, Uppsala, Sweden) was made iso-osmotic with physiological Tyrodes-ACD solution by adding nine parts (vol/vol) of Percoll to one part of 10x concentrated Tyrodes-ACD buffer, pH 6.5. The osmolality of the resulting stock solution was adjusted to 295 mOsm/kg H_2O with deionized water. Uniform mixtures of the platelet preparation (4.5 ml) and stock isoosmotic Percoll (5.5 ml) were poured into polycarbonate centrifuge tubes and centrifugation carried out in a 30° angle-head rotor at 10,000 g for 20 minutes at 22°C . During centrifugation, platelets moved to a position corresponding to the density of the surrounding medium, i.e., platelets were separated on the gradient formed *in situ*. Identical tubes containing density marker beads (Pharmacia) were used to calibrate the gradients. Following centrifugation, gradients were fractionated by removing fractions (0.45 ml) from the top of the gradient with an automatic pipette, and the platelet concentration and radioactivity were determined for each fraction. At 1 hour after injection of the labeled platelet preparations, both the labeled and total platelet populations had equivalent density distributions, as illustrated in Figure 2. Thus, the density distribution of labeled platelet activity was representative of the parent platelet population.

Circulating platelet ^{111}In -activity was determined from 4 ml blood samples drawn before and after graft placement, and collected in 2 mg/ml (ethylenedinitrilo)-tetraacetic acid (EDTA). From each sample, 1.0 ml was used for platelet counting and hematocrit determinations, and 1.0 ml was counted for whole blood ^{111}In activity. The remaining 2 ml were centrifuged at 3000 g for 30 minutes and 1.0 ml of the supernatant (PPP) was counted for plasma ^{111}In -activity. All blood and plasma samples were counted using a gamma spectrometer (Nuclear Chicago, Chi-

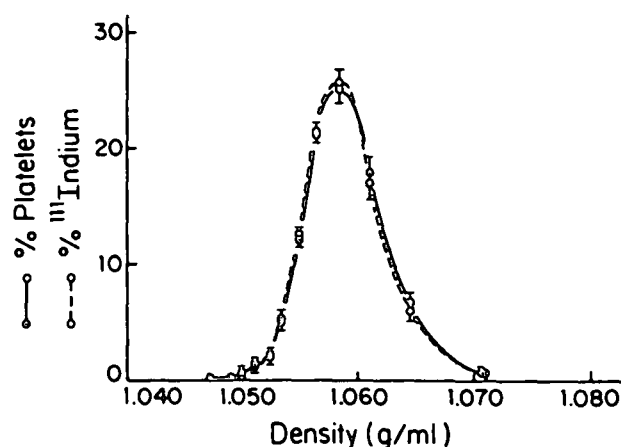


Figure 2. Separation of platelets on Percoll density gradients. One hour after the injection of ^{111}In -labeled platelets into eight baboons, the density distribution of labeled cells ($\circ$) was equivalent to that of the unlabeled parent platelet population ($\square$). Values are means ± 1 SE.

cago, Illinois). Plasma activity per milliliter of whole blood was calculated by multiplying the activity per milliliter of plasma by the factor: (1-hematocrit). Plasma activity averaged $13\% \pm 5\%$ ($n = 14$) of whole blood activity in samples taken 1 hour following injection of the labeled platelet concentrate. Platelet counts were performed on whole blood by using an electronic platelet counter (Clay Adams UF-100, Parsippany, New Jersey).

Mean platelet survival times were calculated by fitting the disappearance curve of platelet radioactivity to gamma functions as described by Murphy et al.^{8,9} The proportion of labeled platelets remaining within the systemic circulation (i.e., recovery) was calculated from the initial platelet activity per milliliter of whole blood, multiplied by the estimated blood volume (65 ml/kg), and divided by the platelet ^{111}In -activity injected.

Platelet survival curves, assessed following ^{111}In -platelet labeling in eight normal animals, displayed a predominantly linear disappearance pattern (Figure 3). Platelet survival times as determined from the computer-fitting procedure averaged 5.59 ± 0.14 days (± 1 SE). Platelet recoveries, determined from samples taken at 1 hour were $79.0\% \pm 2.8\%$. Recoveries determined by extrapolating the best fit survival curves to time zero averaged $80.5\% \pm 2.9\%$. When the recovery of ^{111}In -labeled platelets was calculated from circulating levels of platelet bound isotope measured at 10 minutes, 20 minutes, and 1 hour following injection of the labeled platelet concentrate in seven additional baboons, values were $81.2\% \pm 3.3\%$, $79.3\% \pm 3.4\%$, and $78.9\% \pm 4.2\%$, respectively (Figure 3). Thus, the recovery of the labeled platelets was immediate and equivalent at all early time points, and demonstrated rapid equilibration without transient platelet sequestration. These values for platelet survival and recovery were in

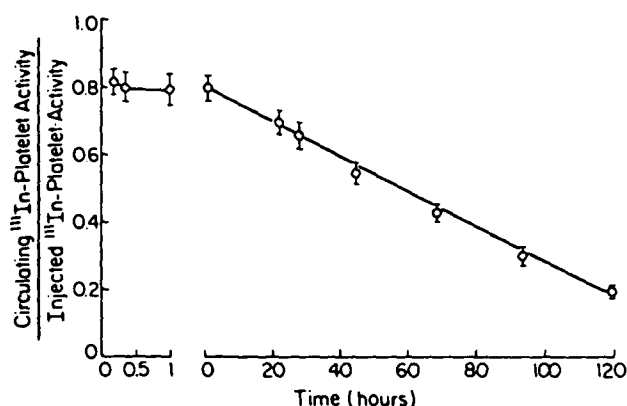


Figure 3. ^{111}In -platelet survival and recovery. Platelet recovery was normal in seven animals at 10 minutes, 20 minutes, and 1 hour after injection of the labeled cells without evidence of transient sequestration ($\circ$). In eight additional animals, recoveries were normal and the platelets exhibited a linear disappearance pattern ($\circ$). Values are mean ± 1 SE.

agreement with those previously obtained in baboons using a ^{51}Cr platelet label.^{7,10}

Platelet suspensions were analyzed for ATP and ADP using neutralized perchloric acid extracts and high performance liquid chromatography (HPLC). Platelet-rich plasma (PRP) was prepared from citrated whole blood (10 ml) by three differential centrifugation steps at 200 g for 10 minutes at 22°C . These platelet preparations have been shown to be essentially free from contaminating leukocytes and representative of the total platelet population.¹¹ Total platelet adenine nucleotides were extracted by the addition of 11.5 M HClO_4 (100 μl) to the platelet suspension (1.0 ml). Cell debris and denatured protein were removed by centrifugation at 2000 g for 15 minutes at 4°C and the supernatant was neutralized by titration with 10.0 M KOH . All neutralized extracts were stored at -80°C prior to analysis by HPLC.

Quantitative nucleotide analysis was carried out using a Spectra-Physics SP8000 liquid chromatograph equipped with a Data System (Spectra-Physics, Santa Clara, California) and coupled to a Gilson variable wavelength UV detector operating at a wavelength of 254 nm. A Partisil 10SAX microparticle anion-exchange column was used (Whatman, Incorporated, Clifton, New Jersey) and optimal resolution of adenine nucleotides was achieved using the operating conditions previously described.¹²

Blood for platelet factor 4 (PF4) and β -thromboglobulin (βTG) was drawn as 5 ml samples from the arteriovenous shunt into precooled syringes containing 1 ml of ACD, acetylsalicylic acid and prostaglandin E_1 , 30 mM and 1 μM final concentrations, respectively.¹³ Blood was collected before and after placement of each graft. Samples were centrifuged at 45,000 g for 20 min at 4°C within 30 minutes of collection. Then 1.0 ml of the platelet-free plasma was removed and stored at -20°C . Assays for PF4 and βTG were performed by competitive radioimmunoassay. βTG determinations were carried out using a commercially available radioimmunoassay (Amersham, Arlington Heights, Illinois). PF4 was measured by the procedure previously reported by us.¹³ To determine the platelet content of PF4 and βTG , the platelets in PRP were lysed with Triton X-100 (1% final concentration). Cell debris was removed by centrifugation at 45,000 g for 20 minutes at 4°C . Samples were then assayed in the same manner as the platelet-free plasma samples.

Analytical Methods

Scintillation camera imaging of both gamma photon peaks of ^{111}In (172 keV and 247 keV) has generally required high energy collimation to prevent image blurring, despite a decrease in both sensitivity and spatial resolution. Since platelet-specific activity was not a limiting factor in the present studies, a high sensitivity ^{99}Tc collimator could be used with good resolution by imaging only the lower energy peak ^{111}In (172 keV peak with a 5% energy window).

ages of the Dacron grafts, including proximal and distal Silastic segments, were acquired with a Picker DC 4/11 Dyna scintillation camera (Picker Corp, Northford, Connecticut) and stored on and analyzed by a Medical Data System SIMUL computer (Medtronic, Ann Arbor, Michigan) interfaced with the camera. This system permitted simultaneous acquisition and analysis of data in 64×64 word mode. Immediately before imaging the graft segments *ex vivo*, 2-minute images were acquired of the 200 μ l sample of platelet concentrate (injection standard) and of a segment of 4.0 mm i.d. Silastic tubing filled with autologous blood and having the same luminal volume as the graft segment (blood standard). All standards and tubings were placed into a groove precisely machined into plexiglass to maintain a linear geometry that was positioned approximately 1 cm from the face of the collimator. The activities of the standards and 10 cm graft segments were counted in the same $3.1 \text{ cm} \times 12.5 \text{ cm}$ region of interest (10×40 pixels) as defined by image analysis software routines. From the time of graft placement, images were acquired continuously with data storage at 2-minute intervals. Deposited ^{111}In -platelet activity was calculated by subtracting the blood standard activity from all dynamic study images.

In several studies, grafts were placed and imaged sequentially for several days following injection of a single preparation of ^{111}In -labeled platelets. Since circulating ^{111}In -platelet activity was lost continuously through normal physiologic mechanisms, and acutely by serial graft placement, measurements of platelet accumulation were expressed as a graft/blood ratio, defined as the ratio of deposited graft activity divided by the whole blood (circulating) platelet activity within the graft lumen measured at the beginning of each experiment. This measurement was chosen since it is independent of the size of the animal, the amount of isotope injected, or the extent to which the isotope may have decayed. The graft/blood ratio will, however, depend upon the timing or sequence of observations if, between periods of graft placement, platelet functional alterations have occurred as a result of aging in the circulation or prior exposure to thrombogenic surfaces.

To determine the graft/blood ratio, the activity of blood within the graft lumen (1.26 ml) was determined by two separate methods. First, it was calculated directly after imaging the blood standard (1.57 ml blood volume). In the second approach, the activity per milliliter of blood present at the beginning of each experiment was calculated by imaging the injection standard before each experiment, multiplying this value by the CPM per ml of whole blood drawn at the time of the experiment (as determined using a gamma counter at some later time t_2), and dividing by injection standard activity (also measured in the gamma counter at t_2). All blood samples and standards were counted simultaneously at the end of each series of experiments. In all calculations, radioactivity values refer to platelet activity only, with total

blood and standard values having been corrected for the fraction of nonplatelet isotope. In the 36 studies involving graft placement, blood activities as determined from the two methods were equivalent $\pm 8\%$ ($\pm 1\%$ SD, $p > 0.5$) demonstrating that either method is appropriate for calculating the graft/blood ratio. Subsequent data are presented according to the second approach since the higher activity of the injection standards (vs blood standards) gave more acceptable counting statistics.

Total platelet deposition (labeled plus unlabeled cells) was estimated by multiplying the graft/blood ratio by the factor: graft blood volume (1.26 ml) $\times$ platelet concentration per milliliter of whole blood. This computation involved the assumption that the labeled and unlabeled platelet populations were equivalent with respect to graft deposition at all times.

Statistical comparisons were made by using Student's *t* test (two-tailed) for paired and unpaired data. All statistical analysis and curve fitting was done using the PROPHET system of the Division of Research Resources, National Institutes of Health. Unless otherwise stated in this report, variance about the mean is given as ± 1 SE.

Results

Effects of Graft Placement on Platelet Viability

Since platelets may undergo biochemical and functional alterations as a result of aging in the circulation, we first evaluated the importance of the delay period between platelet labeling and infusion, and subsequent graft imaging. In six baboons, ^{111}In -platelet deposition was assessed on grafts placed 1, 24, 48, and 72 hours after injection of the labeled cells. All grafts were imaged for 1 hour, and then removed.

Measurements of graft ^{111}In -platelet deposition are shown in Figure 4. Platelet deposition curves were generally sigmoidal, and reached a plateau by 60 minutes. At 1 hour, graft radioactivity was typically 40- to 50-fold greater than the radioactivity circulating within the graft lumen. At all time points, the deposited activity on grafts placed 1, 2, or 3 sequential days after the injection of labeled platelets was equivalent to that observed on grafts studied 1 hour after infusion of the normal labeled platelet population ($p > 0.2$ in all cases), despite platelet aging in the circulation or the prior placement of one, two, or three grafts.

The disappearance of circulating ^{111}In -platelet activity following graft placement in five animals is shown in Figure 5. Although a reduction in circulating platelet radioactivity was consistently observed following the placement of the graft, the remaining platelets appeared to survive normally. The survival curves that would have resulted without the insertion of vascular graft segments were estimated by correcting the measurements of radioactivity taken on Days 2, 3, and 4 for the acute loss of radioactivity

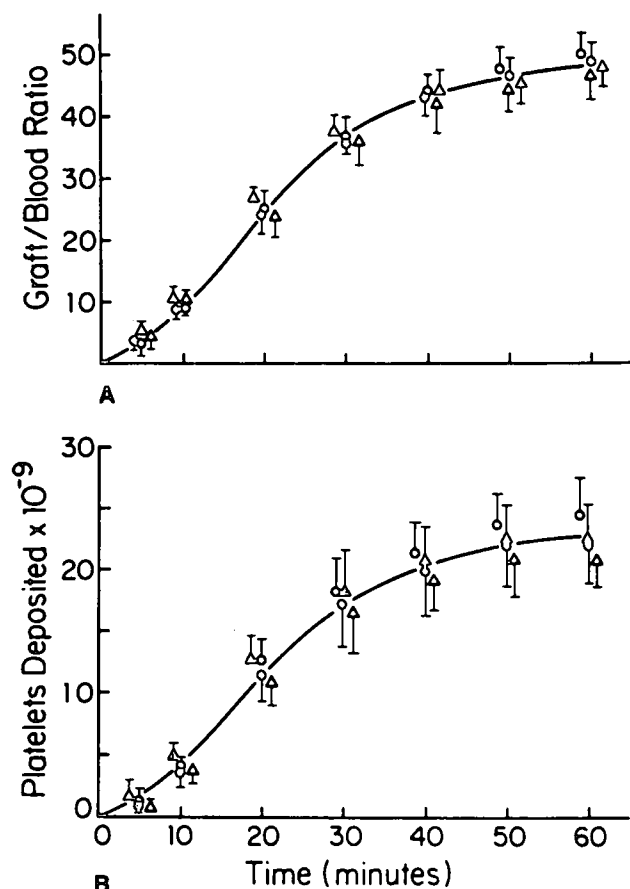


Figure 4. Platelet deposition on grafts placed sequentially. Identical grafts were placed for 1 hour daily in the following sequence: Day 1, ($\circ$); Day 2, ($\square$); Day 3, ($\triangle$); Day 4, ($\diamond$). No significant daily variations ($p > 0.2$) were observed over 4 days when results were expressed as the graft/blood ratio of ^{111}In -platelet activity (A) or as total deposited platelets (B). Values are means ± 1 SE in six animals (Days 1, 2, 3) or four animals (Day 4).

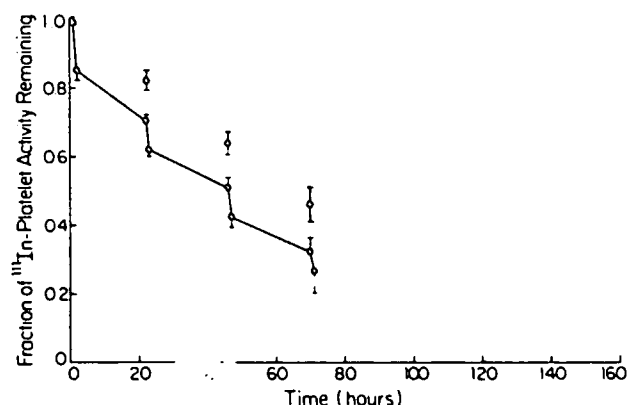


Figure 5. Acute reduction in circulating ^{111}In -platelet activity after graft placement. When the fraction of ^{111}In -platelet activity remaining after graft placement was corrected for the acute reductions, a normal disappearance pattern was observed (symbols in shaded area). Each point represents the mean of at least four determinations ± 1 SE.

resulting from each graft placed previously (Figure 5). All points on the resulting survival curve were within the limits obtained in normal animals.

Reductions in circulating platelet concentration and circulating ^{111}In -platelet activity were determined for the period the graft was in place (Table 1). Circulating platelet number and platelet ^{111}In activity decreased approximately 15% following the placement of each graft, i.e., the reduction values were equivalent ($p > 0.5$), demonstrating that the labeled and unlabeled platelet populations were not measurably different with respect to thrombotic platelet utilization in this model.

In five control studies, a 20×3.125 cm region of interest containing the 10 cm graft segment was imaged, and was subsequently divided into 16 sequential regions with dimensions 1.250×3.125 cm. The activity in each region was calculated as a fraction of total image activity. Although ^{111}In -platelet activity appeared higher in distal graft segments, the relative activities in proximal and distal graft segments did not differ significantly ($44.7\% \pm 7.0\%$ and $55.3\% \pm 7.0\%$ respectively; $p > 0.10$). In general, deposited platelet activity appeared to be uniformly distributed within the graft segment without evidence of localized deposition or thrombus propagation. Morphologic evaluations of grafts exposed to flowing blood for 1 hour demonstrated thrombi rich in platelets with red cells trapped in a fibrin mesh (Figure 6).

Graft-Induced Release of Platelet Granule Constituents

In 14 control studies, measurements of plasma levels of PF4 and βTG were performed on blood samples taken before and after graft placement. Baseline plasma levels of each platelet specific protein showed little variation ($p > 0.3$ in all cases), and the values are shown in Table 1. Following graft placement, PF4 and βTG values increased approximately tenfold over baseline values ($p < 0.001$). However, the total platelet content ($\mu\text{g}/10^8$ platelets) of these proteins as determined from lysed platelet preparations remained unchanged in each case ($p > 0.5$). In addition, the content of ADP and ATP in circulating platelets was not measurably depleted by graft placement ($p > 0.5$, Table 1).

Discussion

The present study was designed to assess directly, in a nonhuman primate model, platelet deposition and acute platelet alterations after exposure to Dacron grafts under arterial flow conditions. Dacron vascular grafts were chosen as the thrombogenic surface since these materials have direct application in humans, and since platelet thrombus formation could be initiated under well-defined conditions of flow and geometry. Thus, in the present studies the graft length (10 cm) and period of exposure (1 hour) were selected to produce measurable platelet accumulation without causing a marked and possibly con-

Table 1. Effect of Dacron Graft on Platelets

Measurements	No. of studies	Baseline	After graft
Platelet count (% baseline)	22	100	84.3 ± 2.0
¹¹¹ In-platelet activity (% baseline)	22	100	85.3 ± 1.6
Platelet factor 4			
plasma (ng/ml)	14	5.2 ± 0.8	58.4 ± 13
platelet content (μg/10 ⁸ platelets)	12	2.7 ± 0.3	2.8 ± 0.2
β-Thromboglobulin			
plasma (ng/ml)	14	3.6 ± 0.9	37.8 ± 2.4
platelet content (μg/10 ⁸ platelets)	12	1.7 ± 0.1	1.9 ± 0.1
Platelet adenine nucleotides (μmol/10 ¹¹ platelets)			
ADP	15	4.64 ± 0.26	4.66 ± 0.28
ATP	15	4.88 ± 0.09	4.50 ± 0.16
ATP/ADP	15	1.117 ± 0.091	1.053 ± 0.115

founding reduction in the circulating platelet count (Table 1).

Since these in vivo studies were performed using platelets labeled with ¹¹¹In-oxine in vitro, it was first necessary to demonstrate that the labeled platelets were functionally normal and representative of the parent population. Thus we observed that the recovery of labeled platelets was normal within 10 minutes

after infusion into the circulation (Figure 3). Platelet survival curves in normal animals were predominantly linear (Figure 3) and equivalent to results obtained previously using a ⁵¹Cr-platelet label.^{7,10} One hour after infusion of the labeled platelet suspension, the labeled and total platelet populations had identical density distributions as determined with Percoll density gradients (Figure 2). These data suggest that the



Figure 6. Scanning electron micrograph of thrombus on graft surfaces. After 1 hour, extensive platelet deposition and fibrin strand formation with trapped erythrocytes was evident in control grafts. Bar = 10 μm.

labeled platelet population was not enriched in either more dense or less dense platelets as a result of platelet harvesting and labeling in vitro. Also, 1 hour after infusion, the labeled platelets were functionally normal with respect to thrombus formation as shown by equivalent reductions in circulating platelet radioactivity and circulating platelet numbers following the insertion of Dacron grafts. Since the labeled and unlabeled platelet populations were equivalent by all tested criteria, total platelet deposition could be calculated from measurements of graft radioactivity. In addition, since these measurements were independent of the timing of graft placement with respect to platelet labeling, this model appears to be an efficient one, (i.e., following a single labeling procedure, grafts may be imaged at daily intervals with equivalent results).

Graft placement, which resulted in the destruction of approximately 15% of the ^{111}In -labeled platelet population, also did not alter the survival, α -granule contents, or dense granule contents of those platelets that continued to circulate. The findings of elevated plasma levels of PF4 and βTG (Table 1) thus imply that α -granule release had occurred only from platelets which had reacted irreversibly with the graft. Since about 75% of platelets removed from the circulation, as determined from platelet counting, were present on the graft after 1 hour (as determined from imaging), the possibility of reversible platelet interactions in addition to graft platelet removal by embolization and lysis cannot be excluded. However, since the viability and function of the circulating platelet pool was not measurably affected, such interactions must be almost totally reversible, or, affect only a small proportion of circulating platelets.

These results are in agreement with previous studies which demonstrated normal platelet ADP/ATP levels in patients and baboons undergoing cardiopulmonary bypass,^{14, 15} but are not in accord with the reported reduction in platelet dense granule contents observed in patients with valvular heart disease and Dacron vascular prostheses, and in dogs with chronic aortic grafts.¹⁶⁻¹⁸ Since our data are consistent with the hypothesis that acute thrombosis in vivo rapidly produces irreversible platelet removal, we speculate that platelet alterations associated with chronic disorders involving elevated platelet turnover may largely reflect the presence of an increased proportion of younger cells rather than an abnormality acquired during circulation. Under these conditions the functional capacity of platelets has been shown to be diminished in rabbits¹⁹ and dogs²⁰ and unchanged in baboons.^{7, 10} The clinical relevance of these observations remains unknown.

In previous studies of ^{111}In -platelet deposition onto vascular grafts in both experimental animals²¹⁻²⁶ and humans²⁷⁻³⁰ the effects of several variables have been studied, including graft type,^{22, 23, 29, 30} blood-flow rate,²³ and the effects of antithrombotic therapy.^{25, 27, 29, 30} We believe that this model of acute thrombosis is suitable for assessing the effects of

other variables and therapies, and for defining systemic blood alterations secondary to platelet thrombus formation on prosthetic surfaces in vivo.

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Index Terms: platelets • vascular grafts • platelet release • indium labeling

FACTORS REGULATING THROMBUS FORMATION IN BABOONS

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ABSTRACT

A baboon arteriovenous shunt model of acute thrombosis induced by Dacron vascular grafts was used to study the role of physical variables regulating thrombus deposition. After labeling autologous baboon platelets with 111-In-oxine, and homologous baboon fibrinogen with 131-I, the deposition of these blood elements was assessed using a gamma camera. When grafts were exposed for periods ranging from 10 - 60 minutes, the net accumulation of platelets was found to depend strongly on the circulating platelet count. This dependence was always greater than first-order, indicating that a doubling in platelet count between individual animals produced a greater than two-fold increase in platelet deposition. Conversely, the dependence of platelet deposition on blood flow rates ranging from 30-220 ml/min was approximately one-half order, indicating that a doubling in flow rate between animals increased platelet deposition by less than 50%. Equivalent results were obtained with untreated grafts, and with grafts which had been preclotted with autologous whole blood. Significant fibrinogen deposition was observed, which, in contrast to the platelet studies, was increased at lower flow rates. Thus, physical variables regulating thrombus composition should be carefully considered in experimental animal models, and perhaps in some clinical settings.

INTRODUCTION

To quantify continuously the overall rate of acute arterial thrombus formation in vivo in a manner that is free of uncontrolled variables, we used a primate model of vascular graft thrombosis (1). This model was developed specifically to produce an acute, significant, and reproducible thrombotic challenge such that the relative importance of in vivo variables could be determined. Thrombosis was initiated using vascular grafts since these materials have widespread applications in man, and since thrombotic occlusion currently limits the usefulness of small caliber prostheses. A baboon animal model was chosen since this species is hemostatically similar to man (1,2). Grafts were studied ex vivo after incorporation into arteriovenous shunts (1,3). This method permits control of graft geometry and blood flow rate, and gamma camera imaging can be performed without attenuation of In-111 emissions by intervening tissues.

Previous 111-In platelet studies in both experimental animals and man have

primarily considered the importance of graft type and the effects of antithrombotic therapy (4-11). The present study was therefore designed to assess the relative importance of the additional variables of blood flow rate and circulating platelet count with respect to the deposition of ^{111}In -platelets and ^{131}I -fibrinogen.

METHODS

Animals studied

Normal male baboons (*papio anubis*) weighing 9-12 kg were used in these studies. All animals had a chronic 3.0 mm i.d. x 50 cm silicone rubber arteriovenous shunt between the femoral artery and vein as described previously (1,3). Vascular graft materials were connected between the two 25 cm cannulae comprising the chronic shunt.

Vascular grafts

External velour knitted Dacron grafts (C.R. Bard, Inc) were rendered impervious by an external wrapping and placement inside Teflon tubing as described previously (1). All grafts were 10 cm in length, 4.0 mm i.d., and were coupled at their proximal and distal ends to 10 cm lengths of 4.0 mm i.d. silicone rubber to provide a uniform flow channel (1). In several studies, the grafts were preclotted with autologous whole blood. Residual clot was stripped from the grafts using a 3.5 mm embolectomy catheter.

Labeling procedures

Autologous whole blood (100 ml) was collected directly into plastic bags (Fenwal TA-3) and labeled with 1 mCi ^{111}In -oxine (Amersham) according to the acid-citrate-dextrose method as described (1). Following a single extraction of platelet rich plasma from whole blood, the platelets were pelleted once to remove residual plasma proteins. Since labeling efficiencies averaged greater than 90%, additional washing procedures were deemed unnecessary. Despite the incomplete harvesting of platelets from whole blood, the labeled platelet population has been previously shown to be functionally normal, with a density distribution equivalent to that of the parent platelet population (1). Baboon fibrinogen was prepared and labeled with 1 mCi ^{131}I according to the iodine monochloride method as described (12). The clottability of the labeled fibrinogen was greater than 93%.

Gamma camera imaging

Images were taken with a Picker DC 4/11 Dyna camera and analyzed with an A³-MDS data processing system (1). Blood standard activities were subtracted from all images to yield deposited activity only. Results were expressed as total (labeled plus unlabeled) platelets or fibrinogen by dividing deposited

activities (CPM) by the blood specific activity (CPM/ml), and multiplying by the blood species concentration per ml (1).

RESULTS

Effect of variable platelet count

The results of 22 studies in which graft platelet deposition was evaluated at 10, 20, and 60 minutes of blood exposure are shown in Figure 1. Curve fitting to the general equation $Y = ax^n$ indicated that the dependence of graft platelet deposition at all time points was greater than first-order. In these studies, blood flow rates were relatively constant, ranging from 170-220 ml/min.

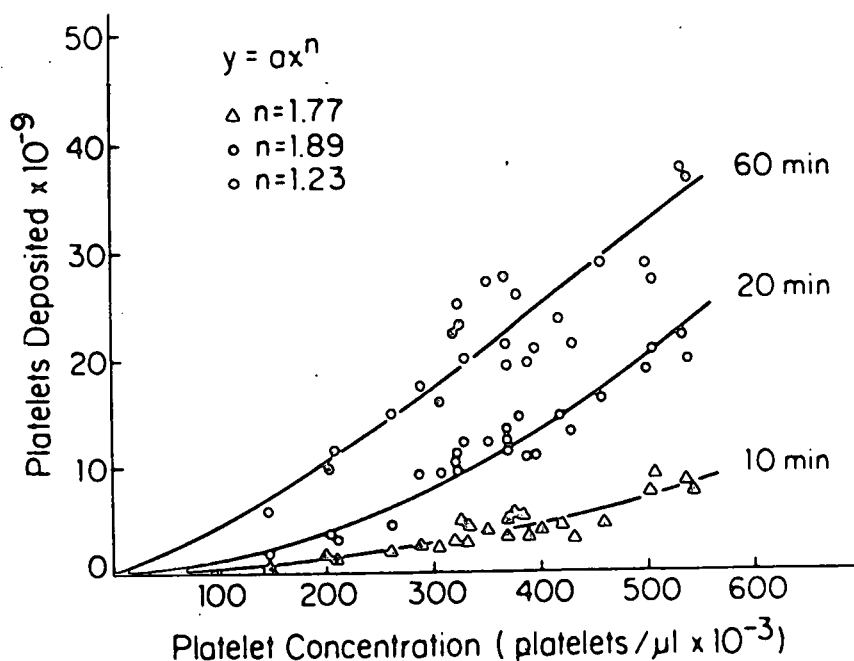


FIGURE 1. Dependence of graft platelet deposition on circulating platelet count.

Effect of blood flow

Separate groups of animals were studied at blood flow rates of 30, 80, and 220 ml/min. As indicated in Figure 2, each group had comparable mean circulating platelet counts so that blood flow rate could be studied as an independent variable. Variations in blood flow rate had only a modest effect on graft platelet deposition. After 60 minutes, this dependence was approximately one-half order according to the curve-fitting analysis described in Figure 1.

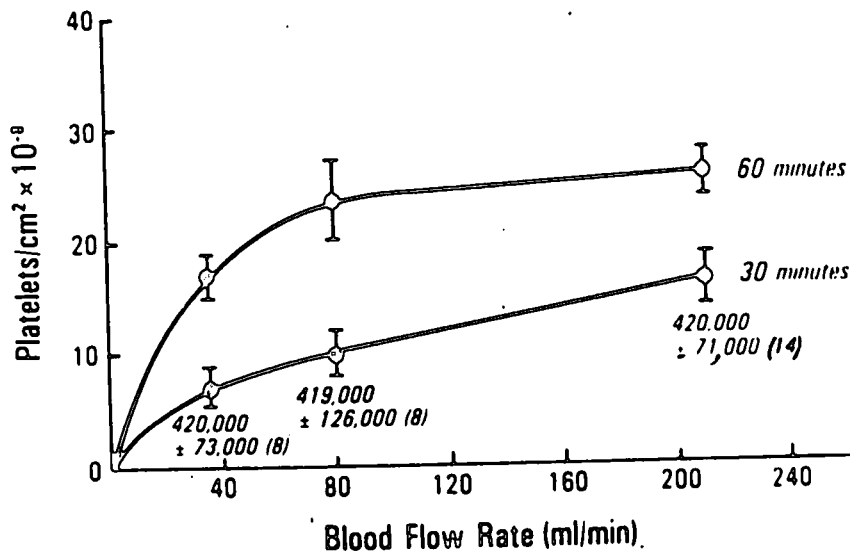


FIGURE 2. Effect of variable blood flow rate on graft platelet deposition.

The dependence of graft platelet deposition on blood flow rate was also studied in animals having grafts preclotted with autologous whole blood. Results are shown in Figure 3, and were equivalent to those obtained with untreated grafts. As indicated, all animals had platelet counts within a fairly narrow range (352,000 - 420,000 platelets per microliter of whole blood).

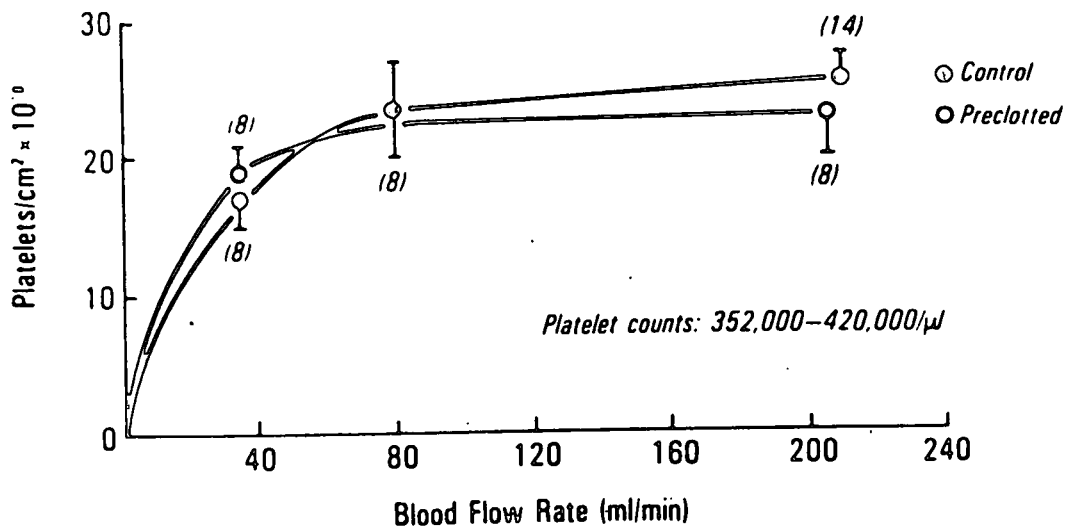


FIGURE 3. Effect of blood flow on platelet deposition onto untreated and preclotted Dacron vascular grafts.

Deposition of ^{131}I -fibrinogen

The results of studies in 7 animals are shown in Figure 4. Deposition appeared to plateau by 2 hours. Between 1 and 2 hours of blood exposure, ^{131}I -fibrinogen accumulation in animals studied at blood flow rates of 30 ml/min, was modestly increased as compared to the group evaluated at flow rates of 150 ml/min. The circulating fibrinogen concentrations in all animals were within a fairly narrow range, 3.6 ± 0.5 mg/ml (± 1 S.D.).

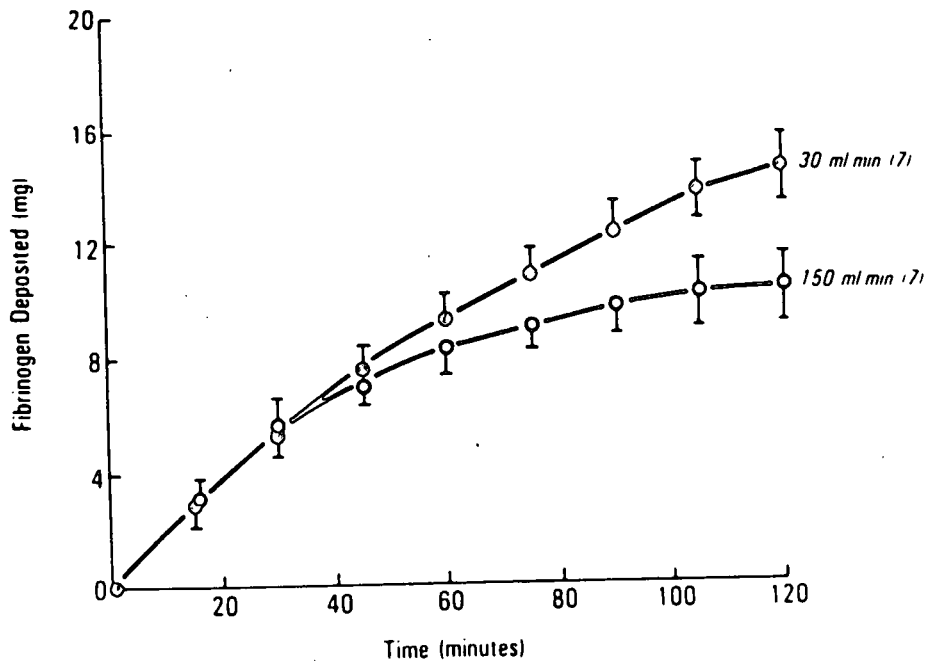


FIGURE 4. Effect of blood flow rate on fibrinogen deposition onto untreated Dacron grafts.

DISCUSSION

The results of the present study suggest that platelet interactions with small caliber prostheses may depend strongly on the circulating platelet count, and to a lesser extent on blood flow rate. While a modest effect of blood flow has been reported in a similar study by Eldrup-Jorgensen, et. al. (13), the possible importance of variations in platelet count between animals was not considered by these investigators. While it may not be possible to extrapolate these results to situations of actual graft implantation, nonetheless, given the striking dependence of platelet thrombus accumulation on platelet count, we believe that this variable should be carefully considered in animal models of experimental thrombosis and perhaps in some clinical settings.

It is of interest that equivalent results were obtained both with untreated grafts, and with grafts which had been preclotted with whole blood as would normally be done in clinical practice. This result suggests that upon blood exposure, untreated grafts may rapidly acquire a flow surface of coagulated blood following filling of the graft interstices. In addition, the preclotting procedure apparently does not reduce graft thrombogenicity acutely.

Finally, this study demonstrates the feasibility of imaging ¹³¹I-fibrinogen which is presumably deposited as fibrin. A modest increase in fibrinogen deposition was observed at lower flow rates, in contrast to the platelet studies. This result may reflect prolonged residence times for coagulation factors at lower flows, giving rise to higher local concentrations of activated species (e.g., thrombin) which promote fibrin formation. In future studies it may be possible to determine the extent to which platelet and fibrinogen deposition are interdependent phenomena, and, by analogy to the platelet studies, to assess the importance of circulating fibrinogen levels.

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Kinetics and in Vivo Redistribution of ^{111}In -Labelled Human Platelets after Intravenous Protamine Sulphate

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Key words

Platelet kinetics – ^{111}In -indium-oxine labelled human platelets – Protamine sulphate – Thrombocytopenia, protamine induced – Neutropenia, protamine induced

Summary

The pathogenesis of thrombocytopenia induced by intravenous protamine sulphate was studied in six patients who underwent cardiopulmonary bypass surgery, and in three normal volunteers. Autologous platelets were labelled with ^{111}In -indium-oxine. Platelet lifespan was determined. *In vivo* ^{111}In -platelet localization, organ redistribution and sites of destruction were quantitated with a scintillation camera and a computer-assisted imaging system. Protamine induced a transient thrombocytopenia, maximal 5–10 min after injection, and 30–40 min in duration. The thrombocytopenia was accompanied by a transient accumulation of platelets in the liver. The splenic platelet pool remained unaltered and no platelets accumulated in the lungs. Platelet survival, measured in two volunteers, was slightly longer than normal and fitted a linear function best. There was a severe transient neutropenia during the period of thrombocytopenia. We conclude that protamine-induced thrombocytopenia is caused by hepatic accumulation of "activated" platelets or platelet aggregates, the process is reversible, and in the two normal volunteers studied, platelet survival was not affected.

Introduction

Protamine sulphate is widely used as a heparin antagonist. This cationic polypeptide aggregates platelets loosely *in vitro* (1, 2) and induces transient thrombocytopenia after intravenous administration (3–5). The approximately 20% fall in platelet count is accompanied by a parallel decrease in systemic vascular resistance, a fall in cardiac output and an increase in pulmonary artery pressure (6–8). In one study it was found that ^{51}Cr -labelled platelets accumulated in the lungs after protamine infusion, a finding interpreted as being due to pulmonary vasoconstriction rather than intravascular obstruction by platelet micro-emboli (5).

^{111}In -8-hydroxyquinoline (^{111}In -oxine) is an efficient platelet label (9) and its physical characteristics permit quantitative gamma imaging of the *in vivo* distribution of labelled cells (10, 11). We have applied this new technology to study the

kinetics, fate and redistribution of ^{111}In -labelled platelets in volunteers after the intravenous administration of protamine sulphate.

Materials and Methods

Reagents

^{111}In -chloride (Radiochemical Centre, Amersham, U. K.) was complexed with 8-hydroxyquinoline [100 μg oxine to 37 MBq (1 mCi) ^{111}In Cl_3] as described by Thakur (11). Protamine-sulphate was obtained from Evans Medical Ltd, Speke, Liverpool, U. K. and heparin (Pularin) from Allen and Hanbury's U. K. Other reagents were supplied by commercial sources and were of analytical grade.

Labelling of platelets. [Modified after (10) and (11), and the recommendations of the ICSH (12)].

All procedures were carried out aseptically at 22°C in plastic or siliconized equipment. After discarding the first 2 ml, collected in a separate syringe, 84 ml venous blood was drawn with a 16 g needle (Butterfly – 16, Abbott Laboratories) sequentially into two 50 ml polystyrene syringes each containing 8 ml acid citrate dextrose, NIH formula A (ACDA), as anticoagulant. Platelets were isolated and labelled with ^{111}In -oxine as follows: the blood was transferred to 50 ml polystyrene conical tubes (Falcon 2070, Oxnard, California U.S.A.) and platelet rich plasma (PRP) prepared by differential centrifugation at 180 g for 10 min; contaminated red cells were removed by further centrifugation of the PRP at 180 g for 5 min; the pH of the PRP was adjusted to 6.2–6.5 by the addition of 5 ml ACD-A per 100 ml PRP; platelets were sedimented into a pellet by centrifugation at 800 g for 15 min; resuspended in ca. 2 ml physiological saline; incubated for 30 min with ^{111}In -oxine at a final concentration of 6.25 μg oxine/ml platelet suspension; washed once with autologous platelet poor plasma (PPP); and finally resuspended in PPP. Radioactivity of the labelled platelets was determined in a source calibrator and 12.95 MBq (350 μCi) or less ^{111}In administered to the subject.

Aggregation of labelled platelets with adenosine diphosphate was assessed *in vitro* under the phase microscope as previously described (13) and ^{111}In -platelets not used for *in vivo* studies unless a normal response was seen.

Platelet survival studies were performed as recommended by the ICSH (13). Mean autologous platelet survival was calculated by least squares analysis of the linear and logarithmic estimates (12) and least squares computer fitting to a gamma function (14). Platelet recovery in the circulation at equilibrium was derived by back extrapolation of the platelet survival curve from days 1 to 10 to zero time.

Image Acquisition and Quantitation of Platelet Distribution

This was done as previously described (10, 11). Briefly, a scintillation camera was interfaced with a PDP-8 based computer-assisted imaging system acquisition matrix and an interpolated display image. The camera was positioned to visualize the heart, lungs, spleen and liver simultaneously. Geometry was maintained by using the xiphisternum and anterior iliac spines as points of reference. Autologous ^{111}In -labelled platelets were injected the day previous to the study to allow equilibrium and re-entry of platelets into the circulation after temporary segregation in the liver (10).

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On the day of the protamine sulphate injection, images of the heart, lungs, liver and spleen were obtained by integrating for 30 s periods for 5 min. The heparin and protamine sulphate were injected sequentially and 30 s images integrated for 45 min. Areas of interest were selected with the computer and time-radioactivity curves of the heart, lungs, liver and spleen generated.

Blood counts were performed with a Coulter Model SR electronic particle counter. Leukocyte differential counts were done on 200 cells.

Patients

Six patients (subjects A to F) were part of a study of platelet kinetics during cardiopulmonary bypass surgery. Autologous platelets were labelled with ^{111}In -oxine and reinjected the day prior to surgery. During extracorporeal circulation patients have a moderate thrombocytopenia due to haemodilution and sequestration of platelets in the bubble oxygenator. The bypass procedure involved administration of 250 U heparin/kg body mass as a bolus and thereafter continuously by intravenous infusion. At the end of the bypass the heparin was neutralized with intravenous administration of 300–400 mg protamine sulphate. ^{111}In -platelet activity in peripheral blood, and blood platelet counts were performed at intervals before, during and for one hour after protamine sulphate infusion.

Three normal volunteers (subjects G, H and I) were investigated in a project approved by the Ethical Committee of the University and the Provincial Administration of the Orange Free State. Autologous platelets were labelled and reinjected. The following day 100 U/kg heparin was administered intravenously. Ten minutes later 50 mg protamine sulphate, diluted in 20 ml physiological saline, was given slowly by intravenous injection over a period of 5–7 min. Two subjects (H and I) had no untoward side effects. Subject G, who previously had received protamine sulphate, had a delayed hypotensive hypersensitivity attack approximately 2 h after the administration of the protamine. The study of this subject was not completed. Since the results in all three volunteers were similar it was not deemed justified to expose other volunteers to the risk of anaphylaxis and the study was terminated.

Results

1. Cardiopulmonary Bypass Surgery Patients

Six patients received protamine sulphate intravenously. The results (subjects A to F) are given in Table 1. In order to compare the results in different patients ^{111}In -radioactivity of labelled platelets just prior to administration of protamine was assumed 100% and subsequent radioactivity related to this value. The results given are those before the protamine administration, and the maximum decrease in absolute platelet count and ^{111}In -activity observed 5 to 10 min after protamine infusion. Blood ^{111}In -activity decreased by a mean (± 1 SD) of $26 \pm 14\%$ and absolute blood platelet counts by $32 \pm 16\%$. The magnitude of the decrease in ^{111}In -activity and platelet counts did not differ significantly ($P > 0.15$; paired *t* statistic).

Table 1 Effect of intravenous protamine on blood platelet count and blood ^{111}In -activity

Subjects	Pre-Protamine ^{111}In -activity (%)	Pre-Protamine Platelet count ¹⁾ ($\times 10^9/\text{l}$)	Post-Protamine ^{111}In -activity (%)	Post-Protamine Platelet count ($\times 10^9/\text{l}$)	Per cent decrease in platelet ^{111}In -activity (%)	Per cent decrease in platelet Platelet count ($\times 10^9/\text{l}$)
A	100	78	80	43	20	45
B	100	106	88	63	12	41
C	100	86	65	58	35	33
D	100	86	83	66	17	23
E	100	68	50	36	50	47
F	100	44	81	42	19	5
A–F, Mean ± 1 SD	100 ± 0	78 ± 21	75 ± 14	51 ± 13	26 ± 14	32 ± 16
G	100	200	–	85	–	56
H	100	280	82	129	18	54
I	100	190	83	140	17	26
G–I, Mean ± 1 SD	100 ± 0	223 ± 49	82 ± 0.71	118 ± 29	17.5 ± 0.7	45 ± 17

¹⁾ Patients have a moderate thrombocytopenia during cardiopulmonary bypass.

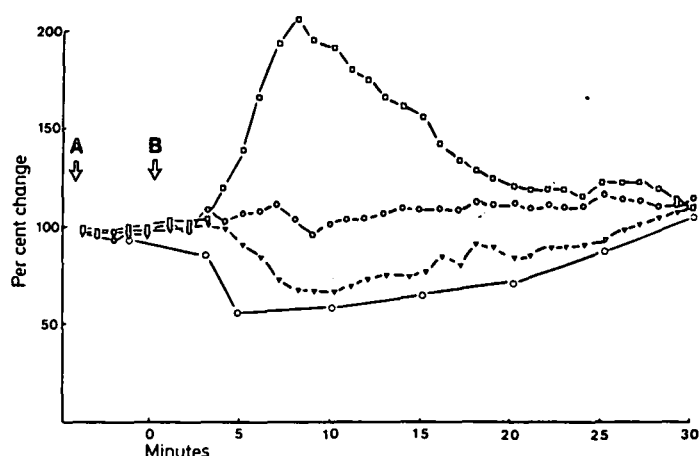


Fig. 1 In vivo organ redistribution of ^{111}In -labelled platelets and blood platelet counts after intravenous administration of protamine sulphate. Changes are expressed as a percentage relative to pre-injection values assumed to be 100%. Transient hepatic accumulation of platelets mirrors the thrombocytopenia. Heparin was administered at time A and protamine at time B. Liver $\square$ – $\square$; spleen $\circ$ – $\circ$; heart ∇ – ∇ ; blood platelet count $\circ$ – $\circ$.

2. Platelet Survival Studies

The relevant data on radioactivity injected, recovery in the circulation, and linear, logarithmic, weighted mean and gamma function analysis of platelet disappearance from the circulation of subjects H and I are given in Table 2. The results of ^{111}In -platelet survival of the same subjects done previously are also given. Normal reference values are from our previous study (10).

Recovery in the circulation was within normal limits but in both subjects less than in the previous study. The disappearance curves of platelets from the circulation fitted a linear function best and there was good correlation between platelet survival determined by the linear, weighted mean and gamma functions. In both subjects platelet survival was slightly longer after protamine sulphate administration compared to the previous study, and the number of "hits" also increased.

3. In Vivo Redistribution of Platelets after Protamine Administration

The subject was placed under the scintillation camera and heparin and protamine sulphate administered sequentially. Changes in organ ^{111}In -radioactivity were monitored for 45 min. Blood platelets were counted before, and 5, 10, 15, 25, 35 and

Table 2 Subject and platelet survival data

Subject	¹¹¹ In-injected MBq (mCi)	Recovery in circulation (%)	Platelet survival (hours)				Weighted mean	Gamma function	Number of "hits"
			T linear T (h)	± 1 SD*	T log T (h)	± 1 SD*			
H Protamine study	8.73 (236)	67	245	2.6	105	8.4	234	247	68
H Previous study	11.39 (308)	92	238	1.6	115	5.5	229	230	13
I Protamine study	10.28 (278)	65	244	2.6	110	8.7	231	243	51
I Previous study	12.95 (350)	83	233	2.5	110	6.0	214	226	16
Normal values (ref. 10)	—	72 ± 16	216 ± 17	2.6 ± 1.6	101 ± 21	9.3 ± 5.7	177 ± 36	179 ± 40	9 ± 6.4

* This standard deviation is the discrepancies between the fitted line and the data.

Table 3 Effect of intravenous protamine sulphate on blood WBC, neutrophil count and haematocrit

Subject	Investigation	Time (minutes) after injection of protamine sulphate						
		Pre-infusion	5	10	15	25	35	45
G	WBC ($\times 10^{-9}/l$)	6.1	1.6	1.9	3.9	5.1	5.5	5.9
	Neutrophils ($\times 10^{-9}/l$)	2.8	0.2	0.1	1.3	2.1	2.6	2.9
	Haematocrit (l/l)	0.48	0.50	0.51	0.51	0.51	0.51	0.51
H	WBC ($\times 10^{-9}/l$)	5.4	4.0	3.5	4.0	4.2	3.9	5.5
	Neutrophils ($\times 10^{-9}/l$)	2.8	0.9	0.8	1.3	1.6	1.9	2.9
	Haematocrit (l/l)	0.39	0.38	0.36	0.36	0.39	0.38	0.38
I	WBC ($\times 10^{-9}/l$)	7.6	5.1	4.4	5.3	6.6	6.9	7.2
	Neutrophils ($\times 10^{-9}/l$)	4.4	2.0	0.8	2.2	3.5	3.9	4.2
	Haematocrit (l/l)	0.41	0.40	0.41	0.40	0.41	0.40	0.39

45 min after protamine infusion. The results obtained in subject H are illustrated in Fig. 1 and 2. The results in all three normal subjects were similar but the time lag and magnitude of change differed somewhat, making construction of an aggregate curve impossible. Between 3 and 5 min after protamine sulphate was administered the platelet count started to decrease and reached a nadir at 5 min. In subjects G, H and I the minimum platelet count was respectively reached 10, 5 and 5 min after the injection. Platelet counts returned to baseline levels 30–45 min after protamine administration. Organ radioactivity distribution changed after protamine. The disappearance of platelets with time from the circulation was mirrored by an increase in hepatic

¹¹¹In-radioactivity. Splenic ¹¹¹In-activity remained constant. No increase in radioactivity occurred in the lungs.

4. Effect of Protamine Sulphate Administration on the Blood Neutrophil Count

Peripheral blood neutrophils were counted before, and at frequent intervals after protamine administration to subjects G, H and I. The results are given in Table 3. In all three subjects a marked transient neutropenia was more pronounced than the thrombocytopenia. Neutrophil counts were maximally depressed 10 min after injection and had returned to normal levels at about 45 min. There was no change in the blood haematocrit of subjects H and I, whereas that of subject G, who later had a hypotensive reaction, increased somewhat.

Discussion

Protamine is a strongly polycationic polypeptide which is widely used to neutralize heparin in cardiopulmonary bypass surgery and in blood cell separator cell component donation procedures. Protamine aggregate platelets *in vivo*, probably by a direct effect on the platelet membrane. Intravenous infusion of protamine causes transient thrombocytopenia (3–5), a decrease in systemic vascular resistance, a fall in cardiac output and an increase in pulmonary artery pressure. It is not clear how the platelets bring about their effect. It has been suggested that these vascular changes are mediated by the release of vasoactive platelet substances (8). Some researchers think the increase in pulmonary vascular resistance is caused by obstruction of the pulmonary vascular bed by platelet microthrombi (15), whereas others consider the release of smooth-muscle-active substances from platelets more important (5, 8).

Adverse reactions to protamine, similar to that seen in subject G, are occasionally encountered. The clinical effect may in part be

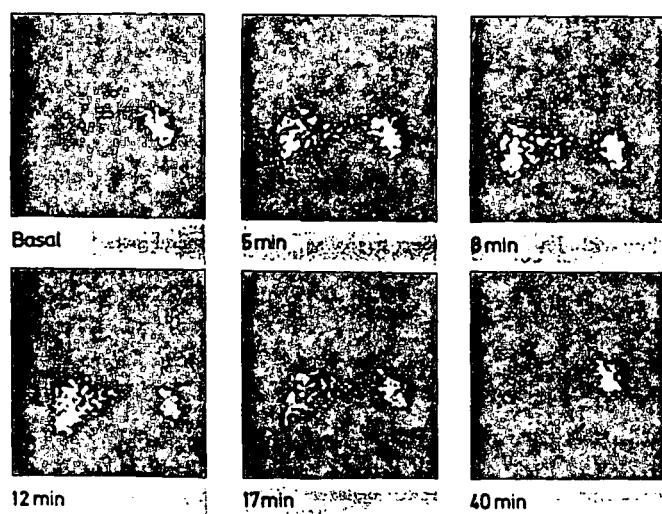


Fig. 2 Scintillation camera images illustrating the transient accumulation of ¹¹¹In-labelled platelets in the liver after intravenous administration of protamine sulphate. The splenic pool of platelets and the heart are clearly visualized.

ascribed to release of vasoactive substances from the platelets (8). In view of the severity of the reaction our subject G had, we did not feel it justified to expose other volunteers to the risk. Although the series is small the results in all three subjects were virtually identical, thus confirming the validity of the study.

^{111}In , a cyclotron-produced isotope, 2.8 d half-life, complexed to 8-hydroxyquinoline is an efficient platelet label (9). The physical characteristics of the radionuclide permit quantitative computer-assisted scintillation camera imaging of platelets *in vivo* (10, 11). We have applied these techniques to study the effect of protamine sulphate infusion on the *in vivo* redistribution of platelets and on platelet survival to elucidate the nature of the thrombocytopenia and to determine whether platelets accumulate in the lungs. We confirm the finding that intravenous protamine sulphate causes a rapid but transient thrombocytopenia. In both the cardiopulmonary bypass surgery group and the normal volunteers the magnitude of the decrease in ^{111}In -labelled and total blood platelet counts was similar (Table 1). This indicates that the labelled platelets responded to the protamine sulphate in the same manner as did the unlabelled platelets. Platelet recovery in the circulation and platelet survival was within normal limits (Table 2). The finding that platelet survival after protamine administration was slightly longer in both normal subjects compared to the findings in the same subjects in a previous study, is of interest. The fewer "hits" in the gamma function model (14) indicates that the platelet survival curves in both subjects were also more linear after protamine compared to that of the previous control studies (Table 2). It is possible that the platelets exposed to, and presumably reversibly aggregated by the protamine, become less reactive and are not removed from the circulation until the end of their lifespan. This series is however far too small for any firm conclusions in this regard.

In vivo platelet accumulation in the lungs after protamine infusion has been studied in dogs (5). These investigators observed a transient tenfold increase in ^{51}Cr -labelled platelets in the lungs but not in the skeletal muscles. In our study protamine induced thrombocytopenia was accompanied by a transient accumulation of ^{111}In -activity in the liver (fig. 1 and 2). No increase in ^{111}In -platelet activity was observed in the lungs or in the spleen. It is not apparent why our results differ from the findings of Rådegran (5), but it may be due to greater aggregation response of canine platelets to protamine (16). The hepatic ^{111}In -platelet accumulation resembles that seen in the first 90 min after reinjection of ^{111}In -labelled platelets in normal man and dog (10, 11). We have ascribed this phenomenon to a transient hepatic pooling of platelets injured by the collection and labelling procedure. The liver thus seems able to temporarily sequester "activated" platelets. A similar phenomenon is apparently observed here. The protamine presumably causes *in vivo* aggregation of activation release of the platelets. This is a reversible process and the platelets, after from the liver, have at least a normal life span.

The protamine induced severe neutropenia in all three normal subjects studied (Table 3). The neutropenia was maximal within 5–10 min after injection, and seemed to parallel the kinetics of the thrombocytopenia. That this was not due to changes in plasma volume was reflected by the constancy of the haematocrit. Transient neutropenia is known to occur in anaphylaxis, presumably due to rapid intravascular redistribution of granulocytes, especially in the pulmonary capillary bed (17), but our subjects H and I did not show any evidence of anaphylaxis. Platelets contain

histamine which is secreted during the release reaction. Histamine may cause a temporary pool shift of neutrophils by inducing them to stick to vascular endothelium (18). Protamine may by this mechanism cause a pseudoneutropenia. It should be possible to study this phenomenon with ^{111}In -oxine labelled granulocytes.

This study illustrates the advantages of ^{111}In -oxine as a platelet label. *In vivo* quantitative studies of platelet redistribution and sites of destruction are possible with the techniques we have developed. The interaction between a drug and platelets could thus be studied with a new approach and the pathogenesis of the transient thrombocytopenia elucidated.

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A comparison between the use of recombinant hirudin and heparin during hemodialysis

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A comparison between the use of recombinant hirudin and heparin during hemodialysis. The purpose of this study was to determine the anticoagulant and antithrombotic potential of hirudin during hemodialysis by comparing the efficacy of dialysis with heparin to that of dialysis with recombinant hirudin (r-hirudin). Eleven patients with chronic renal failure and on maintenance hemodialysis were included in this open cross-over study. Conventional doses of heparin were administered during the first dialysis of the study. Two days later r-hirudin, at a dose of 0.15 mg/kg, was given as a bolus at the start of the second dialysis. The mean decreases in plasma levels of urea, uric acid and creatinine were approximately 50% after dialysis with both anticoagulants. Dialysis was therefore equally effective. However, effective dialysis with r-hirudin was achieved with a shorter activated partial thromboplastin time (APTT; range 65 to 103 seconds) compared to that with heparin (> 120 seconds), thereby decreasing the risk of bleeding. Markedly less ^{111}In -labeled platelets accumulated at the inlet of the artificial kidney when r-hirudin was used, suggesting a smaller loss of hollow fiber volume. The results indicate that hirudin may be a suitable alternative anticoagulant for use during hemodialysis and it thus warrants further investigation.

Extracorporeal thrombogenesis is a major problem associated with hemodialysis. The composition of the artificial membrane in the extracorporeal system and the large surface area to which the blood is exposed, contribute significantly to activation of the coagulation cascade; white blood cells and blood platelets [1]. The use of an anticoagulant is therefore a prerequisite to prevent thrombotic occlusion of the artificial kidney to ensure effective dialysis. Standard unfractionated heparin is currently the anticoagulant of choice. Its anticoagulant effect is achieved through interaction with the natural thrombin inhibitor, antithrombin III (AT III). Heparin potentiates the inactivation of thrombin by forming a complex with its cofactor AT III, which serves as a template to which thrombin binds [2]. Heparin does, however, not completely prevent thrombogenesis [3], and there are several complications associated with its long-term use. These include thrombocytopenia, increased bleeding tendency, osteoporosis, increased lipolytic activity, and changes of lipid patterns [4–8]. Activation of lipolysis by heparin during hemodialysis also leads to immunosuppressive effects [7]. Therefore a safer and more effective anticoagulant would be beneficial.

Hirudin, the most potent natural inhibitor of thrombin, seems

to be a promising alternative anticoagulant for heparin. Hirudin is a direct thrombin inhibitor and does not require endogenous cofactors. It reacts with thrombin in a 1:1 molar ratio to form a noncovalent complex. The carboxy terminal region of hirudin, which is rich in acidic residues, binds ionically to the anion binding exosite of thrombin. The amino-terminal region binds via hydrophobic interaction to the apolar binding site and the Pro 46-Lys 47-Pro 48 region occupies the basic specificity pocket of the active site [9]. Through this mechanism hirudin inhibits all the actions of thrombin and so effectively inhibits coagulation and prevents heparin-resistant arterial-type thrombosis when given in large enough dosages [10]. Hirudin has no adverse effects when infused into humans, because it is pharmacologically virtually inert [11]. Hirudin is also a weak immunogen [12–14]. Hirudin was first used during hemodialysis in 1926 [15]. However, impurities in the crude preparation and the uncertainty about its anticoagulant activity and side effects limited its clinical use. Purified hirudin is now made in sufficient amounts by recombinant technology, and its use during hemodialysis has already been studied in dogs [16] and man [17]. We evaluated the use of recombinant hirudin as an anticoagulant during hemodialysis in humans. We assessed the efficacy of dialysis by comparing r-hirudin with heparin. To achieve this, we measured the biochemical profile, platelet count, hematocrit, platelet function, and coagulation before, during, and after dialysis.

Methods

Subjects

Eleven patients (median age 37 years, range 22 to 51), with chronic renal failure (creatinine clearance < 10 ml/min) and on maintenance hemodialysis were included. The patients gave informed consent to participate. The study was approved by the Ethics Committee of the Provincial Administration and the University of the Orange Free State. The use of r-hirudin was approved by the South African Medicines Control Council. All patients refrained from taking drugs that may influence platelet function and fibrinolysis for at least 10 days prior to, and during the study.

Study design

This was an open cross-over study. Standard unfractionated heparin from porcine intestinal mucosa (Heparin Sodium, Labetthica, SA) and r-hirudin (Hoechst AG, Frankfurt and Behringwerke AG, Marburg, Germany) were used. Because of the prolonged elimination of hirudin in terminal renal failure [18],

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Table 1. The mean decrease in serum levels of solutes during dialysis and the percent efficacy of dialysis with heparin and hirudin respectively

	Hirudin		Heparin	
	Decrease	% Efficacy	Decrease	% Efficacy
Urea	13.2 ± 1.9 mmol/liter	50 ± 7	15.7 ± 3.7 mmol/liter	51 ± 7
Uric acid	0.2 ± 0.1 mmol/liter	48 ± 14	0.3 ± 0.1 mmol/liter	50 ± 11
Creatinine	423 ± 152 µmol/liter	45 ± 6	439 ± 149 µmol/liter	44 ± 6

r-hirudin was administered during the second dialysis of the study. This was to avoid possible effects of residual r-hirudin during the second dialysis. Cuprammonium rayon type artificial kidneys (Terumo, Japan) were used once and then discarded. Dialysis was done against an acetate buffer on a Gambro AK 10 artificial kidney machine (Gambro, Sweden). Each dialysis session lasted four hours.

The day before the first dialysis of the study (Day 0), blood (53 ml in 7 ml ACD-A) was collected, the platelets isolated and labeled with ^{111}In -tropolone [19] and reinjected. One day later (Day 1), dialysis with heparin was done. It was administered according to the protocol in use at the Dialysis Unit. The total intra-dialysis heparin dose during the four-hour dialysis session varied between 5,000 and 10,000 IU. Two days later (Day 3), the second dialysis was done with r-hirudin. A single bolus dose of r-hirudin, 0.15 mg/kg dissolved in sterile pyrogen-free water, was given at the start of blood flow through the extracorporeal circuit. The heparin and r-hirudin were infused into the arterial side of the extracorporeal circuit, between the pump and the artificial kidney.

In a dose finding study dosages of 0.08 mg/kg ($N = 2$) and 0.12 mg/kg ($N = 2$) r-hirudin was found to be inefficient to prevent occlusion.

Study variables

Platelet accumulation at the inlet of the artificial kidney. The accumulation of ^{111}In -labeled platelets was measured with a NaI-scintillation probe. The probe was fixed in a position at the inlet of the dialyzer. The radioactivity counts were expressed as the increase over blood radioactivity (baseline). The latter was measured after the first minute of blood flow through the dialyzer.

Laboratory analyses

Blood was collected at the arterial side of the extracorporeal system. Serum levels of creatinine, urea and uric acid were determined using a Technicon DAX analyzer (Bayer Diagnostics, Basingstoke, UK). The platelet count and haematocrit were determined with a Technicon H * 1 blood cell analyzer (Bayer Diagnostics) on blood collected in K $_3$ EDTA. Blood was collected in 3.2% tri-sodium citrate (9:1 vol/vol) for the coagulation and *ex vivo* platelet aggregation studies. The activated partial thromboplastin time (APTT; Actin FS, Dade, Miami, FL, USA) and thrombin time (TT; bovine thrombin; Dade) were measured with a Cobas Fibro semi-automated optical system (Roche, Switzerland) [20]. The fibrinogen levels (derived fibrinogen) were measured with an Automated Coagulation Laboratory instrument (Instrumentation Laboratory, Italy) according to the instructions of the manufacturers [21]. The plasma antithrombin III (AT III) levels were measured by means of a standard chromogenic assay kit (Instrumentation Laboratory) with an Automated Coagulation Laboratory instrument (Instrumentation Laboratory) [22]. *Ex vivo*

aggregation of platelets in response to ADP (0.5, 1.0, 1.5, 2.0, 10, and 20 µM) and epinephrine (10 µM) was measured as described in detail [23]. Spontaneous platelet aggregation was also measured. The circulating platelet aggregate ratio (CPAR) was determined as described [24]. The plasma levels of heparin and r-hirudin were determined in blood collected in 3.2% and 3.8% tri-sodium citrate (9:1 vol/vol), respectively. The concentration of heparin in the plasma and dialysate was determined with a chromogenic assay kit (Instrumentation Laboratory) using the Automated Coagulation Laboratory instrument. [25] The plasma and dialysate concentrations of r-hirudin were determined with a chromogenic substrate method [26].

Statistics

The effect of the specific anticoagulant on a variable within a treatment regimen was determined by calculating the mean difference (post-dialysis value – pre-dialysis value), and the 95% confidence interval for the difference. The difference in effect between treatments was determined by subtracting the change during dialysis with r-hirudin from that measured during dialysis with heparin. The 95% confidence interval for this difference was calculated to compare the effect of the anticoagulants during dialysis. The areas under the ^{111}In -labeled platelet accumulation curves were calculated. The mean effects of the anticoagulant, reflected by the relative sizes of the areas, were compared by analysis of variance (ANOVA). The corresponding 95% confidence interval was calculated for the difference between treatments.

A double (hirudin) or single (heparin) exponential function was fitted to the r-hirudin and heparin concentration in time profiles of each individual to determine the plasma distribution and elimination half-lives.

The results are given as a mean ± 1 SD if the results were normally distributed. If not, the median and range are given.

Results

Biochemistry

The mean decrease in urea, uric acid and creatinine after dialysis, and the efficacy of dialysis with heparin and r-hirudin are summarized in Table 1. Dialysis with r-hirudin was as effective as dialysis with heparin since none of the values differed significantly ($P > 0.05$).

Coagulation and plasma levels of heparin and r-hirudin

The changes in activated partial thromboplastin time (APTT) and thrombin time (TT) during dialysis with heparin and r-hirudin, respectively, are summarized in Table 2. Plasma levels of fibrinogen and AT III are given in Table 3. The difference between the decrease in AT III levels during dialysis with heparin,

Table 2. Activated partial thromboplastin time (APTT) and thrombin time (TT) during dialysis with heparin and r-hirudin

	Pre-dialysis	10 min	60 min	120 min	180 min	240 min
APTT, seconds						
Hirudin	30 to 38	65 to 103	53 to 82	51 to 75	48 to 76	46 to 70
Heparin	30 to 39	> 120	> 120	58 to > 120	36 to > 120	28 to > 120
TT, seconds						
Hirudin	21 to 36	> 180	> 180	> 180	> 180	> 180
Heparin	24 to 38	> 180	> 180	> 180	55 to > 180	35 to > 180

Values are given as a range at each time interval.

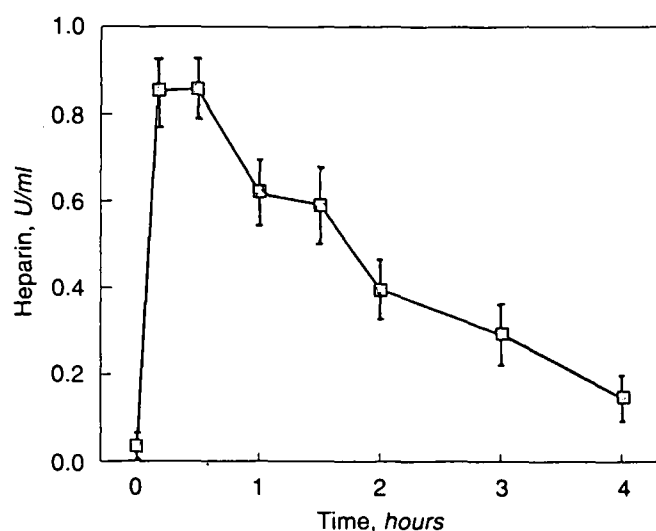
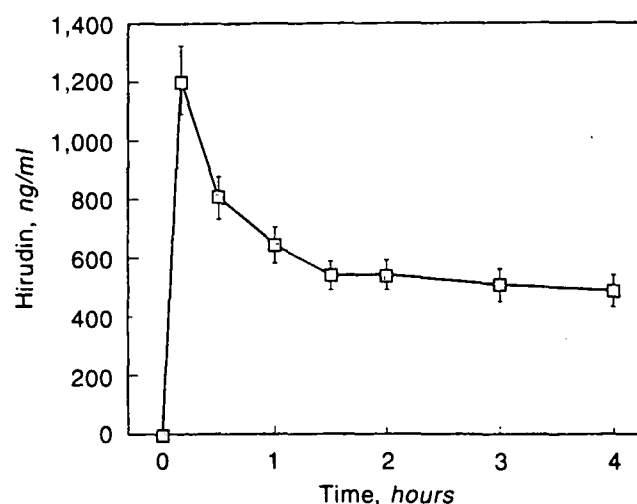
Table 3. Changes in fibrinogen and antithrombin III levels during hemodialysis

	Heparin			Hirudin			^b Hep - Hir $\pm$ 1 SD	95% CI
	Pre $\pm$ SD	^a MD $\pm$ 1 SD	95% CI	Pre $\pm$ 1 SD	^a MD $\pm$ 1 SD	95% CI		
Fibrinogen, g/liter	4.0 $\pm$ 1.5	0.4 $\pm$ 0.7	-0.1; 0.8	3.7 $\pm$ 1.2	0.2 $\pm$ 0.4	-0.1; 0.5	-0.2 $\pm$ 0.6	-0.5; 0.2
AT III, %	83 $\pm$ 20	-7 $\pm$ 27	-25; 11	84 $\pm$ 23	12 $\pm$ 23	-3; 29	20 $\pm$ 17	8; 31 ^c

^a The effect of the anticoagulant on a variable within a treatment regimen was determined by calculating the mean difference (MD; post-dialysis value minus pre-dialysis value) and the 95% confidence interval (CI).

^b The difference between treatments was determined by subtracting the change during dialysis with r-hirudin (Hir) from that measured during dialysis with heparin (Hep). The 95% CI for this difference was also calculated to compare the anticoagulants during dialysis.

^c $P \leq 0.05$

**Fig. 1.** Changes in the plasma concentrations of heparin during the four hours of dialysis. Results are given as a mean $\pm$ 1 SEM.**Fig. 2.** Changes in the plasma concentrations of r-hirudin during the four hours of dialysis. Results are given as a mean $\pm$ 1 SEM.

and the increase during dialysis with r-hirudin, was significant ($P < 0.05$).

The changes in the plasma levels of heparin and r-hirudin are summarized in Figures 1 and 2, respectively. The half-life of heparin in the seven patients who did not receive maintenance doses was 66 minutes (median; range from 42 to 225 min). The distribution half-life of r-hirudin was 11 minutes (median; range from 5 to 24 min) and the apparent elimination half-life was nine hours (median; range from 2 to 107 hr). In eight of the patients the elimination half-life ranged from 2 to 12 hours. In the remaining three it was 20, 36 and 107 hours, respectively. No r-hirudin or heparin was measured in the dialysate.

Accumulation of ¹¹¹In-labeled platelets

The accumulation of ¹¹¹In-labeled platelets at the inlet of the artificial kidney is given in Figure 3. The median for the areas

under the curves (AUC) for r-hirudin (0.66 hr $\times$ change from baseline; range -0.22 to 3.88) was smaller than that for heparin (1.22 hr $\times$ change from baseline; range -0.36 to 8.09). The 95% confidence interval (CI) for the difference between treatments was from 1.0 to 2.49 hours $\times$ change from baseline.

Hematological measurements

Neither the platelet count nor the hematocrit changed significantly within or between treatments.

Platelet function studies

The results are summarized in Table 4. There were no significant differences within or between treatment regimens. However, platelet aggregation in response to the higher concentrations of ADP tended to be slightly inhibited after dialysis with heparin, but not after dialysis with r-hirudin. The finding that there were no

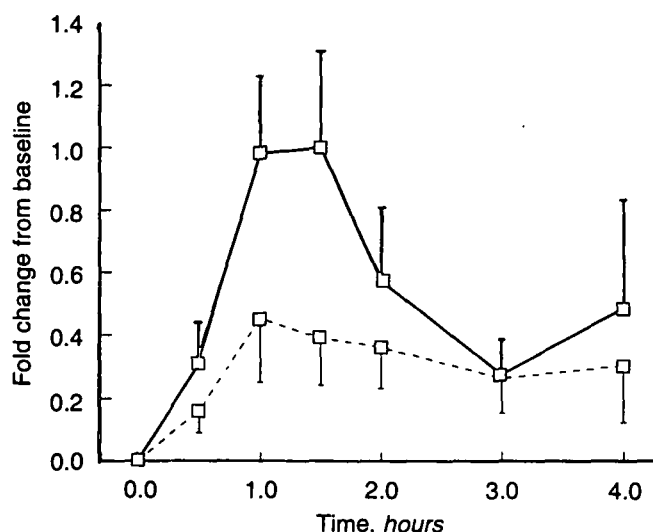


Fig. 3. The relative accumulation of ^{111}In -labeled platelets, expressed as the fold increase from baseline at the inlet of the artificial kidney during dialysis with heparin (—□—) and r-hirudin (---□---), respectively. Results are given as a mean $\pm$ 1 SEM.

increases in circulating platelet aggregates is surprising, but might have been influenced by the site of sample collection.

Discussion

This study in patients clearly demonstrates that r-hirudin can be used successfully as anticoagulant during hemodialysis. The percent decrease in plasma creatinine, urea and uric acid were equivalent for both anticoagulants, indicating that dialysis with r-hirudin was as effective as dialysis with heparin. When compared to heparin, effective dialysis with r-hirudin was achieved at a shorter APTT (Table 2). This is of particular importance since a prolonged APTT is associated with an increased risk of bleeding [26, 27]. The changes observed in the TT is probably of no clinical relevance because the TT is an extremely sensitive test of thrombin inhibition. Therefore it is not indicative of the anticoagulation status or of a bleeding risk [28, 29].

The longer elimination half-life of r-hirudin is a matter of concern. However, one must take into account that it is perhaps not ideal to derive the long half-lives from the course of plasma levels determined from samples collected only for four hours. Our results does compare favorably with that measured in patients with chronic renal failure and chronic renal disease [18], and is considerably longer than that measured in normal humans and animals [30–34]. The prolonged half-life provides a long-lasting anticoagulant effect and the potential for drug accumulation. This needs to be investigated. The reason for the large variation in elimination times of r-hirudin from the plasma of individual patients is not clear. There was no correlation between the elimination times and the baseline creatinine, urea or uric acid values. The large variation may require that the dosages be individually adjusted when repeated treatments are given. A possible solution may be to treat the dialyzer and not the patient, in which case a dialyzer with a larger cut-off value will have to be used, together with a constant infusion of r-hirudin at the inlet of the dialyzer. In this study the dialyzers had a cut-off value of 5 kD which prevented the removal of r-hirudin by the dialysis process.

The elimination half-life of heparin (90 min) was similar to that measured in normal humans [35, 36]. Heparin is partially depolymerized by the cells of the monocyte-macrophage system and the degradation products excreted by the kidneys [36, 37]. The potential for accumulation of degradation products may therefore explain, at least in part, some of the complications of the long-term use of heparin [4–8].

Markedly less ^{111}In -labeled platelets accumulated at the inlet of the artificial kidney during dialysis with r-hirudin. Excessive platelet deposition, especially during the first three hours of dialysis, was prevented by r-hirudin (Fig. 3). This may have resulted in a smaller loss of hollow fiber volume because there is a strong relationship between the number of platelets deposited and loss of volume [38]. In this case dialysis with r-hirudin could have been more effective during this time. Unfortunately we did not measure it. The fact that deposition of platelets after three hours of dialysis were similar for both anticoagulants suggests that a minimum number of platelets will always accumulate in the artificial kidney. This assumption is supported by the findings that D-Phe-Pro-Arg CH_2Cl , a synthetic and direct thrombin inhibitor, completely prevents platelet deposition onto native Dacron vascular graft material [38], but was not able to do the same in artificial kidneys [39].

There are several possible reasons why heparin is not as effective as hirudin in inhibiting arterial thrombus formation. These include: (a) heparin may contain fractions that activate platelets directly, thereby promoting thrombogenesis [40]; (b) activated platelets release proteins, such as platelet factor 4 and beta-thromboglobulin, that can inactivate heparin locally at the surface of thrombi [41]; (c) the inactivation of thrombin bound to other components in a thrombus may be prevented by steric or ionic hindrance of the heparin-AT III complex [42]; and (d) platelets within a thrombus may be activated by meizothrombin which is not inactivated by the heparin-AT III complex [43].

The AT III levels decreased during dialysis with heparin and increased during dialysis with r-hirudin. The decrease after heparin dialysis is not surprising and can be attributed to consumption of AT III by heparin to inactivate thrombin [44]. However, the decrease was not substantial and is possibly of little clinical significance. Moreover, the decrease was transient since the AT III levels before dialysis with r-hirudin were similar to that before dialysis with heparin. The increase in AT III levels after dialysis with r-hirudin is difficult to explain. It cannot result from hemoconcentration [45] since the hematocrit did not change markedly. AT III is an acute phase protein. The slight increase during dialysis with r-hirudin may indicate that there was a slight consumption of AT III. That can be explained by the pre-phase enzyme activation of Factor IX, Factor X, and others. When r-hirudin is used, these factors are activated and bound by AT III.

To our knowledge only one study investigated the use of r-hirudin during hemodialysis in humans [17]. In that study a dose of 0.08 mg/kg was effective to prevent occlusion of the extracorporeal circuit. In our dose finding study this dose and even a higher dose of 0.12 mg/kg was too low to prevent thrombus formation in the extracorporeal circuit. We therefore used a dose of 0.15 mg/kg.

In conclusion, this study demonstrates the feasibility of using r-hirudin in patients undergoing hemodialysis. The results indicate that an intravenous bolus of 0.15 mg/kg r-hirudin was safe and effective. Since it is administered as a single pre-dialysis dose,

Table 4. Changes in the circulating platelet aggregate ratio (CPAR) and platelet aggregation during hemodialysis

	Heparin			Hirudin			^b Hep - Hir $\pm$ 1 SD	95% CI
	Pre $\pm$ 1 SD	^a MD $\pm$ 1 SD	95% CI	Pre $\pm$ 1 SD	^a MD $\pm$ 1 SD	95% CI		
CPAR	0.81 $\pm$ 0.14	0.00 $\pm$ 0.11	-0.07; 0.07	0.78 $\pm$ 0.12	0.05 $\pm$ 0.13	-0.04; 0.13	0.04 $\pm$ 0.19	-0.08; 0.17
Aggregation, %								
Spontaneous	4.6 $\pm$ 1.6	1.0 $\pm$ 1.7	-0.03; 2.2	4.5 $\pm$ 2.0	0.6 $\pm$ 2.7	-1.3; 2.5	-0.4 $\pm$ 3.5	-2.9; 2.2
Adrenalin 10 μ M	55.9 $\pm$ 9.6	-3.2 $\pm$ 10.5	-1.07; 4.3	58.9 $\pm$ 8.4	-7.3 $\pm$ 16.0	-18.7; 4.1	-4.1 $\pm$ 18.5	-17.3; 9.1
ADP: 0.5 μ M	9.0 $\pm$ 5.6	2.2 $\pm$ 3.9	-0.8; 5.2	15.9 $\pm$ 14.8	-4.5 $\pm$ 6.9	-8.6; 3.6	-3.8 $\pm$ 6.3	-9.1; 1.5
1.0 μ M	26.9 $\pm$ 16.2	-4.5 $\pm$ 17.2	-16.8; 7.8	25.1 $\pm$ 15.7	-4.0 $\pm$ 10.1	-11.2; 3.2	0.5 $\pm$ 23.9	-16.6; 17.6
1.5 μ M	46.5 $\pm$ 16.4	10.0 $\pm$ 19.0	-23.7; 3.5	44.6 $\pm$ 13.5	0.0 $\pm$ 19.5	-13.9; 13.9	10.1 $\pm$ 16.6	-2.0; 22.1
2.0 μ M	52.6 $\pm$ 16.2	-6.9 $\pm$ 13.4	-16.5; 2.7	52.0 $\pm$ 10.8	-1.0 $\pm$ 16.9	-13.1; 11.1	5.9 $\pm$ 20.7	-8.9; 20.7
10.0 μ M	61.6 $\pm$ 9.8	-5.8 $\pm$ 9.1	-12.2; 0.8	61.6 $\pm$ 10.7	1.8 $\pm$ 12.1	-6.9; 10.5	7.6 $\pm$ 15.4	-4.5; 17.6
20.0 μ M	63.0 $\pm$ 9.3	-3.6 $\pm$ 8.5	-9.7; 2.5	65.4 $\pm$ 12.4	2.9 $\pm$ 12.1	-5.7; 11.6	6.6 $\pm$ 15.4	-4.5; 17.6

^a The effect of the anticoagulant on a variable within a treatment regimen was determined by calculating the mean difference (MD; post-dialysis value minus pre-dialysis value) and the 95% confidence interval (CI).

^b The difference between treatments was determined by subtracting the change during dialysis with r-hirudin (Hir) from that measured during dialysis with heparin (Hep). The 95% CI for this difference was also calculated to compare the anticoagulants during dialysis.

it will simplify the dialysis procedure. The shorter APTT indicates a reduced risk of developing a bleeding tendency. The fact that less platelets accumulated at the inlet of the dialyzer indicates that the dose was sufficient to protect against thrombus formation. In addition no side effects, bleeding complications or thrombotic events occurred with the use of r-hirudin. These findings, together with the fact that r-hirudin can be used successfully in patients with an AT III deficiency and in patients with heparin-induced thrombocytopenia [35], indicate that r-hirudin may be a suitable and better alternative for heparin as anticoagulant. In this study r-hirudin was used in only one dialysis session in each of a relatively small number of patients. Therefore, final conclusions and recommendations cannot be made. It will be worthwhile to investigate the use of r-hirudin in a larger number of patients who are undergoing serial dialysis two to three times per week.

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The effect of r-hirudin vs. heparin on blood-membrane interactions during hemodialysis

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Abstract. The aim of this study was to determine if recombinant (r-)hirudin, used as anticoagulant during hemodialysis, has favourable effects on blood-membrane interactions. The results were compared with that obtained when standard unfractionated heparin, the anticoagulant of choice, was used. **Material and methods:** Eleven patients with chronic renal failure and on maintenance hemodialysis were included in this open cross-over study. Heparin was administered according to the existing protocol in use at the Dialysis Unit during the first dialysis of the study (5,000 to 10,000 IU). r-Hirudin, 0.15 mg/kg, was given as a bolus at the start of the second dialysis, two days later. The effect of the anticoagulant on leukocyte and complement activation, thrombogenesis, release of platelet activating factor and pulmonary gas exchange was studied. **Results:** In most cases after dialysis with heparin (8 of 11), but not with r-hirudin, macroscopically visible thrombi formed at the inlet of the artificial kidneys. Irrespective of the anticoagulant used, a transient leukopenia (neutropenia) developed ten minutes after dialysis was started. Heparin anticoagulation resulted in a significant increase in plasma levels of complement C3a at all time points, whereas with r-hirudin the increase was significant after only 30 and 240 min. The O₂ saturation decreased significantly during the first two hours of dialysis with heparin. The partial O₂ pressure decreased significantly during the first two hours of dialysis, irrespective of the anticoagulant used. **Conclusions:** We conclude that r-hirudin is superior to heparin with regard to inhibition of thrombus formation in the dialyzer during hemodialysis. Slight, but favourable effects on complement activation, O₂ saturation and lung CO diffusing capacity were also found with r-hirudin.

Key words: hemodialysis – recombinant hirudin – heparin – platelets – complement activation

Introduction

The artificial membranes of the extracorporeal circuit which are used during hemodialysis are all bio-incompatible to variable degrees. This causes blood-membrane interactions which results in activation of especially the coagulation cascade and the alternative pathway of the complement system [Andrade et al. 1981, Hakim et al. 1984]. Activation of the complement system can cause an increased margination and sequestration of neutrophils in the pulmonary capillaries, which results in a marked but transient leukopenia during the first two to ten minutes of dialysis [Jensen et al. 1973, Kelly et al. 1989]. This, together with trapping of micro-aggregates in the pulmonary bed, as a result of activation of the

coagulation cascade, may cause dialysis-induced pulmonary dysfunction [Biscel et al. 1975, Craddock et al. 1977, Mahajan et al. 1977, Davenport and Williams 1988].

An anticoagulant is used to reduce these blood-membrane interactions and to ensure safe and effective dialysis. Standard unfractionated heparin is most commonly used. Its action is mainly through potentiation of its cofactor, antithrombin III, which in turn binds to and inhibits thrombin [Rosenberg 1978]. However, heparin is neither entirely safe [Swartz 1981, Rupp et al. 1982] nor is it totally effective in reducing membrane-blood interactions [Ansell and Deykin 1980, Kelly et al. 1989, Schulman 1993]. An alternative more effective and perhaps safer anticoagulant would therefore be beneficial.

Hirudin, a potent direct and specific inhibitor of thrombin, was first isolated from the salivary glands of the medicinal leech and is now made by recombinant technology [Markwardt 1991]. r-Hirudin has several advantages over heparin. It does not need a cofactor to express its activity, it has a shorter half-life, and it has the

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ability to bind to and inactivate thrombus bound thrombin [Weitz et al. 1990]. r-Hirudin also prevents heparin-resistant arterial-type thrombosis when used in large enough dosages [Kelly et al. 1991]. No serious side effects due to its use have been documented. Its effectiveness as anticoagulant during hemodialysis has been shown in the dog [Bucha et al. 1990] and man [Vanholder et al. 1994, Van Wyk et al. 1995].

The objective of this study was to determine whether r-hirudin has more favourable effects, possibly superior to that of heparin, on the blood-membrane interactions, subsequent activation processes and adverse downstream events, during hemodialysis in humans.

Materials and methods

Subjects: We included eleven patients (median age 37 years, range 22 to 51), with chronic renal failure (creatinine clearance <10 ml/min) and on maintenance hemodialysis. The patients gave informed consent to participate in this study which was approved by the Ethics Committee for Clinical Studies of the University of the Free State. The use of r-hirudin was approved by the South African Medicines Control Council. All patients refrained from taking drugs that may influence coagulation and platelet function for at least 10 days prior to, and during the study.

Study design: It was an open cross-over study with two consecutive dialysis procedures. The first dialysis was done with heparin from porcine intestinal mucosa (Heparin Sodium, Labethica, SA) as anticoagulant. It was administered according to the existing protocol in use at the Dialysis Unit, i.e. an initial bolus infusion when blood flow through the extracorporeal circuit was started, followed by maintenance doses if necessary [Lindsay 1983]. The initial dose of heparin was calculated from the body mass of the patient to prolong the activated clotting time (ACT) to 80% longer than normal. The ACT was monitored on site and maintenance infusions were given as a bolus if necessary. Two days later, the second dialysis was done with r-hirudin, 0.15 mg/kg (HBW 023, Hoechst AG, Frankfurt and Behringwerke AG, Marburg, Germany) dissolved in sterile pyrogen-free water. It was given as a bolus simultaneous with the start of blood flow through the extracorporeal circuit. The heparin and r-hirudin were infused into the arterial side of the extracorporeal circuit, between the pump and the artificial kidney. r-Hirudin has a prolonged elimination in patients with end-stage renal failure [Nowak et al. 1992, Van Wyk et al. 1995]. Therefore, to avoid possible effects of residual r-hirudin, it was administered during the second dialysis of the study. Cuprammonium rayon type artificial kidneys (Terumo, Japan) were used once and then discarded. Dialysis was done against an acetate buffer on a Gambro AK 10 artificial kidney machine (Gambro, Sweden). Each dialysis session lasted four hours.

Study variables: Blood was collected at the arterial side of the extracorporeal system at various time intervals during dialysis. Serum levels of creatinine, urea and uric acid were determined before and after dialysis, using a Technicon DAX analyzer (Bayer Diagnostics, Basingstoke, UK). The plasma levels of heparin and r-hirudin and the leukocyte and neutrophil counts were determined before dialysis and again at 10, 60, 120, 180 and 240 min after the start of dialysis. Heparin concentrations were determined in blood collected in 3.2% tri-sodium citrate (9:1 vol/vol) with a chromogenic assay kit (Instrumentation Laboratory) using the Automated Coagulation Instrument [Larsen et al. 1978]. r-Hirudin concentrations were determined in blood collected in 3.8% tri-sodium citrate (9:1 vol/vol) using the chromogenic substrate method [Grötsch et al. 1991]. The leukocyte and neutrophil counts were determined on blood collected in K₃EDTA using a Technicon H*1 blood cell analyzer (Bayer Diagnostics, Basingstoke, UK). Blood samples were also collected before dialysis and at 10, 30 and 240 min in complement sample collection tubes (C3a_{desArg}, Amersham, UK). Plasma concentrations of complement C3a_{desArg} were measured with an ¹²⁵I assay system according to the specifications of the manufacturers. Blood samples to determine the concentrations of platelet activating factor (PAF) were collected at the same time intervals as those for complement measurement. The preparation of these blood samples was done as described [Yamada et al. 1986]. A platelet activating factor ³H-scintillation proximity assay system (Amersham, UK) was used to determine the plasma levels of PAF.

The arterial oxygen saturation level and partial pressures of O₂ and CO₂ (pO₂ and pCO₂) were measured in heparin anticoagulated blood before dialysis and at 120 and 240 min using an ABL 500 instrument (Radiometer, Copenhagen). Lung function tests were measured before and after dialysis with a Medical Graphic 1070 lung function system (Medical Graphics Corporation, USA). Measurements conformed to the standards set by the American Thoracic Society [Gardner et al. 1987]. The flow volume loop was measured by using the forced spirometric technique, and the single breath technique was used to measure the CO diffusing capacity [Wanger 1992].

At the end of each dialysis session the dialyzer was removed and opened at the inflow side. The thrombi, if present, were carefully removed, fixed in a 3% 0.2 M phosphate buffered glutaraldehyde solution (pH 7.2) and viewed with an electron microscope (Philips Transmission Electron Microscope, EM 301) at 60 kV.

Statistics: The effect of the specific anticoagulant on a variable within a treatment regimen was determined by calculating the mean difference (MD; post-dialysis value minus pre-dialysis value), and the 95% confidence interval (CI) for the difference. The difference in effect between treatments was determined by subtracting the

change during dialysis with r-hirudin (Hir) from that measured during dialysis with heparin (Hep). The 95% confidence interval for this difference was calculated to compare the effect of the anticoagulants.

Results

Creatinine, urea and uric acid: The baseline serum levels of creatinine, urea and uric acid were $423 \pm 152 \mu\text{mol/l}$, $0.2 \pm 0.1 \text{ mmol/l}$ and $13.2 \pm 1.9 \text{ mmol/l}$ respectively. Dialysis efficiency, calculated as the per cent decrease in serum levels was $44 \pm 6\%$ for creatinine, $50 \pm 11\%$ for urea and $51 \pm 7\%$ for uric acid when heparin was used. The corresponding values for r-hirudin was $45 \pm 6\%$, $48 \pm 14\%$ and $50 \pm 7\%$ respectively. None of the values differed significantly.

Plasma levels of anticoagulant: The plasma levels of r-hirudin and heparin are summarised in Table 1.

Electron microscopy: Arterial type thrombi containing platelets, leukocytes and red blood cells were found at the inlet of 8 of 11 dialyzers after dialysis with heparin (Fig. 1). No deposits were seen after dialysis with r-hirudin.

Leukocytes: The changes in the leukocyte and neutrophil counts are summarised in Figure 2. During the first ten minutes of dialysis with heparin the leukocyte and neutrophil counts decreased by approximately 66% and 82% respectively. The corresponding decreases during dialysis with r-hirudin, 72% and 88%, were not significantly different from that found with heparin. The counts then increased to pre-dialysis values and remained so throughout dialysis.

Pulmonary gas exchange: The diffusing capacity of carbon monoxide (DLCO) decreased by $1.9 \pm 2.5 \text{ ml/min/mmHg}$ (95% CI from -4.2 to 0.4 ml/min/mmHg) during dialysis with heparin. During dialysis with r-hirudin the DLCO increased by $1.6 \pm 2.9 \text{ ml/min/mmHg}$ (95% CI from -1.1 to 4.3 ml/min/mmHg). These changes were not statistically significant within or between treatments. The other lung function tests that were measured were not affected and the results are therefore not given. The changes in O_2 saturation and partial pressures of O_2



Fig. 1 A representative electron micrograph of a thrombus that formed at the inlet of a dialyzer during dialysis with heparin. Platelets predominate and a few red blood cells and white blood cells can be seen (magnification $\times 6850$).

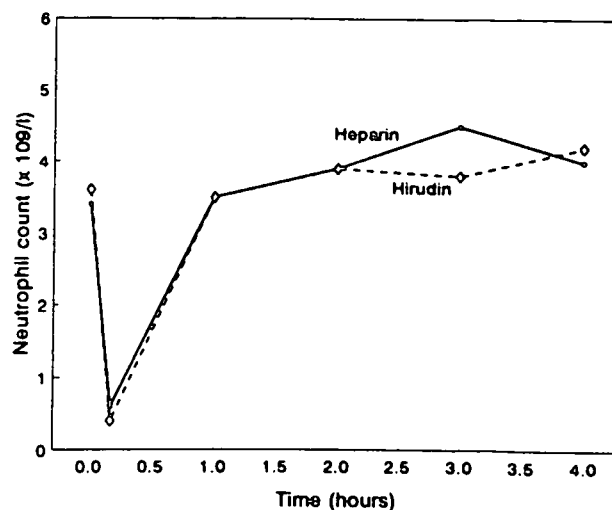
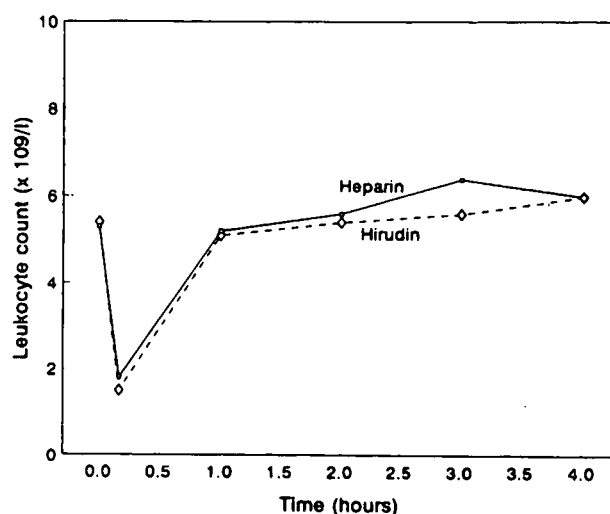


Fig. 2 The mean changes in the leukocyte and neutrophil counts during dialysis with heparin and r-hirudin respectively.

Table 1 Plasma levels of heparin and r-hirudin during dialysis.

	Heparin (U/ml)	r-Hirudin (ng/ml)
Pre-dialysis	0.00	<15
10 min	0.85 ± 0.28	1208 ± 388
60 min	0.62 ± 0.25	645 ± 207
120 min	0.40 ± 0.22	543 ± 168
180 min	0.29 ± 0.23	507 ± 187
240 min	0.15 ± 0.17	487 ± 183

Table 2 Changes in oxygen saturation, partial oxygen (pO₂) and -carbondioxide (pCO₂) pressures during dialysis with heparin and r-hirudin respectively.

	Heparin			r-Hirudin			^b Hep – Hir	
	^a MD ± 1 SD	95% CI		^a MD ± 1 SD	95% CI		± 1 SD	95% CI
<i>O₂ saturation (%)</i>								
Pre-dialysis	95.0 ± 3.0			95.0 ± 3.0				
120 minutes	94.0 ± 3.0	-1.2 ± 1.1	-1.9; -0.5*	95.0 ± 4.0	-0.4 ± 3.9	-3.0; 2.2	-0.8 ± 4.0	-3.7; 2.1
240 minutes	95.0 ± 3.0	0.0 ± 1.3	-0.9; 0.9	93.0 ± 5.0	-1.7 ± 4.1	-4.5; 1.1	1.8 ± 4.4	-5.6; 9.2
<i>pO₂ (mmHg)</i>								
Pre-dialysis	86.7 ± 18.3			83.3 ± 18.3				
120 minutes	74.7 ± 12.9	-12.1 ± 9.8	-18.7; -5.5*	78.7 ± 16.5	-9.9 ± 12.8 ^c	-19.2; -0.6*	-3.0 ± 5.0	-13.4; 7.4
240 minutes	80.7 ± 12.8	-6.0 ± 10.4	-13.0; 1.0	73.6 ± 17.9	-9.7 ± 16.3	-20.7; 1.3	3.7 ± 19.6	-9.5; 16.8
<i>pCO₂ (mmHg)</i>								
Pre-dialysis	29.8 ± 4.9			31.7 ± 4.4				
120 minutes	30.2 ± 4.3	0.4 ± 2.6	-1.3; 2.1	30.9 ± 3.9	-0.9 ± 2.5	-2.6; 0.8	1.3 ± 2.2	-0.2; 2.8
240 minutes	29.3 ± 3.3	-0.5 ± 2.7	-2.3; 1.3	31.4 ± 4.5	-0.4 ± 3.8	-2.9; 2.1	-0.1 ± 3.3	-2.3; 2.1

^a The effect of the anticoagulant on a variable within a treatment regimen was determined by calculating the mean difference (MD; 120 or 240 minutes value minus pre-dialysis value) and the 95% confidence interval (CI);

^b The difference between treatments was determined by subtracting the change during dialysis with r-hirudin (Hir) from that measured during dialysis with heparin (Hep). The 95% CI for this difference was also calculated to compare the anticoagulants during dialysis;

^c One outlier excluded in statistical analyses

*: $p \leq 0.05$ (vs. pre-dialysis)

and CO₂ are summarized in Table 2. The pCO₂ was not significantly affected by dialysis or the anticoagulant used. The O₂ saturation decreased significantly during the first two hours of dialysis with heparin, but not with

r-hirudin. The pO₂ decreased significantly during the first 2 hours of dialysis, irrespective of the anticoagulant used. There were no significant differences between treatments.

Table 3 Changes in complement (C3a_{derivate}) and platelet activating factor (PAF) during dialysis with heparin and r-hirudin respectively.

	Heparin			r-Hirudin			^b Hep – Hir	
	^a MD ± 1 SD	95% CI		^a MD ± 1 SD	95% CI		± 1 SD	95% CI
<i>C3a_{derivate} (μg/ml)</i>								
Pre-dialysis	0.45 ± 0.14			0.53 ± 0.29				
10 minutes	0.97 ± 0.26	0.51 ± 0.27	0.32; 0.70*	0.84 ± 0.44	0.29 ± 0.54	–0.09; 0.67	0.21 ± 0.52	–0.16; 0.58
30 minutes	0.85 ± 0.33	0.40 ± 0.40	0.11; 0.69*	0.92 ± 0.28	0.38 ± 0.33	0.15; 0.61*	0.02 ± 0.40	–0.27; 0.31
240 minutes	0.60 ± 0.10	0.15 ± 0.13	0.06; 0.24*	0.73 ± 0.19	0.20 ± 0.28	0.01; 0.39*	–0.05 ± 0.30	–0.25; 0.15
<i>PAF (pg/ml)</i>								
Pre-dialysis	265 ± 101			261 ± 304				
10 minutes	181 ± 149	75 ± 160	–197; 48	203 ± 187	–59 ± 277	–272; 155	–16 ± 330	–272; 261
30 minutes	240 ± 160	16 ± 176	–155; 123	181 ± 181	–75 ± 325	–325; 176	59 ± 405	–251; 368
240 minutes	261 ± 213	5 ± 256	–192; 203	213 ± 149	–48 ± 336	–314; 219	53 ± 389	–261; 352

^a The effect of the anticoagulant on a variable within a treatment regimen was determined by calculating the mean difference (MD; 10, 30 or 240 minutes value minus pre-dialysis value) and the 95% confidence interval (CI);

^b The difference between treatments was determined by subtracting the change during dialysis with r-hirudin (Hir) from that measured during dialysis with heparin (Hep). The 95% CI for this difference was also calculated to compare the anticoagulants during dialysis;

^c One outlier excluded in statistical analyses

*: $p \leq 0.05$ (vs. pre-dialysis)

Complement activation and platelet factor: The changes in the plasma complement $C3a_{desArg}$ and PAF levels are given in Table 3. Heparin anticoagulation resulted in a significant increase in $C3a_{desArg}$ at all time intervals. With r-hirudin the increase was significant only after 30 and 240 min. The circulating PAF levels decreased ten minutes after dialysis was started, irrespective of the anticoagulant used. During dialysis with heparin it then increased to approximate pre-dialysis levels. With r-hirudin it decreased even more after 30 min. Thereafter it also increased but tended to remain low. None of these changes were significant.

Discussion

In a previous study [Van Wyk et al. 1995] we showed that a bolus injection of 0.15 mg/kg r-hirudin was sufficient to permit clinically effective dialysis at a markedly shorter activated partial thromboplastin time than with heparin. This implies that dialysis was performed at a lower risk of bleeding [Swartz 1981]. With this study we compared the effect of r-hirudin (0.15 mg/kg) with that of heparin on blood-membrane interactions during hemodialysis. Macroscopically visible thrombi formed at the inlet of the artificial kidney during dialysis with heparin (Fig. 1). This was not observed when r-hirudin was used. Thus, even though total occlusion was prevented by the heparin administered, it was not enough to fully prevent activation of coagulation and of platelets. These results are not unique. Similar results have been reported previously [Bischel et al. 1975, Lindsay and Clark 1982]. It is important to note that high shear conditions (high blood flow rate) exist in the extracorporeal circuit. This results in platelet-dependent arterial type thrombus formation. It is known that dosages of heparin which are adequate to inhibit coagulation do not affect platelet-dependent thrombosis [Kelly et al. 1989, 1991]. Thrombus formation can result in occlusion of the fibre bundle and subsequent loss of hollow fibre volume in the dialyzer with the resultant loss of surface area available for dialysis [Kelly et al. 1989]. Furthermore, embolization of the thrombi could cause adverse downstream effects [Bischel et al. 1975].

The pO_2 pressure decreased during both heparin and r-hirudin dialysis (Table 2). This can possibly be explained by the fact that an acetate dialysate buffer was used. It is known that the acetate buffer, due to its chemical nature, causes a loss of CO_2 through the dialyser. This can result in hypoventilation in an attempt to maintain the arterial pressure of CO_2 . In such cases the hypoventilation will decrease the pO_2 [Blanchet et al. 1984]. It is possible that dialysis with a bicarbonate dialysate could prevent this effect [Blanchet et al. 1984]. There was a slight decrease in DLCO during dialysis with heparin.

This could possibly be due to trapping of micro-aggregates in the pulmonary capillaries [Bischel et al. 1975], although we have no proof that it happened. However, this can possibly in part explain why the O_2 -saturation decreased during dialysis with heparin and not with r-hirudin. A decrease in DLCO has previously been ascribed to pulmonary leukocyte sequestration and complement activation [Blancet et al. 1984, Vanholder et al. 1987]. Our results do not fully agree with such a hypothesis, since an early transient neutropenia occurred with both anticoagulants (Fig. 2), while comparable results for the DLCO with heparin and r-hirudin were not found. The inability to observe changes in the other lung function tests was possibly due to the fact that it was only measured before and after dialysis. It was not possible to repeat these measurements during the dialysis procedure. Even though, from a clinical point of view, the changes in the pulmonary function seem insufficient to cause symptoms, it may have significant effects in patients with preexisting marginal cardiopulmonary dysfunction [Brautbar et al. 1980].

Hemodialysis with both heparin and r-hirudin was associated with a precipitous but short lasting fall in the peripheral leukocyte count ten minutes after the start of dialysis. Differential counts indicated that this decrease was largely due to a decrease in the neutrophil count (Fig. 2). The neutropenia indicates a certain initial level of bioincompatibility of the dialyzer membranes, and is not affected by the anticoagulant, since similar changes were observed for heparin and r-hirudin. Transient neutropenia is common during hemodialysis and may result from complement activation and subsequent margination of neutrophils to the vascular endothelium and adhesion to the artificial surfaces [Jensen et al. 1973, Dodd et al. 1983]. It has previously been argued that the hypoxia and hypoxemia during hemodialysis are caused by sequestration of leukocytes in the capillary bed of the lungs [Mahajan et al. 1977]. The results of this study may indicate that possible lung damage as a result of leukocyte sequestration did not induce a significant dysfunction in gas exchange by the lungs. Since intradialysis lung function was not measured, and the patient group was relatively small, final conclusions in this regard cannot be made.

Activation of the alternative pathway of the complement system [Hakim 1986] and the release and activity of platelet activating factor [Sakaguchi et al. 1991] are factors which may play a role in neutrophil activation during hemodialysis. The changes in the plasma concentrations of $C3a_{desArg}$ (Table 3) were almost mirror images of the changes in the circulating neutrophil count in both treatment regimes. This suggests that activation of the alternative pathway of the complement system may play a role in the activation of the neutrophils and subsequent neutropenia. The use of r-Hirudin had an inhibiting effect on complement activation at the start of dialysis. It has

been reported that during the first minutes of hemodialysis an early release of PAF occurs [Tetta et al. 1993]. We could not confirm these findings (Table 3). Although there was a tendency for circulating PAF levels to decrease after the start of dialysis, it was not significant. The large variation in individual values further complicates the interpretation of these results.

We were able to show that r-hirudin is superior to heparin with regard to inhibition of thrombus formation in the dialyzer. Slight, but favourable effects on complement activation, lung CO diffusing capacity and O₂-saturation were also found when r-hirudin was used. Even though these effects were slight, they could be clinically relevant because in patients on chronic dialysis there is repetitive exposure of blood to the artificial dialysis membranes. Therefore even mild effects, on a chronic basis may be important. However, since only 11 patients were included in this cross-over study, and r-hirudin was administered during only one dialysis session on each patient, it is not possible to make final conclusions and recommendations. These results warrant investigation in a larger group of patients undergoing serial dialysis two to three times per week. The prolonged half-life of r-hirudin in patients with renal failure [Nowak et al. 1992, Van Wyk et al. 1995] will have to be taken into account when such studies are done. In future this problem could possibly be solved by neutralizing the residual r-hirudin with an antagonist at the end of the dialysis session.

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Abstract □ Lepirudin has a short half-life, and only 50–60% of the intravenously administered dose is excreted by the kidneys. The fate of the remainder is unknown. We designed a study to determine the fate of this lepirudin. In each of six baboons, [¹³¹I]lepirudin was given intravenously as a bolus or infused over 30 min, 24 h apart. The in vivo redistribution of [¹³¹I]lepirudin was determined and quantified by scintillation camera imaging. In all studies, the half-life of [¹³¹I]lepirudin, as determined from the disappearance of radioactivity, was 21 ± 3 min. The half-life determined from the disappearance of lepirudin, measured by the Ecarin Clotting Time (ECT) method, was similar at 23 ± 8 min. Results obtained with the labeled lepirudin are therefore comparable with those obtained using the plasma concentration of lepirudin. When lepirudin was administered as a bolus, the half-life was 18 ± 4 min, and lepirudin was cleared from the plasma at a rate of 42 ± 12 mL/min and by the kidneys at 23 ± 2 mL/min. Following infusion over 30 min, the half-life and total and renal clearances were not significantly different. In both studies, between 50 and 60% of the administered lepirudin was excreted by the kidney. Studies on sacrificed baboons showed that appreciable amounts of lepirudin were present in the bile, indicating the liver as a contributor to the elimination of lepirudin.

Introduction

Hirudin is regarded as the most potent direct inhibitor of thrombin and its recombinant form, lepirudin ([Ileu¹, Thr²]-63-disulfatohirudin), has the same physiochemical characteristics and biochemical properties as the native protein.^{1,2} Lepirudin is a 65 amino acid polypeptide (7 kDa) produced by transfected yeast cells.¹ The interaction between hirudin and thrombin results in the formation of a stable, noncovalent stoichiometric 1:1 complex that inhibits all functions of thrombin.^{1,2} Apart from its inhibition of coagulation, it also inhibits platelet-dependent arterial-type thrombosis when given in high enough dosages.^{3–5} Although the pharmacokinetics of lepirudin is well-known, there are still some unresolved issues. It is generally accepted that lepirudin is mainly excreted in an unchanged form by the kidneys.^{1,6} However, approximately 40 to 50% of injected protein cannot be accounted for in the urine. The sites of metabolism and/or uptake of this lepirudin are unknown. We designed this study to investigate the metabolism and fate of lepirudin in baboons.

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Subjects and Methods

Experimental Animals—Eight male baboons (*Papio ursinus*) supporting permanent femoral arteriovenous shunts (A-V shunts) of silicone rubber tubing^{7,8} were used. The animals were sedated with intramuscular ketamine hydrochloride (10 mg/kg body mass, Centaur Laboratories, South Africa) to enable handling. Anesthesia was maintained with subsequent administrations when necessary. One hundred milliliters of saline were given intravenously (iv) to each baboon 30 min before the start of a study to ensure that they were normovolaemic.

Experimental Protocol—Six baboons received [¹³¹I]lepirudin as an iv bolus of 0.3 mg/kg as well as an infusion at a rate of 0.01 mg/kg/min for 30 min on separate days. The treatment requirements were such that an equal number of baboons received the bolus or infusion on each day. The lepirudin (HBW 023, Hoechst AG, Frankfurt and Behringwerke AG, Marburg, Germany) was labeled with 131-iodine (¹³¹I; Radiochemical Centre, Amersham, UK) using the IODO-GEN method.⁹ Labeling efficiency was $98 \pm 1\%$. It is important to note that only labeled lepirudin was infused and that we determined the plasma concentration of the labeled lepirudin (see later). Therefore, when reference is made to lepirudin, it refers to the results obtained from the plasma concentration as determined using the ECT method.¹⁰ Similarly, reference to labeled lepirudin refers to results obtained from radioactive count rates of [¹³¹I]lepirudin.

Scintillation Camera Imaging—Imaging of the in vivo distribution of labeled lepirudin was done with a Large Field of View Scintillation Camera fitted with a high-energy collimator. Image analysis was done with an A³-MDS data processing system that was interfaced with the camera. Imaging was done in two phases; they are, a dynamic image acquisition phase and a static acquisition phase.

Dynamic Image Acquisition—The baboons were positioned in front of the detector of the camera so as to include the kidneys, bladder, liver, and spleen in the field of view. Dynamic image acquisition, consisting of 3-min images (64 × 64 word mode) for 120 min (infusion study) and 90 min (bolus study), started simultaneously with the infusion of [¹³¹I]lepirudin. For analysis, a region of interest was selected for the kidneys, the bladder, the spleen, and the liver, and the radioactivity in each region was expressed as a percentage of injected [¹³¹I]labeled lepirudin, which was estimated by the geometrical mean method of quantification (see Static Image Acquisition). The radioactive count rates of the left and right kidneys were summed to obtain total kidney radioactivity.

Static Image Acquisition and Quantification of In Vivo Distribution of [¹³¹I]Lepirudin—After the dynamic image acquisition, static anterior and posterior images of 3 min (64 × 64 word mode) were acquired of the head, thorax and abdomen, and legs. A background image was also acquired to correct whole body and region of interest (organ) radioactivity for background radioactivity. Before treatment on the second day, anterior and posterior images of the thorax and abdomen were acquired to determine and correct for residual ¹³¹I radioactivity as a result of infusion of [¹³¹I]lepirudin on the previous day.

To obtain anterior and posterior whole body radioactivity, the radioactivity in the head, thorax and abdomen, and legs was

corrected for background radioactivity and/or residual radioactivity and summed. Regions of interest for the kidneys, bladder, spleen, and liver were selected to determine organ radioactivity. On the first day of the experiment, these radioactivities were corrected for background radioactivity. On the second day, organ radioactivity was corrected for background radioactivity and residual radioactivity, measured before the infusion of [131 I]lepirudin. The in vivo organ distribution of [131 I]lepirudin was quantified by the geometric mean method.¹¹

Blood Collection and Analysis—Blood, 4.5 mL in 0.5 mL of a 3.2% sodium citrate solution, was collected before a study was started and then every 15 min thereafter. The radioactivity in 1 mL of plasma was determined in a gamma counter. Plasma levels of lepirudin were determined by the ECT method, which was specifically developed to measure hirudin levels in plasma and body fluids.¹⁰ The activated partial thromboplastin time (aPTT) was measured with a fibrinometer (Clotex II, Hyland Division, Travenol Laboratories, Costa Mesa, CA), and reagents were supplied by the same company. The aPTT was measured to give an indication of the level of anticoagulation achieved and to show that the labeled lepirudin was functional.

To determine if the labeling procedure affected the function of [131 I]lepirudin, unlabeled and labeled lepirudin were added to plasma obtained from 4 baboons. Plasma concentrations were 2.5 and 5.0 μ g/mL of lepirudin. In addition, a 50/50 mixture of labeled and unlabeled protein was also used. The aPTT was determined on all samples.

Urine Collection and Analysis—Urine was collected into a urine-collecting bag with a Teflon-coated latex Foley catheter (GRS Medicals, Kelvin, SA) for up to 24 h and analyzed for lepirudin by the ECT method.¹⁰ The total amount of lepirudin was calculated from the total urine volume and the concentration in urine.

Urine was also analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples analyzed were [131 I]lepirudin, urine without [131 I]lepirudin (native urine), [131 I]lepirudin added to native urine, and urine collected from the treated baboons. The results strongly suggest that [131 I]lepirudin was excreted in an unchanged form. The results are not given.

Calculations—Plasma Half-Lives of Lepirudin and [131 I]-Lepirudin—The elimination half-lives of lepirudin and [131 I]-lepirudin were calculated by adjusting a one-phase exponential function to the appropriate phases of the log-linear plasma concentration-time and radioactive count rate-time profiles by the method of least-squares analysis. The value k , in the function $C_{\max} \times e^{-kt}$, was thus found, where $C_{\max}$ is the maximum lepirudin concentration or maximum radioactive count rate in plasma. The half-lives were calculated as $t_{1/2} = 0.693/k$.

Total Clearance of Lepirudin and [131 I]Lepirudin—For the constant infusion, total clearance (CL_{total}) was calculated by dividing the rate of infusion (mg/min or radioactive count rate/min) by the maximum concentration of lepirudin or radioactive count rate in the plasma. To estimate the maximum plasma concentration or plasma radioactive count rate, an exponential association function was fitted to the data points obtained during the infusion period and extrapolated to maximum (i.e., an estimate of steady-state levels).¹²

For the bolus injection, CL_{total} was calculated by dividing the total dose, or the total counts injected, by the area under the curve ($AUC_{0-\infty}$) of the plasma lepirudin concentration (μ g/mL)-time or plasma radioactivity (radioactive count rate/mL)-time profiles. The area under the plasma concentration-time and plasma radioactive counts rate-time curves up to 90 min (AUC_{0-90}) were calculated using the trapezoid rule. The curves were extrapolated to ∞ using the formula $AUC_{0-\infty} = AUC_{0-90} + C_p^*/k_e$, where C_p^* is the plasma lepirudin concentration or plasma radioactive count rate at the last sampling time (90 min). The terminal rate constant (k_e) was determined from the slope of the terminal portion of the log-linear C_p versus time curve.

Renal Clearance of [131 I]Lepirudin—For the constant infusion, renal clearance (CL_{renal}) was calculated by dividing the rate of appearance of [131 I]lepirudin in the bladder (radioactive count rate/min) by the maximum counts in the plasma. The latter was estimated by fitting an exponential association function to the available radioactive count rate/mL-time profiles and extrapolated to estimate the maximum value where a steady state was reached.

For the bolus injection, CL_{renal} was calculated by dividing the maximum radioactive count rate in the bladder by $AUC_{0-\infty}$ of the plasma radioactivity (radioactive count rate/mL)-time profiles.

Extravascular Clearance—The plasma clearance of lepirudin by the extravascular compartment was estimated by calculating the difference between the total clearance and the renal clearance.

Determination of Sites of Lepirudin Accumulation in Sacrificed Baboons—Two baboons were used. The one baboon was sacrificed after 30 min of infusion of [131 I]lepirudin. The other one received no lepirudin and was sacrificed to obtain organs and body fluids that served as controls for immunohistochemistry and lepirudin determinations, respectively. The baboons were deeply anesthetized. The test baboon was exsanguinated through the arterial side of the A-V shunt. About 2 L of saline at 37 °C were simultaneously infused under pressure (140 mmHg) into the venous side of the shunt to replace the blood that was lost.

Ex Vivo Quantification of the Distribution of [131 I]Lepirudin—The liver, spleen, kidneys, and bladder of the sacrificed baboons were immediately excised by careful dissection. Residual radioactivity in the body and that present in the liver, spleen, kidneys, and bladder were determined by scintillation camera imaging.¹¹ Images of 3 min (64 $\times$ 64 word mode) were acquired. Organ radioactivity was then expressed as a percentage of total body radioactivity, obtained by summation of residual and organ radioactivities.

Organ Biopsies—Biopsies of the kidneys (cortex and medulla), spleen, and liver were collected to establish the location of the lepirudin by using immunohistochemistry techniques. A primary antibody, rabbit anti-hirudin antibody (diluted 1:100 in PBS; Celsus Laboratories, Cincinnati, OH) was added to thin processed sections of the tissues and incubated for 60 min. A secondary goat anti-rabbit antibody (Signet kit, Johnson & Johnson, Johannesburg, Republic of South Africa) was added to bind to the primary antibody and a third antibody complex, a peroxidase-antiperoxidase immune complex, was used as the marker complex. These antibody complexes become visible when the tissues are exposed to the substrate diaminobenzidine. By adding hydrogen peroxide to the diaminobenzidine solution, a brown precipitate forms where lepirudin is present. Light microscopy was used to interpret the stains. Lightmicrographs of all tissues were enlarged 200 times and examined for the presence of lepirudin in cells.

Biopsies of the kidneys, liver, and spleen were also homogenized in saline and centrifuged at 8000 g for 24 h. The ECT method¹⁰ was used to test for the presence of lepirudin in the supernatant of the homogenates.

Collection and Analysis of Duodenal and Gastric Juices and Bile—Duodenal and gastric juices and bile were collected from the sacrificed baboons to test for the presence of lepirudin by the ECT method.¹⁰

Statistics—Statistical comparison was done using Student's t test for paired data. Values of $p < 0.05$ were considered significant. The results are expressed as mean $\pm$ 1 standard deviation (SD).

Results

Changes in aPTT and Pharmacokinetics of Lepirudin—In the in vitro studies, concentrations of 2.5 and 5.0 μ g/mL lepirudin lengthened the aPTT to 87 ± 4 and 174 ± 5 s. [131 I]Lepirudin, at the same concentrations, lengthened the aPTT to 92 ± 4 and 180 ± 6 s. When labeled and unlabeled lepirudin were mixed, the aPTT lengthened to 90 ± 3 and 178 ± 4 s. The difference in measurements was not significant. The labeling process therefore does not affect the function of lepirudin.

The in vivo changes in aPTT are summarized in Table 1. The labeled hirudin was biologically active because it caused a 2–3-fold lengthening in the aPTT. When lepirudin infusion was stopped and following the bolus injection, the aPTT rapidly shortened to reach near pre-infusion values after 90 min.

The changes in circulating lepirudin and labeled lepirudin are summarized in Figure 1. Following infusion or the bolus injection, the plasma levels of lepirudin and [131 I]-lepirudin decreased rapidly. In all studies, the plasma half-life of lepirudin was 24 ± 9 min. When calculated from the

Table 1—Changes in APTT^a

time (min)	infusion	bolus
-30	40 ± 4	—
-10	—	—
0	131 ± 20	42 ± 6
30	79 ± 10	98 ± 16
60	64 ± 9	74 ± 13
90	56 ± 8	64 ± 12

^a Results are given in seconds and are expressed as mean ± 1 SD.

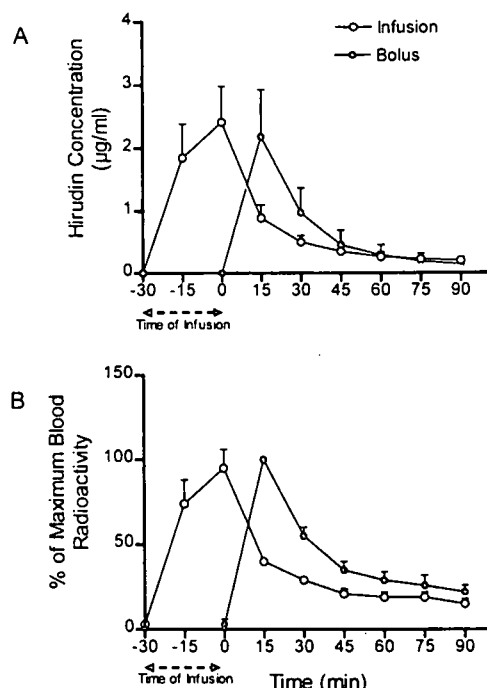


Figure 1—Dynamic changes of (A) plasma lepirudin and (B) [¹³¹I]lepirudin following infusion (open circles) or bolus administration (black circles). Values are given as a mean ± 1 SD.

Table 2—Total, Renal, and Extravascular Clearance of Lepirudin and [¹³¹I]Lepirudin

route	clearance	r-Lepirudin	[¹³¹ I]Lepirudin
infusion	total	49 ± 16	52 ± 10
	renal	—	32 ± 10
	extravascular	—	20 ± 8
bolus	total	42 ± 12	45 ± 12
	renal	—	23 ± 2 ^b
	extravascular	—	22 ± 12

^a Values are given in mL/min and are expressed as mean ± 1 SD. ^b $p < 0.05$, infusion versus bolus (Student's *t* test for paired data).

disappearance of radioactivity from the blood, it was 21 ± 6 min. The difference was not statistically significant ($p > 0.05$). After infusion of [¹³¹I]lepirudin was stopped, lepirudin was eliminated from the plasma with a half-life of 23 ± 8 min ([¹³¹I]lepirudin = 21 ± 3 min). When the same amount of [¹³¹I]lepirudin was given as a bolus, the half-life was 18 ± 4 min ([¹³¹I]lepirudin = 19 ± 8 min). The mean difference in the half-lives between infused and bolus injected lepirudin was 5 ± 10 min. The 95% confidence interval of between -5 and 16 min indicates that the difference was not statistically significant.

Total, renal, and extravascular clearance of lepirudin is summarized in Table 2. Total clearance was ≈45 mL/min, whether it was calculated from the data obtained from the concentration of lepirudin or from labeled lepirudin. Between 51 and 61% of this lepirudin was cleared by the kidneys.

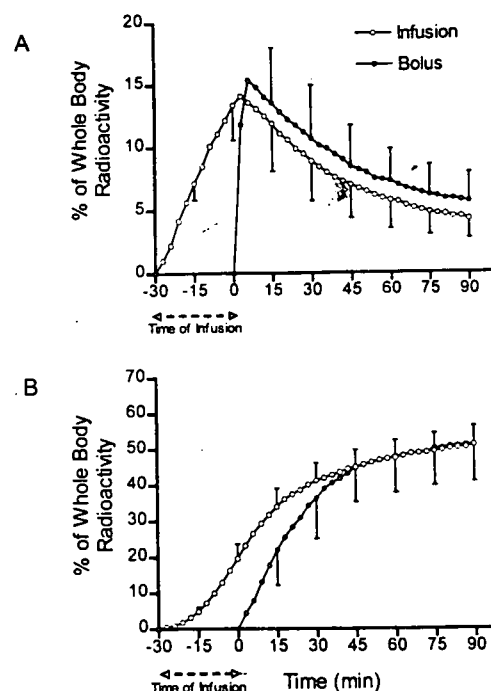


Figure 2—Dynamic changes of [¹³¹I]lepirudin in the (A) kidneys and (B) bladder following infusion (open circles) and bolus administration (black circles). The radioactivity is expressed as a mean percentage ± 1 SD of whole body radioactivity at the end of each study.

Table 3—Organ Distribution of [¹³¹I]Lepirudin at the End of Treatment

organ	infusion	bolus
kidneys	4.3 ± 1.5	5.7 ± 2.3
bladder	51.2 ± 5.2	51.2 ± 10.3
liver	1.7 ± 0.8	1.8 ± 0.9
spleen	3.6 ± 2.4	3.0 ± 2.7

^a Percent of whole body radioactivity at the end of each study. Values are given as mean ± 1 SD.

Table 4—ECT of Bile and Duodenal and Gastric Juices, and of the Supernatant of Homogenized Kidney, Liver, and Spleen Tissue^a

sample	ECT, s
bile	229
gastric juice	49
duodenal juice	39
kidney	77
liver	44
spleen	49

^a Control values for the ECT method are between 38 and 40 s.

In Vivo Distribution of [¹³¹I]Lepirudin—The changes in radioactivity in the kidneys and bladder are summarized in Figure 2, and the in vivo organ distribution at the end of the study in Table 3. Only ≈5% of total injected [¹³¹I]lepirudin was quantified in the liver and the spleen. The results obtained in the sacrificed baboon, 0.1% of the injected radioactivity in the spleen and 1.5% in the liver, also showed that the liver and spleen contained little [¹³¹I]lepirudin. Between 50 and 60% of the injected labeled lepirudin could be accounted for in the bladder. At the end of infusion or shortly after the bolus was injected, the kidneys contained ≈15% of the injected radioactivity (Figure 2A).

The presence of lepirudin in the bile and gastric and duodenal juices of the sacrificed baboon was measured by the ECT method, and the results are shown in Table 4. Of these, only the bile contained appreciable amounts as indicated by the markedly longer than normal ECT results.

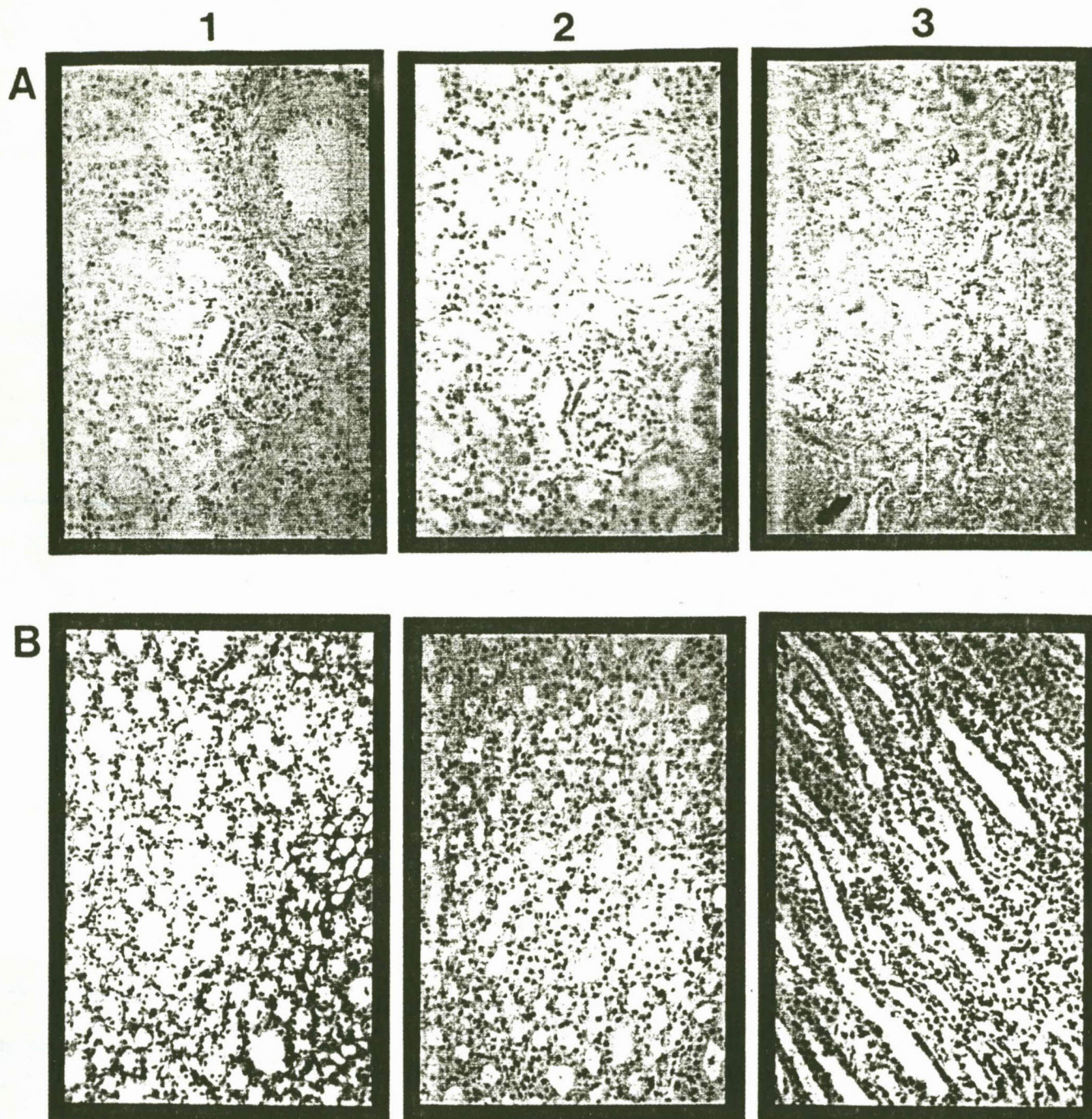


Figure 3—Light micrographs (enlarged 200X) of the (A) renal cortex and (B) medulla. A1 and B1 are from baboons that received lepirudin. A2 and B2 are negative controls where no anti-hirudin antibody was used. A3 and B3 show the renal cortex and medulla of the control baboon that did not receive lepirudin but was treated with anti-hirudin antibody.

The amount of lepirudin excreted by the kidneys was calculated from the total urine volume and the concentration of r-hirudin in urine. In two cases ($1 \times$ bolus study and $1 \times$ infusion study), very little urine could be collected. The results are nevertheless given. After infusion of labeled lepirudin, 2.2 ± 1.9 mg of lepirudin was excreted, after the bolus 2.2 ± 1.5 mg was excreted. This amount was $\approx 54\%$ of the total dose of lepirudin given.

Immunohistochemistry—The results are presented in Figures 3 and 4. The kidney, liver, and spleen of the baboon that was not treated with lepirudin stained negative when the tissues were incubated with the anti-hirudin antibody (Figure 3, A3 and B3, and Figure 4, A3 and B3). This result indicates that these tissues did not contain proteins that interact with the antibody. Kidney, spleen, and liver tissue

from baboons treated with lepirudin also stained negative when the tissues were not incubated with the antibody (Figure 3, A2 and B2, and Figure 4, A2 and B2), indicating that the positive staining obtained in the test samples was not a result of the staining procedure.

In the renal cortex, the cytoplasm of the epithelial cells of the convoluted tubules, the cytoplasm of the endothelial cells of the blood vessels, and the connective tissue stained for lepirudin. The glomeruli stained negative (Figure 3, A1). The renal medulla stained intensely. It is likely that lepirudin was present in the epithelium of the thick and thin segments of the loop of Henle and the collecting tubes. The cytoplasm of the endothelial cells of the vas recta and the connective tissues between the cells stained strongly (Figure 3, B1).

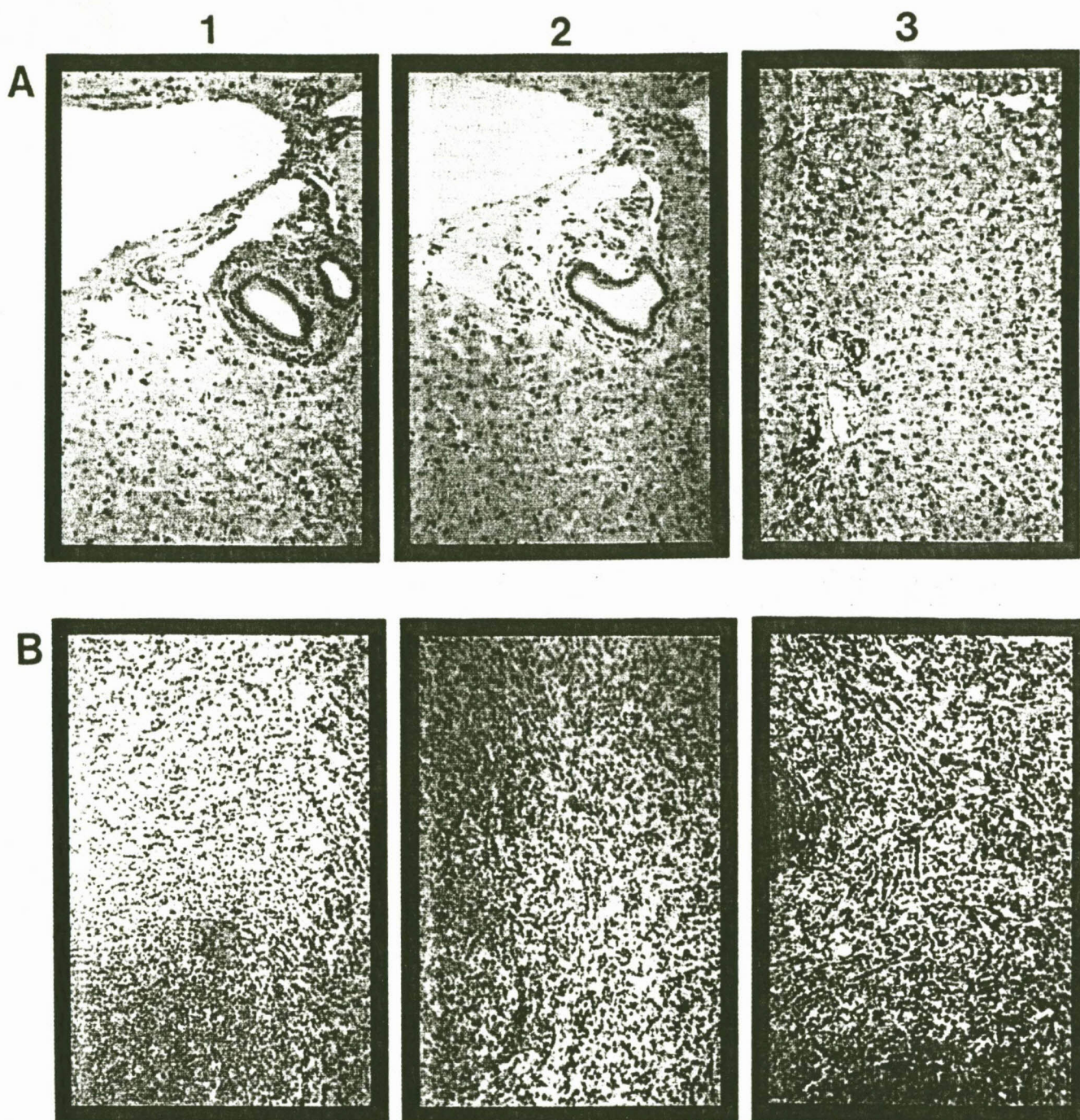


Figure 4—Light micrographs (enlarged 200X) of the (A) liver and (B) spleen. A1 and B1 are from baboons that received lepirudin. A2 and B2 are negative controls where no anti-hirudin antibody was used. A3 and B3 show the liver and spleen of the control baboon that did not receive lepirudin but was treated with anti-hirudin antibody.

The spleen did not stain for lepirudin (Figure 4, A1). On the other hand, staining of liver is ambiguous (Figure 4, B1). The cytoplasm of the hepatocytes did not stain strongly, but the cytoplasm of the endothelial cells of the hepatic artery and portal vein and that of the epithelial cells of the bile duct stained strongly.

Discussion

In a study of this nature, where the pharmacokinetics and mechanism of clearance of a compound labeled with a radioisotope is studied, it is vital that the labeled compound reacts in the same way as the native compound. There are several lines of evidence to show that the behavior of

lepirudin, when labeled with ^{131}I , was not markedly affected by the labeling procedure. First, treatment with the labeled lepirudin lengthened the aPTT 3–4-fold (Table 1). In addition, when unlabeled and labeled hirudin, either alone or as a 50/50 mixture, were added to plasma in vitro, the lengthening in aPTT was equivalent. This result indicated that the labeled lepirudin was functional. Second, when the half-life was measured from the radioactive count rate in blood, it was 21 ± 6 min, which was not significantly different from that estimated for lepirudin (24 ± 9 min). These estimates were also not significantly different from that obtained in another study.⁵ Third, after 90 min of treatment with labeled lepirudin, no accumulation of ^{131}I was imaged in the thyroid or spleen, both of which rapidly

remove free iodine in plasma.¹³ It is therefore reasonable to conclude that labeling of lepirudin with ¹³¹I did not adversely affect the function and plasma clearance of lepirudin and that the ¹³¹I remained bound to the lepirudin. The results obtained with the labeled lepirudin can therefore be compared with those of lepirudin, and conclusions relating to the pharmacokinetics of lepirudin can be made from the results obtained with labeled lepirudin.

It is evident that lepirudin was mainly excreted by the kidneys (Figures 2A and B). Between 50 and 60% of the injected lepirudin was detected in the bladder 90 min after it was injected (Table 3), which agrees with results obtained in humans and rhesus monkeys.^{6,14,15} Because the clearance of lepirudin approximates that of creatinine in humans, it was proposed that excretion be by glomerular filtration.^{14,15} We used immunohistochemical techniques in an attempt to verify this proposal (Figure 3). The results were rather confusing and do not confirm glomerular filtration. Glomeruli in the renal cortex did not stain for lepirudin. Tissue surrounding the glomerulus and the blood vessel did stain. Even more surprising was the fact that the tubuli in the medulla, and especially the epithelial cells lining the tubules, stained intensely, which suggests tubular reabsorption and secretion. A well-planned study to investigate glomerular filtration and possible tubular reabsorption and secretion is called for. Such a study will provide definitive answers about the mechanisms by which the kidneys excrete lepirudin.

It is generally accepted that the fraction of lepirudin that is not excreted by the kidneys is distributed in the extravascular compartment.¹⁴ The *in vitro* results in this study however showed that the liver plays a part in the catabolism of lepirudin and that lepirudin was excreted in the bile. Although we could not quantify appreciable quantities of [¹³¹I]labeled lepirudin in the liver (Table 3), immunohistochemical studies showed that lepirudin was present in the hepatocytes and the tissues surrounding the blood vessels and bile ducts. The finding that lepirudin was present in the bile means that it has to be excreted into the digestive system. We explain the absence of lepirudin in the duodenal juices (Table 4) and the finding that lepirudin was not present in the feces of normal humans 24 h after it was administered¹⁵ by digestion and absorption of lepirudin by the enzymes present in the duodenum. It is reasonable to assume that the 40% of administered lepirudin that was not excreted by the kidneys, could be excreted by the liver into the bile. However, a well-planned study is needed to quantify the contribution of the liver to the clearance of lepirudin from plasma. It is further evident that the spleen plays no part in the catabolism of lepirudin (Table 3, Figure 4).

The plasma elimination half-life of lepirudin was 18 ± 4 min when it was injected as a bolus. When the same quantity was infused over 30 min, the estimated half-life was not significantly different (23 ± 8 min). This result is similar to the estimated half-life that we determined in baboons where different dosages of lepirudin were infused over 30 min.⁵ The half-lives estimated in the baboons are shorter than the 55–70 min measured in humans, rhesus monkeys, rats, rabbits, and dogs.^{6,15–17} We have no easy explanation for this discrepancy.

The total clearance of lepirudin from plasma was ≈45 mL/min, whether the same amount was infused or given as a bolus. Similar results were obtained when clearance was calculated from the radioactive count rates (Table 2). This value is not very different from that determined in rhesus monkeys,¹⁸ but differs greatly from the results reported for humans, rats, rabbits, and dogs.^{6,15} Labeled lepirudin, infused over 30 min, was cleared by the kidneys at a significantly higher rate than that adminis-

tered as a bolus (Table 2). However, when renal clearance was expressed as a percentage of plasma clearance, 62 ± 16% of infused lepirudin was cleared by the kidneys. The corresponding value for bolus administered lepirudin, 55 ± 15%, was not significantly different (*p* > 0.05, Student's *t* test for paired data). Thus, although the rate of clearance was different, the relative contribution of the kidneys to plasma clearance was the same. It is important to note that in rhesus monkeys that received a bolus of lepirudin, renal clearance also contributed ≈50% to plasma clearance.¹⁸ These results are similar to those obtained in this study. The method of administration of the labeled lepirudin did not affect the extravascular clearance (Table 2).

In summary, whether 0.3 mg/kg of labeled lepirudin was infused over 30 min or given as a bolus, it did not influence its plasma half-life and clearance. This result suggests that the study was performed with plasma concentrations below the maximum threshold levels of excretion of lepirudin by the kidneys. The mode of administration did not affect the clearance of lepirudin from plasma, nor that by the kidneys or the extravascular compartment. The kidneys were the main sites of excretion of lepirudin, where between 50 and 60% was excreted, probably by tubular secretion. It has been speculated that the remainder may be catabolized by the kidneys and that the methods used to determine lepirudin are unable to recognize the degradation products.^{6,14,18,19} The quantification of the percentage labeled lepirudin found in the bladder argues against renal catabolism. If the labeled lepirudin was catabolized, much more than 50 to 60% of administered labeled lepirudin would have been detected in the bladder at the end of the study. In addition, SDS-PAGE suggested excretion in an unchanged form. A surprising finding was that the bile contained appreciable amounts of lepirudin.

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r-Hirudin inhibits platelet-dependent thrombosis during cardiopulmonary bypass in baboons

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Background. Systemic anticoagulation is required during cardiopulmonary bypass (CPB) to inhibit the activation of platelets, the coagulation system and ultimately thrombus formation. Unfractionated heparin is most commonly used, but it is neither entirely safe nor completely effective. The use of protamine sulphate to reverse the anticoagulant effect further complicates the use of heparin. The clinical need for a heparin substitute is therefore obvious. We evaluated the efficacy of r-Hirudin, a potent and specific inhibitor of thrombin, as anticoagulant in a baboon model of cardiopulmonary bypass.

Methods. Ten baboons, divided into two groups of five each, were used. The one group received 0.7 mg/kg r-Hirudin as a bolus before CPB was started, followed by a constant infusion of 1.4 mg/kg/hr for the 90 min of CPB. The other group received a bolus of 2.5 mg/kg heparin before the start of CPB, followed by maintenance dosages to maintain the activated clotting time (ACT) >400 sec.

Results. Adequate anticoagulation was obtained with both anticoagulants. Haemodilution due to priming the extracorporeal system with Ringer's lactate and appropriately anticoagulated donor blood, was equivalent in both groups. During CPB with heparin, but not with hirudin, there was a significant increase in the number of circulating platelet aggregates, thrombin-antithrombin (TAT) complexes and ¹¹¹In-labelled platelet accumulation in the oxygenator. After the initial decrease in platelet count due to haemodilution, it further decreased significantly during CPB with heparin but remained relatively constant when r-Hirudin was used.

Conclusions. Our results strongly suggest that r-Hirudin is superior to heparin especially with respect to its inhibitory effect on platelet dependent thrombogenesis caused by the biomembranes of the oxygenator.

KEY WORDS: Cardiopulmonary bypass - Hirudin therapeutic use - Thrombosis - Blood platelets - Papio.

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The use of extracorporeal circulation during cardiopulmonary bypass (CPB) involves extensive contact between blood and artificial surfaces. Exposure of blood to artificial surfaces leads to the activation of blood platelets and the coagulation system with subsequent thrombin generation and thrombus formation. Therefore, systemic anticoagulation is required. Standard unfractionated (SU) heparin is most commonly used during CPB, but it is neither entirely safe nor completely effective. Heparin markedly enhances the inhibition of thrombin by antithrombin III (AT III).¹ However, the heparin-AT III complex is unable to inactivate thrombin which is bound to fibrin in a thrombus. This thrombin remains active to act as an ongoing source of thrombogenesis and increases heparin requirements.^{2,3} The large doses of heparin which are required to perform successful CPB may induce serious side effects such as heparin-induced thrombocytopenia⁴ and bleeding.⁵ In addition, despite anticoagulation with heparin, thrombin is still produced, fibrin formation still occurs and thrombi are still formed.^{6,7} The use of protamine sulphate (PS) to reverse the anticoagulant effect of heparin further complicates its use. Besides the fact that the use of PS may cause adverse events, it can by itself increase postoperative bleeding.⁸⁻¹² Furthermore, inadequate heparin reversal or heparin rebound can also cause hypocoagulability.^{13,14} The clinical need for a heparin substitute is obvious and has stimulated the development of a variety of new anticoagulants. With this study we chose to evaluate r-Hirudin as an alternative anticoagulant for use during CPB.

r-Hirudin is the most potent and specific known natural inhibitor of thrombin.¹⁵ It produces a dose dependent inhibition of arterial thrombus formation that greatly exceeds the minimal antithrombotic effects produced by heparin.¹⁶ The major advantages of r-Hirudin over heparin are that it does not depend on a cofactor to express its activity, it has a very short half life which may make neutralisation of its anticoagulant activity unnecessary,¹⁷ it has no fibrinolytic activity,¹⁸ it decreases the risk of developing a bleeding tendency^{19,20} and it inhibits both free thrombin and thrombus-bound thrombin more effectively than heparin.³ r-Hirudin has already been evaluated in dogs and pigs on CPB,^{21,22} and in two patients undergoing open heart surgery.^{23,24} In all instances it proved to more adequately anticoagulate the blood and ensure effective CPB.

We used a baboon model to evaluate the efficacy of r-Hirudin as an anticoagulant during CPB. The baboon was chosen as experimental animal because its vascular anatomy closely resembles that of humans and because the molecular and cellular composition and function of the haemostatic system is very similar to that of humans.²⁵

Materials and methods

Animals and anticoagulants

Ten adult healthy baboons, *Papio ursinus*, weights ranging between 14 and 18 kg, were included in this study which was approved by the Ethics Committee for Animal Experimentation of the University of the Orange Free State in accordance with the National Code for animal use in research, education, diagnosis and testing of drugs and related substances in South Africa (based on the "Guide for the care and use of Lab. Animals; NIH 85-23, revised 1985). The baboons did not receive any medication that could affect platelet function or coagulation for at least two weeks prior to the study.

The animals were divided into two groups. In the one group (n=5) each baboon received 0.7 mg/kg r-Hirudin (HBW 023; Hoechst AG, Frankfurt and Behringwerke AG, Marburg, Germany) as a bolus immediately before starting the bypass procedure. This was followed by a constant infusion of 1.4 mg/kg/hr for the 90 min duration of CPB. The r-Hirudin was infused at the inlet of the oxygenator. The other group received 2.5 mg/kg

SU heparin (porcine intestinal mucosa, heparin sodium, Labethica, S.A.) as a bolus immediately before starting the bypass procedure. This was followed by maintenance dosages, when needed, to maintain activated clotting times (ACT) of longer than 400 sec.

Surgical preparation and cardiopulmonary bypass

The animals were sedated with intramuscular ketamine hydrochloride (10 mg/kg; Anaket V, Centaur Laboratories, RSA), and anaesthetised with 30 mg/kg intravenous sodium pentobarbital (Sagatal, Rhône-Poulenc, RSA). Thereafter they were intubated and ventilated with a volume-controlled Penlon 2000 respirator. Anaesthesia was maintained with a mixture of oxygen and halothane (Fluothane; Wyeth-Ayerst Laboratories, Philadelphia, PA).

A midline sternotomy was performed and the aorta cannulated with an 18F Argyle cannula. Both the superior and inferior venae cavae were cannulated and snared for venous return. Cardiopulmonary bypass was established with the use of a paediatric membrane oxygenator (Dideco, Italy) gassed with 95% oxygen and 5% carbon dioxide. Blood flow through the extracorporeal system was maintained with a Travenol roller pump (Travenol Labs, Inc, Ann Arbor MI). The extracorporeal circuit was primed with 300 ml lactated Ringer's solution (Adcock Ingram, S.A.) and 200 ml of donor baboon blood which was anticoagulated with either r-Hirudin (1.2 mg) or heparin (9.2 mg). During the CPB procedure the baboons were cooled to 32°C. Rewarming started ten minutes before the end of CPB.

Blood gases were measured periodically throughout the procedure to ensure proper ventilation. Arterial pH was maintained between 7.35 and 7.45, the carbon dioxide tension between 35 and 45 mmHg and the oxygen tension >120 mmHg.

At the end of the 90 minute CPB, the baboons were sacrificed with an overdose of pentobarbital and potassium chloride.

Study variables

The day before CPB, blood (53 ml in 7 ml ACD-A) was collected, the platelets were isolated by differential centrifugation, labelled with ¹¹¹In-tropolone and re-injected.²⁶ A sodium iodide crystal gamma detector, fixed in position at the lower part of the oxygenator, was used to determine the accumulation of ¹¹¹In plate-

let radioactivity during CPB. The radioactivity counts were corrected for blood radioactivity and used to construct time-radioactivity curves. After CPB the oxygenators were flushed at low pressure with saline and imaged with a computer assisted scintillation camera. A 3 ml blood sample, which was collected before the CPB procedure, was also imaged to determine the radioactivity in 1 ml of blood. This count rate and the platelet count were used to calculate the number of platelets deposited onto the membranes of the oxygenator. Quantification of the liver and spleen radioactivity before and after CPB was done as described.²⁷

Blood was collected before, during and after CPB from the venous and arterial side of the extracorporeal circuit. The platelet count and hematocrit were determined with a Technicon H*1 blood cell analyser (Bayer Diagnostics) on blood collected in K₃EDTA. To determine circulating platelet radioactivity, blood was collected in K₃EDTA, the hematocrit (hct) was determined, the radioactivity in 1 ml of blood and 1 ml of plasma were counted in a scintillation counter (Packard Auto-gamma 5650, Packard Instruments International S.A., Switzerland). Circulating platelet radioactivity (RA) was calculated by using the following formula: Blood RA - [(1 - hct) x Plasma RA]. Blood was collected in 3.2% tri-sodium citrate (9:1 vol/vol) for coagulation studies. The activated partial thromboplastin time (aPTT; Actin FS, Dade, Miami, FL, USA) and 10 U/ml calcium thrombin time (CaTT; bovine thrombin reagent reconstituted in 0.025 M CaCl₂, Dade, Miami, FL, USA) were measured with a Cobas Fibro semi-automated optical system (Roche, Switzerland). The plasma antithrombin III (AT III) levels were measured by means of a chromogenic assay kit (Instrumentation Laboratory, Italy) with an Automated Coagulation Laboratory instrument (Instrumentation Laboratory, Italy). The thrombin-antithrombin (TAT)-complex levels were determined with an ELISA kit (Behringwerke, Marburg, Germany). The circulating platelet aggregate ratio (CPAR) was determined as described.²⁸ The plasma concentrations of heparin and r-Hirudin were determined in blood collected in 3.2% and 3.8% tri-sodium citrate (9:1 vol/vol), respectively. The plasma concentration of r-Hirudin was determined with a chromogenic substrate method²⁹, and that of heparin with a chromogenic assay kit

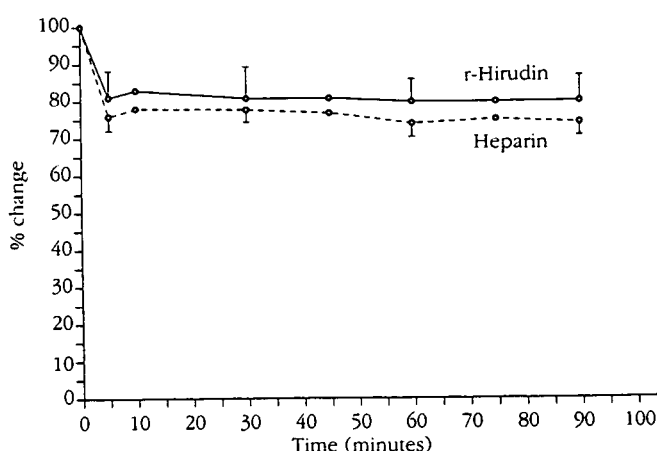


Fig. 1.—The per cent change in the hematocrit during CPB with heparin (n=5) and r-Hirudin (n=5) respectively. The preCPB level was taken as 100%. Results are given as mean ± SEM.

(Instrumentation Laboratory, Italy) using the Automated Coagulation Laboratory instrument.

The celite activated clotting time (ACT) (Hemo-chron, International Technidyne, Edison, NJ) was measured on site throughout the 90 min of CPB.

Statistical analyses

Student's "t"-test for paired data was used to test for differences within a treatment group (preCPB *vs* post-CPB). Differences between treatment groups were tested with Student's "t"-test for unpaired data. Repeated measures ANOVA was used to compare curves. The mean effects of the anticoagulant as reflected by the relative sizes of the areas under the curves, were compared by ANOVA. Values of $p < 0.05$ were considered to be statistically significant.

Results

Hematocrit

The changes in hematocrit are summarised in Figure 1. There was an initial decrease of $24 \pm 9\%$ with heparin and $19 \pm 17\%$ with r-Hirudin. Thereafter it remained relatively constant.

Coagulation

The changes in the plasma concentration of heparin and r-Hirudin are illustrated in Figure 2. In both cas-

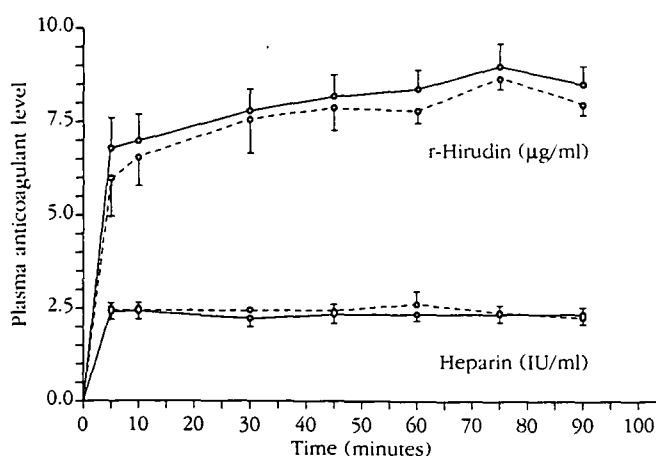


Fig. 2.—The plasma levels of heparin (n=5) and r-Hirudin (n=5) in oxygenated (solid line) and venous blood (broken line) respectively. Results are given as mean $\pm$ 1 SEM.

es the concentration in oxygenated and venous plasma remained at a relatively constant level following the initial bolus of anticoagulants. The aPTT was longer than 300 sec and the CaTT longer than 200 sec, throughout the CPB procedure irrespective of the anticoagulant used and the site of blood collection. The ACT was between 500 and 600 sec during CPB with heparin, but could not be used to monitor r-Hirudin since it is too sensitive and was therefore not measurable.

The plasma AT III levels decreased significantly during CPB with heparin and increased significantly during CPB with r-Hirudin (Table I). TAT complexes increased markedly in both oxygenated and venous blood when heparin was used. When r-Hirudin was used, TAT complexes remained unchanged in oxygenated plasma and increased slightly, but significantly in venous plasma (Table I).

Circulating platelet aggregates

There was a significant decrease in the circulating platelet aggregate ratio (CPAR) in oxygenated blood during CPB with heparin (Table I). This indicates an increase in the number of circulating platelet aggregates as a result of exposure to the artificial oxygenator membranes. In contrast, there was a slight, but not significant, increase in the CPAR in oxygenated blood when r-Hirudin was used, which indicates that r-Hirudin inhibited aggregate formation in the oxygenator.

Platelet count and circulating platelet radioactivity

The results are summarised in Figure 3. The initial decrease in platelet count as a result of haemodilution was 41.8% with heparin and 34.16% with r-Hirudin. The corresponding decreases at the end of CPB were $58 \pm 11\%$ and $35 \pm 11\%$ respectively. The decrease in platelet count during CPB with heparin was significantly more than with r-Hirudin (repeated measures ANOVA). The decreases in circulating platelet radioactivity followed the same pattern as that of the platelet count. The fact that the decrease in platelet radioactivity was slightly more than that of the platelet count may be due to the fact that the platelets in the donor blood, with which the extracorporeal system was primed, were not labelled with $^{111}\text{Indium}$.

^{111}In -labelled platelet accumulation in the oxygenator

The kinetics of deposition of ^{111}In -labelled platelets onto the membranes of the oxygenator, expressed as the fold increase from baseline, are illustrated in Figure 4. Significantly more labelled platelets accumulated when heparin was used

TABLE I.—Changes in antithrombin III (AT III), thrombin-antithrombin complex (TAT) and circulating platelet aggregate ratio (CPAR) in oxygenated and venous blood before and after CPB with heparin and r-Hirudin, respectively. Data with a normal distribution are given as mean $\pm$ 1 SD, otherwise the ranges are given (n=5 in each case).

Parameter	Heparin				r-Hirudin			
	Oxygenated blood		Venous blood		Oxygenated blood		Venous blood	
	Before	After	Before	After	Before	After	Before	After
AT III (%)	79.8 $\pm$ 9.4	49.6 $\pm$ 10.1*	76.8 $\pm$ 8.5	49.8 $\pm$ 10.7*	61.6 $\pm$ 3.7	81.2 $\pm$ 4.3*	64.8 $\pm$ 8.9	81.2 $\pm$ 3.8*
TAT ($\mu\text{g/ml}$)	10 to 26	40 to >200	11 to 22	32 to >200	23.7 $\pm$ 12.1	25.4 $\pm$ 13.6	10.8 $\pm$ 1.6	25.5 $\pm$ 14.6*
CPAR	0.90 $\pm$ 0.05	0.83 $\pm$ 0.03*	0.83 $\pm$ 0.07	0.76 $\pm$ 0.05	0.83 $\pm$ 0.17	0.89 $\pm$ 0.13	0.87 $\pm$ 0.08	0.82 $\pm$ 0.09

*p<0.01: Student's "t"-test for paired data. (pre-CPB vs post-CPB)

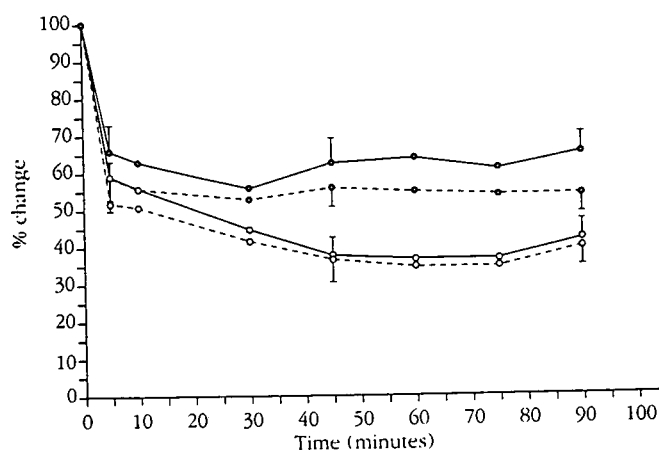


Fig. 3.—The per cent change in the peripheral platelet count (solid line, $n=5$) and the circulating ^{111}In -labelled platelets (broken line, $n=5$) during CPB with heparin (open circles) and r-Hirudin (solid circles), respectively. The preCPB counts were regarded as 100%. Results are given as mean $\pm$ 1SEM.

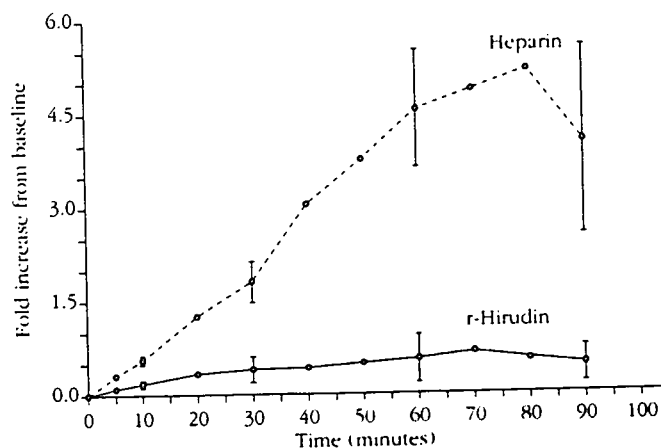


Fig. 4.—The deposition of ^{111}In -labelled blood platelets onto the oxygenator membranes during CPB with heparin ($n=5$) and r-Hirudin ($n=5$) respectively. The results are expressed as the mean $\pm$ 1SEM fold increase from baseline. The mean areas under the curves were 276 min $\times$ change from baseline for heparin and 38 min $\times$ change from baseline for r-Hirudin.

($p<0.05$). The mean areas under the curves were 276 min $\times$ change from baseline for heparin and 38 min $\times$ change from baseline for r-Hirudin. At the end of the study, the oxygenator contained labelled platelets to the equivalent of 105 ml (median, range from 59 to 129 ml) of blood after CPB with heparin. The corresponding value with r-Hirudin was 3 ml (median, range from 1 to 55 ml).

Distribution of ^{111}In -labelled platelets in the spleen and liver

Platelet radioactivity in the spleen decreased by $32\pm14\%$ during CPB with heparin and by $24\pm16\%$ when heparin was used. There were no significant differences. Hepatic platelet radioactivity remained relatively constant in all cases irrespective of the anticoagulant used.

Discussion and conclusions

Our results indicate that effective CPB can be performed with r-Hirudin as anticoagulant. No obvious adverse reactions were observed in cardiac or respiratory function. The administration regimen of a bolus followed by a constant infusion was adequate to ensure continual protection against coagulation and thrombus formation during the procedure, thus the extracorporeal circuit remained patent for the 90 min of CPB. Optimal plasma levels of r-Hirudin to inhibit platelet deposition onto the artificial oxygenator membranes was achieved (Fig. 4). Unfortunately the ACT which is used to monitor the anticoagulation response on site during CPB, is too sensitive to use for r-Hirudin. In future the approach towards monitoring r-Hirudin on site would be to add recombinant tissue factor to the celite ACT system.³⁰ Bacher *et al.*³¹ found that the CaTT is the most accurate assay to monitor r-Hirudin in the laboratory. This did not prove to be the case in this study, possibly because of the relatively high r-Hirudin concentration in the plasma.

It is important to note that haemodilution by the priming solution, which is represented by the decrease in the hematocrit (Fig. 1), was equivalent for the treatment groups, and that the hematocrit remained at a constant level thereafter. It is therefore reasonable to assume that the changes observed in the other measurements were as a result of the CPB and the anticoagulant used.

Following the initial decrease in platelet count and circulating platelet radioactivity due to haemodilution, the platelet count decreased further when heparin was used, indicating platelet consumption by the biomaterials. This is supported by the results of accumulation of ^{111}In -labelled platelets in the oxygenator (Fig. 4) and the fact that markedly more platelets were trapped in the oxygenator after it was rinsed at the end of CPB. The increase in circulating platelet aggregates

and the marked increase in TAT complex formation, suggestive of thrombin formation, further support these findings. In contrast, when r-Hirudin was used, the platelet count and circulating platelet radioactivity remained unchanged after the initial decrease. In addition, only a small amount of platelets accumulated in the oxygenators, very few platelets were present after the oxygenators were rinsed at the end of the procedure and there was no significant change in the number of circulating platelet aggregates. Although statistically significant, the increase in TAT complex levels in venous blood after CPB with r-Hirudin has no clinical relevance, and may be the result of *in vivo* activation of the coagulation system.

There are several possible reasons why heparin is not as effective as r-Hirudin in inhibiting thrombin formation and platelet deposition. These include: (a) heparin may contain fractions that activate platelets directly, thereby promoting thrombogenesis;³² (b) the release of heparin neutralising proteins, such as heparinase and platelet factor 4, may inactivate heparin locally at the surface of thrombi;³³ (c) the inactivation of thrombin which is bound to other components in a thrombus may be prevented by steric or ionic hindrance of the heparin-AT III complex;³⁴ fibrin monomers can protect thrombin from inactivation by the heparin-AT III complex;² (d) binding of heparin to vitronectin, fibronectin and other plasma proteins limits the amount available for anticoagulation;³ and (e) platelets within a thrombus can be activated by meizothrombin, which is not inactivated by the heparin-AT III complex.³⁵

It is of interest to note that the radioactivity quantified in the spleen decreased almost equivalent to the decrease in blood radioactivity and by haemodilution. This was expected, because platelets in the circulation are in equilibrium with those pooled in the spleen and pooled platelets can be mobilised freely.³⁶ Since platelets in the liver are present in the blood compartment of the liver,³⁷ it was expected that the radioactivity in the liver should decrease in parallel with blood radioactivity. However, hepatic radioactivity remained relatively constant, thereby indicating that labelled platelets accumulated in the liver. It is possible that activated circulating platelets were removed from the circulation by the liver. These platelets can be sequestered and permanently removed from the circulation. It is also possible that at least some of the platelets transiently accumulate in the liver, similar to

the "collection injury" that is seen when freshly labelled platelets are re-injected.³⁸ This "activation injury" can be reversed by an unknown mechanism and "rejuvenated" platelets are again released into the circulation.

The decrease in AT III levels during CPB with heparin is not surprising and can be attributed to the consumption of AT III by heparin to inactivate thrombin.³⁹ The increase in AT III levels after CPB with r-Hirudin is difficult to explain. Similar results were obtained in patients undergoing haemodialysis with r-Hirudin as anticoagulant.⁴⁰ It might be caused by a response of the coagulation system to an intervention or trauma.

In conclusion, we demonstrate here that r-Hirudin is superior to heparin during CPB. Although adequate anticoagulation was achieved with both anticoagulants, we showed that thrombin formation and platelet deposition in the oxygenator were significantly inhibited with r-Hirudin. The effective inhibition of platelet-mediated thrombotic processes has important implications: there is a decreased risk of end organ damage by micro-aggregates and of oxygenator occlusion by micro-emboli which assures more effective oxygenation because of a larger surface area available for gas exchange. In addition, r-Hirudin can be useful in patients with an AT III deficiency, heparin-induced thrombocytopenia, heparin-provoked thrombosis, heparin resistance, or patients who are allergic to protamine sulphate. It is also possible that patients who exhibit the heparin rebound phenomenon or who bleed excessively from heparin may better tolerate r-Hirudin as an anticoagulant. We conclude therefore that r-Hirudin has the potential of being a useful alternative and superior anticoagulant to heparin. In the case of an overdose of r-Hirudin or during renal failure it is possible to lower the r-Hirudin plasma level quickly and effectively by means of hemofiltration.²³

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Kinetics and Sites of Destruction of ^{111}In -Oxine-Labeled Platelets in Idiopathic Thrombocytopenic Purpura: A Quantitative Study

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Kinetics and quantification of the sites of destruction of ^{111}In -indium-oxine-labeled autologous platelets were investigated in eight patients with idiopathic thrombocytopenic purpura. The mean platelet count was $17 \pm 9 \times 10^9/\text{liter}$; platelets were separated by differential centrifugation and labeled with $5.6 \pm 2.5 \text{ MBq } ^{111}\text{In}$. Whole body and organ ^{111}In -platelet distribution was quantitated with a scintillation camera and a computer-assisted imaging system acquisition matrix. Areas of interest were selected with the computer and organ ^{111}In -radioactivity expressed as a percentage of whole body activity. Mean platelet survival was $49.5 \pm 29.6 \text{ hr}$ and the survival curves were exponential. Equilibrium percentage organ ^{111}In -radioactivity was (normal values in parentheses): spleen 33.7 ± 8.8 (31.1 ± 10.2); liver 16.1 ± 9.5 (13.1 ± 1.3); thorax 22.8 ± 3.7 (28.2 ± 5.6). Percentage organ ^{111}In -activity at the time when labeled platelets had disappeared from the circulation was: spleen 44.5 ± 16.4 (40 ± 16); liver 16.0 ± 11.5 (32.4 ± 7.2); thorax 19.7 ± 6.0 (17.7 ± 10.3). Thorax activity corresponds to bone marrow radioactivity. Three patterns of platelet sequestration were evident. Three patients had mainly splenic sequestration, two mainly hepatic sequestration, and three diffuse reticuloendothelial system sequestration with a major component of platelets destroyed in the bone marrow. Splenectomy was performed in two patients. The pattern of ^{111}In -platelet sequestration was not predictive of response of glucocorticoid therapy or indicative of the necessity for splenectomy. Quantitative ^{111}In -labeled autologous platelet kinetic studies provide a new tool for the investigation of platelet disorders.

Key words: platelet kinetics, Indium, immune thrombocytopenic purpura

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INTRODUCTION

Chronic idiopathic (or immune) thrombocytopenic purpura (ITP) is a disease of unknown etiology, not associated with platelet alloantibodies or drugs, characterized by thrombocytopenia caused by the patient's production of an antibody directed against a platelet-associated antigen. In normal subjects, platelets cleared from the circulation are sequestered in the liver, spleen [1], and reticuloendothelial component of the bone marrow [2]. In ITP ^{51}Cr -labeled platelet destruction in vivo is mainly splenic, indeterminate, or hepatic, as assessed by relative changes in surface radioactivity [1]. None of the isotope techniques that have previously been employed have permitted quantitative studies of the absolute percentage of labeled platelets sequestered in the spleen and liver. This is a result of both the low labeling efficiency of ^{51}Cr for platelets and the low gamma emission of 9% of this radionuclide, which make it unreliable for external organ localization with a scintillation camera.

$^{111}\text{Indium-8-hydroxyquinoline}$ ($^{111}\text{In-oxine}$) is an efficient platelet label [3], and the isotope has 89 and 94% gamma emission per disintegration for the 173 and 247 keV photon energies, respectively. These physical characteristics permit quantitative scintillation camera imaging of the in vivo distribution of $^{111}\text{In-oxine}$ -labeled platelets [2,4].

We report on the exploitation of the technology of a scintillation camera interfaced with a computer-assisted imaging system for the quantitative study of kinetics, distribution, and sites of destruction of $^{111}\text{In-oxine}$ -labeled autologous platelets in eight patients with ITP.

MATERIALS AND METHODS

Patients

Eight patients (subjects 1 to 8) with ITP, and not on any treatment, gave informed, written consent for an investigation that was approved by the Ethical Committee of the Province and the University of the Orange Free State. ITP was diagnosed on the basis of thrombocytopenia of undetermined etiology, normal or increased megakaryocytes in the bone marrow, and the absence of a demonstrable cause of platelet destruction or utilization. The acute self-limiting entity seen in children was excluded.

Patients were treated with methylprednisolone, 48 mg per day, and if they responded, the glucocorticoid dose was gradually reduced over several weeks. Splenectomy was performed if improvement did not occur within 2 weeks (patient 4) or if the maintenance dose of methylprednisolone was more than 8 mg per day (patient 8).

Relevant details of the patients are given in Table I. These patients have been observed for less than 2 years, so a final verdict on the permanence of the remissions and final outcome or requirement for splenectomy is not possible. The present state is as follows. The two splenectomized patients are in complete clinical and hematologic remission. The results in the patients treated only with methylprednisolone are variable. Patients 1, 5, and 6 have normal platelet counts and are not on maintenance steroid therapy. Patients 2, 3, and 7 have no significant haemorrhagic symptoms and are in partial hematologic remission on low doses of prednisolone.

Reagents

$^{111}\text{In-oxine}$ (Radiochemical Centre, Amersham, U.K.) supplied as a 1 ml isotonic solution of 0.75% NaCl containing 37 MBq (1 mCi) of ^{111}In complexed with 50 μg

TABLE 1. Relevant Patient, Laboratory and ¹¹¹In-Platelet Kinetic Data

Subject	Sex/Age (years)	Blood platelets ($\times 10^{-9}$ /liter)	Serum platelet antibody	¹¹¹ In-oxine administered MBq (μ Ci)	Platelets injected ($\times 10^{-9}$)	Recovery in circulation (%)	Platelet survival (hr)	Platelet turnover (μ l/hr)	Length of follow-up (months)	Recent blood platelet count ($\times 10^{-9}$ /liter)	Therapy
1	Male, 32	24	Positive	5.6 (150)	26.5	47	20.1	2,286	15	166	Steroids
2	Female, 16	15	Negative	5.6 (150)	44.9	60	24.1	934	13	90	Steroids
3	Male, 15	35	Negative	4.6 (123)	6.9	51	30.7	2,012	12	110	Steroids
4	Female, 16	15	Positive	4.0 (108)	25.9	30	93.0	484	21	452	Splenectomy
5	Male, 19	< 10	Positive	11.4 (309)	7.3	47	64.9	295	16	265	Steroids
6	Female, 16	< 10	Negative	4.8 (130)	41.1	30	91.4	328	22	242	Steroids
7	Female, 57	20	Negative	3.7 (100)	28.4	72	31.9	784	6	68	Steroids
8	Female, 26	< 10	Positive	4.9 (131)	11.0	92	40.1	244	24	414	Splenectomy
Mean (± 1 SD)											
		17 $\pm$ 9	—	5.6 $\pm$ 2.5 (150 $\pm$ 67)	24 $\pm$ 14.6	54 $\pm$ 21	49.5 $\pm$ 29.6	921 $\pm$ 799			—
Reference values (± 1 SD)											
		270 $\pm$ 50	Negative	—	—	72 $\pm$ 15.7	232 $\pm$ 17	1,594 $\pm$ 244			—

8-hydroxyquinoline, 6 mg Hepes buffer, and 100 μ g Polysorbate 80, was kindly donated by the manufacturers. Other reagents were obtained from commercial sources and were of analytical grade.

Labeling of Platelets

This was performed as previously described [2]. Briefly, 475 ml of blood was collected in a double-pack closed bag system containing ACD NIH formula A as anticoagulant. Platelet-rich plasma (PRP) was obtained by centrifugation at 300g for 15 min, and the PRP was transferred to polystyrene tubes. After acidifying to pH 6.2–6.5 with ACD, platelets were sedimented by centrifuging at 800g for 15 min, resuspended in physiological saline, incubated for 30 min with ^{111}In -oxine, washed once with platelet-poor autologous plasma (PPP), and finally resuspended in PPP.

Less than 11.4 MBq (309 μCi) of ^{111}In was administered to a patient (Table I). Labeled platelets were used for in vivo studies only if they aggregated satisfactorily with collagen in vitro [5].

Platelet Survival Studies

Platelet survival studies were performed on whole blood specimens as recommended by ICSH [6]. Blood samples were collected at 5, 15, 30, 45, 60, 90, and 180 min after reinjection of labeled platelets and thereafter twice daily. Blood volume was estimated [7]. Platelets were counted by phase microscopy [8]. Mean autologous platelet survival was calculated by least-squares computer fitting to a gamma function [9]. All data points were used until 20% of the zero time radioactivity was reached. Platelet recovery in the circulation at equilibrium was derived by back extrapolation of the platelet survival curve to zero time. Platelet turnover was calculated from the blood platelet count and the platelet intravascular life span and corrected for the exchangeable splenic platelet pool [10].

Image Acquisition and Quantitation of Platelet Distribution

This was performed with a large field of view Searle Pho Gamma scintillation camera interfaced with a Medical Data Systems A² computer-assisted imaging system acquisition 64 $\times$ 64-matrix. The methods were modified from a previous study with another scintillation camera system [2].

Geometry was maintained by using the xiphisternum and anterior iliac spines as reference points. Anterior and posterior whole body ^{111}In -radioactivity was measured at equilibrium and daily by obtaining four images by positioning the camera so as to encompass the whole of the patient. Regions of interest were selected by computer analysis to determine specific organ or regional activity from the scintillation camera images. The thorax and upper abdominal region were subdivided into the heart, liver, and spleen. The thorax radioactivity is that of the thoracic region remaining after subtraction of cardiac radioactivity and would thus include the lungs and bony cage.

Whole body and region of interest radioactivity counts were corrected for attenuation by calculating the geometrical mean—ie, the square root of the product of the anterior and posterior counts. The corrected counts calculated by the geometrical mean method represent the radioactivity of the organ as if it were situated in the midanterior-posterior plane of the body.

The initial (equilibrium) radioactivity was derived by back extrapolation of least-square regression analysis of data, and the final organ radioactivity was determined at the patient's platelet survival time.

RESULTS

Platelet Recovery and Survival in the Circulation and Turnover

As shown in Table I, platelet recovery in the circulation was variable, and the mean of 54% did not differ significantly ($0.1 > P > 0.05$; Student's *t* test) from normal. Platelet survival was shortened, varying from 20.1 to 93.0 hr with a mean of 49.5 ± 29.6 hr. The shapes of the survival curves were exponential, and in all patients the platelets were removed from the circulation after only one "hit" in the gamma function model. Mean platelet turnover was 58% that of the reference value and was markedly lower than normal in all but patients 1 and 3.

Early Distribution of ¹¹¹In-Platelets in Organs

At equilibrium mean ¹¹¹In-platelet activity in the liver, spleen, and in the thoracic area corresponded to that of normal subjects (Table II). However, hepatic radioactivity varied widely between patients. Two patients (2 and 8) had considerably less and three patients (1, 4, and 5) had more liver radioactivity than normal. Splenic ¹¹¹In-activity of the ITP patients was very similar to that of the normal group.

Final Organ ¹¹¹In-Platelet Sequestration Pattern

The results are given in Table II. Organ ¹¹¹In-radioactivity is expressed in two ways: as a percentage of equilibrium whole body ¹¹¹In-activity assumed to be 100%, and as a percentage of ¹¹¹In-radioactivity present in the whole body at the patient's platelet survival time (figures in parentheses).

The patients could be separated into three groups on the basis of the major organ or region of ¹¹¹In-radioactivity accumulation: three with a splenic, two with a hepatic, and three with a reticuloendothelial system (RE system) pattern of platelet destruction. These distribution patterns are illustrated with scintillation camera images (Figs. 1-3).

Whole body ¹¹¹In-radioactivity decreased in six of the eight patients and reached a mean of $89.4 \pm 7\%$ at the time of platelet survival in the circulation. This is significantly ($0.01 < P < 0.02$; Student's *t* test) less than in normal volunteers.

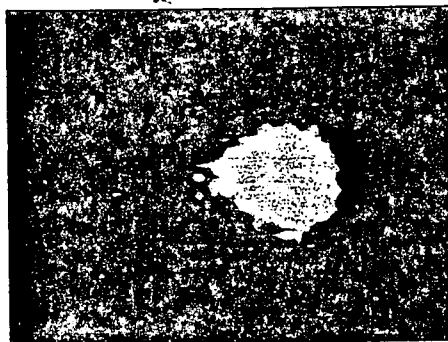


Fig. 1. A scintillation camera image of ¹¹¹In-oxine platelet distribution at the time of platelet disappearance from the circulation (patient 1). The pattern is that of splenic sequestration of labeled platelets. The liver is hardly visible.

TABLE II. Equilibrium and Final Regional and Organ ¹¹¹In-Radioactivity Distribution

Subject and pattern of sequestration	Equilibrium (% activity distribution)				Final (% activity distribution)				
	Liver	Spleen	Thorax	Whole body	Liver	Spleen	Thorax	Non-spleen (non-liver)	Whole body
Splenic									
1	20.4	39.3	22.5	100	10.7 ^a (11.4) ^b	55.6 (59.5)	18.3 (19.6)	33.7 (22.5)	93.4
2	5.4	27.7	19.2	100	4.6 (4.6)	61.1 (61.1)	23.8 (23.8)	34.4 (33.9)	99.6
3	14.1	48.5	22.9	100	10.8 (12.7)	72.4 (85.0)	1.9 (2.2)	16.8 (12.5)	85.2
Hepatic									
4	19.4	33.4	23.6	100	28.0 (33.0)	31.1 (36.6)	15.6 (18.4)	40.9 (15.4)	85
5	36.2	30.0	19.2	100	38.5 (39.1)	35.0 (35.5)	14.5 (14.7)	26.5 (23.9)	98.5
RE system									
6	13.1	27.7	19.2	100	17.2 (19.8)	30.0 (34.5)	24.2 (27.8)	52.8 (32.7)	87
7	11.4	21.5	27.6	100	10.5 (13.0)	30.2 (37.4)	21.3 (26.4)	59.3 (30.3)	80.7
8	9.4	41.3	28.5	100	8.0 (9.3)	40.3 (46.9)	29.1 (33.9)	51.7 (29.7)	85.9
Mean ± 1 SD	16.1 ± 9.5	33.7 ± 8.8	22.8 ± 3.7	100 ± 0	16.0 ± 11.5 (17.9 ± 12.1)	44.5 ± 16.4 (49.6 ± 17.9)	19.7 ± 6.0 (20.9 ± 9.7)	39.5 ± 14.5 (22 ± 15.2)	89.4 ± 7
Reference ± 1 SD	13.1 ± 1.3	31.1 ± 10.2	28.8 ± 5.6	100	32.4 ± 7.2 (33 ± 7.4)	40.0 ± 16 (40.7 ± 16.2)	17.7 ± 10.3 (18.1 ± 10.5)	27.6 ± 16.2 (24.3 ± 16.3)	98 ± 1

^aPercentages are expressed relative to equilibrium whole body ¹¹¹In-radioactivity assumed to be 100%.^bPercentages in parentheses are expressed relative to the whole body ¹¹¹In-radioactivity present at the patient's survival time.

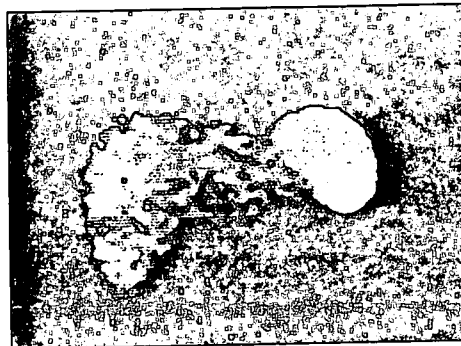


Fig. 2. A scintillation camera image of ^{111}In -oxine platelet distribution at the time of platelet disappearance from the circulation (patient 5). The pattern shows prominent hepatic sequestration of labeled platelets. The spleen is clearly visible.

DISCUSSION

The exploitation of ^{111}In -oxine as a platelet label, in conjunction with the modern technology of a computerized scintillation camera system, allows a new quantitative approach to the study of platelet kinetics and organ distribution of labeled platelets [2,4]. Some aspects of the technique, such as preparation of platelets, variation in size of viscera, and blood volume, are difficult or impossible to control and may influence the results. We acknowledge that geometrical complexities make surface counting as an estimate of organ radioactivity subject to error. We have minimized geometrical and attenuation errors by adopting the geometrical mean method. With this method we have shown that quantifications of organ radioactivity in vivo and in removed postmortem specimens correspond within 5% in baboons [11]. Although our method is now more accurate than that previously described [2], the interpretation of our data should take the technical problems into account.

Platelet survival in our patients was shortened, the disappearance curves fitted that of an exponential function, and the random destruction is reflected by the sequestration of platelets after one "hit." The advantage of ^{111}In -oxine over ^{51}Cr -sodium chromate as a platelet label is evident in this study. Autologous platelets could be labeled, despite the severe thrombocytopenia. This eliminates the effect of alloantibodies, present in 19% of patients, induced by pregnancy and transfusion, on allogenic platelet survival [12].

Platelet recovery in the circulation at equilibrium was low in our patients (Table I). This is probably due to rapid destruction of a population of the transfused platelets, since the pooling of platelets in the spleen remains normal in ITP [13,14]. Platelet turnover in all our patients but two was markedly reduced. This is at variance with the results of other investigators, who have reported normal or increased platelet turnover [13,14]. We have no ready explanation for this discrepancy. The labeled platelets were not significantly contaminated with plasma, red cells, or lymphocytes. It is possible that the difference is due to the interpretation of the platelet disappearance curves, but this is unlikely. In our patients the curves fitted an exponential function and there was no evidence of a long "tail" which may have influenced the calculation of platelet sur-

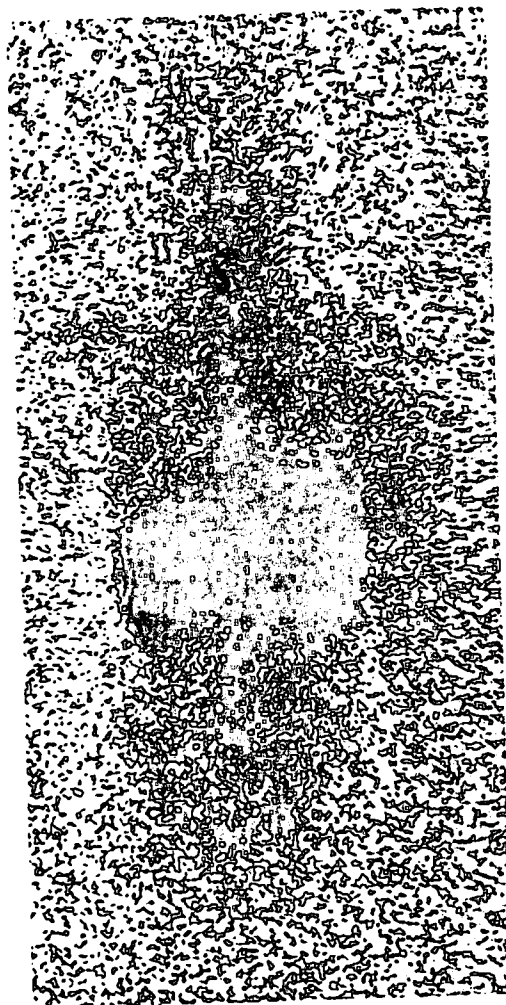


Fig. 3. A whole body scintillation camera image of ^{111}In -oxine platelet distribution at the time of platelet disappearance from the circulation (patient 8). The pattern is that of diffuse reticuloendothelial system sequestration of labeled platelets. The spleen and liver are evident. The presence of radioactivity in the areas of bone marrow distribution is prominent.

vival. Baldini [15] has pointed out that antiplatelet antibodies may technically alter results of ^{51}Cr -labeled platelet survival. The antibody can cause release of the label in vivo in rabbits [16], as well as in man [15]. These results suggest that platelet survival measured by the ^{51}Cr method may be falsely reduced in some patients with ITP and thus give a relatively high estimate of platelet turnover. A possible explanation for our results may be that less ^{111}In -oxine than ^{51}Cr is released by antiplatelet antibody. Our study was done with autologous platelets, a technique that is not possible with ^{51}Cr in severe thrombocytopenia. One may speculate that these circulating platelets are relatively resistant to antibody injury and thus have longer survival times. Obviously, our finding needs confirmation and further investigation.

This study provides some quantitative data on the sequestration pattern of platelets in ITP. Whole body ¹¹¹In-radioactivity decreased significantly in six of our eight patients (Table II), whereas in normal subjects this remained virtually constant [2]. We have referred to the possible release of radiolabel by antiplatelet antibody, and these results would support the *in vivo* loss of some ¹¹¹In-oxine in ITP. Unfortunately, we did not collect urine and stools in these studies, and the avenue of ¹¹¹In loss from the body remains unknown. This *in vivo* loss of radionuclide influences quantification of organ ¹¹¹In-radioactivity if this is expressed relative to the initial whole body activity. We have thus also calculated organ radioactivity relative to whole body radioactivity at the time of platelet disappearance from the circulation (Table II). It is, however, evident that one cannot accurately interpret these data unless it is known whether the ¹¹¹In-label is released from platelets in the circulation or if it is released after they have been sequestered in the reticuloendothelial system.

In studies with ⁵¹Cr-tagged platelets, it has been shown that the spleen is the apparent site of platelet destruction in 59%, and the liver in 14% of cases with ITP. In 27% the site of destruction is indeterminate [1]. None of the techniques that have been employed in the past permitted calculation of the absolute percentage of injected labeled platelets sequestered in the liver or spleen. We recognize three patterns of platelet sequestration in our patients.

In three patients the major site of platelet destruction was the spleen (Table II, Fig. 1). Splenic sequestration in these patients occurred at the expense of hepatic destruction (patients 1 and 2) or the RE system (patient 3). In two patients hepatic ¹¹¹In-radioactivity accumulation was prominent (Table II, Fig. 2), and thoracic and especially splenic platelet destruction was relatively slight. The third pattern of platelet destruction was that of diffuse RE-system sequestration and was observed in three patients (Table II, Fig. 3). We suggest that in this group the bone marrow is the major site of platelet destruction. The accumulation of platelets in the thorax at the end of the study consists almost exclusively of radioactivity present in the bony cage of the thorax. In postmortem and *in vivo* studies in baboons we found no ¹¹¹In-radioactivity in the lungs at the end of the platelet life span, and all residual radioactivity of this region was present in the bone marrow [11]. This is in accord with the findings of other workers who have not been able to show destruction of platelets in the lung [17,18]. The scintillation camera data and percentage of radioactivity accumulation thus reflects the phagocytic activity of the reticuloendothelial component of the bone marrow. This is evident on examination of the scintillation camera image (Fig. 3). It is clear that our methodology of quantification would underestimate this component of platelet sequestration, since the activity of the pelvic bones, skull, and proximal long bones are not taken into account. The diffusely spread nature of the bone marrow makes quantitative studies in these regions impossible, since we have not been able to obtain high enough radioactivity count statistics in these organs for quantitative data. We have shown that there is no significant utilization of platelets in normal vasculature [2], but this may be different in thrombocytopenic subjects [19]. It does, however, seem reasonable to assume that the major sites of platelet destruction are the spleen, liver, and bone marrow. The calculated nonsplenic, nonhepatic component of platelet destruction in our patients (Table II) shows that the bone marrow is apparently a major site of platelet sequestration in ITP. This is clearly so in the diffuse RE-system destruction pattern group of patients, where more than 50% of platelets are sequestered in bone marrow. The importance of the bone marrow as a site of platelet sequestration in ITP will also be

underestimated in a study with reinfused labeled platelets, since the possible intramedullary destruction of platelets immediately after release from megakaryocytes is not taken into account.

The clinical importance of ^{51}Cr -labeled platelet studies in the therapeutic evaluation of ITP is controversial. Najean et al [18,20] concluded that in patients where splenic sequestration can clearly be demonstrated, splenectomy will lead to remission. Aster and Keene [21], in contrast, suggest that the site in which labeled cells are sequestered appears related to the severity of the disease at the time of the study. Our series is far too small for evaluation of therapeutic response relative to the platelet sequestration pattern, but some generalizations may be made (Tables I and II). Splenectomy was not necessary in any of the three patients with a splenic pattern of platelet sequestration; all responded to glucocorticoid therapy. The two patients (patients 4 and 8) in whom splenectomy was performed had a hepatic and a RE-bone marrow sequestration pattern, respectively. Platelet survival, recovery in the circulation, and the presence of a PF3 releasing serum antibody could not be related to the platelet sequestration pattern or the therapeutic response in our patients. Our findings are thus in agreement with the conclusions of others [1,22,23] that isotope studies are of little value in predicting the benefit of splenectomy in ITP and that the decision for or against splenectomy must be based upon clinical criteria such as the response to glucocorticoids and the duration and severity of the thrombocytopenia.

The development of these new techniques for the study of platelet kinetics in ITP provides new insights into the patterns of platelet sequestration in this disorder. It is especially evident that the role of the bone marrow in this regard has been underestimated in the past. Exploitation and refinement of this methodology will lead to a better understanding of platelet kinetics in health and disease.

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Platelet Turnover and Kinetics in Immune Thrombocytopenic Purpura: Results With Autologous ^{111}In -Labeled Platelets and Homologous ^{51}Cr -Labeled Platelets Differ

By Anthon du P. Heyns, Philip N. Badenhorst, Mattheus G. Lötter, Henry Pieters, Paula Wessels, and Harry F. Kotzé

Mean platelet survival and turnover were simultaneously determined with autologous ^{111}In -labeled platelets (^{111}In -AP) and homologous ^{51}Cr -labeled platelets (^{51}Cr -HP) in ten patients with chronic immune thrombocytopenic purpura (ITP). In vivo redistribution of the ^{111}In -AP was quantitated with a scintillation camera and computer-assisted image analysis. The patients were divided into two groups: those with splenic platelet sequestration (spleen-liver ^{111}In activity ratio >1.4), and those with diffuse sequestration in the reticuloendothelial system. The latter patients had more severe ITP reflected by pronounced thrombocytopenia, decreased platelet turnover, and prominent early hepatic platelet sequestration. Mean platelet life span estimated

with ^{51}Cr -HP was consistently shorter than that of ^{111}In -AP. Platelet turnover determined with ^{51}Cr -HP was thus overestimated. The difference in results with the two isotope labels was apparently due to greater in vivo elution of ^{51}Cr . Although the limitations of the techniques should be taken into account, these findings indicate that platelet turnover is not always normal or increased in ITP, but is low in severe disease. We suggest that this may be ascribed to damage to megakaryocytes by antiplatelet antibody. The physical characteristics in ^{111}In clearly make this radionuclide superior to ^{51}Cr for the study of platelet kinetics in ITP.

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PLATELET turnover is generally regarded as normal or increased in chronic immune thrombocytopenic purpura (ITP).¹⁻⁴ However, the estimate of the extent of the increase in platelet turnover varies from a mean of 4.9 times normal¹ to 2.3 times normal.² This difference has been ascribed to difficulties to accurately estimate of platelet life span in ITP.⁵ Because the calculation of the platelet turnover is dependent on the estimate of the platelet life span, it is clear that the result of the latter is critical.

The ^{51}Cr labeling technique⁶ is used in most laboratories for the determination of platelet survival. This radionuclide has a low binding efficiency for platelets and, in ITP with severe thrombocytopenia, it is impossible to determine the life span of autologous labeled platelets by this method. ^{111}In -oxine is a superior platelet label,⁷ and its high gamma photon yield facilitates quantitative imaging of the in vivo distribution of labeled platelets.⁸ ^{111}In labels platelets with high efficiency, permitting kinetic studies with autologous platelets even in the presence of severe thrombocytopenia.^{9,10}

We have further investigated our previous finding that platelet turnover is not always increased in ITP⁹ and now relate this to the pattern of platelet sequestration in the disease. We compared the results of estimates of platelet life span with ^{111}In -oxine-labeled autologous platelets (^{111}In -AP) with that determined by ^{51}Cr -labeled homologous platelets (^{51}Cr -HP). The results differed; this influenced the estimates of platelet turnover. Turnover estimated with ^{111}In -AP was usually increased in those patients with a splenic sequestration pattern, but was decreased in patients in whom ^{111}In -AP were destroyed diffusely in the reticuloendothelial system

(RES). In ITP it seems that the ^{51}Cr labeling method spuriously reduces the estimate of mean platelet survival time, and platelet turnover is therefore erroneously overestimated.

MATERIALS AND METHODS

Patients

Ten patients (patients 1 to 10) with ITP, and not on any treatment, gave informed, written consent to participate in a study approved by the Ethical Committee of the University of the Orange Free State. ITP was diagnosed on the basis of conventional clinical criteria: adult-onset thrombocytopenia of undetermined etiology, normal or increased numbers of bone marrow megakaryocytes, and the absence of a demonstrable cause of platelet destruction or utilization such as systemic lupus erythematosus, drug ingestion, or hypersplenism. Relevant patient details are given in Table 1.

Platelet Studies

Autologous platelets were labeled with ^{111}In -oxine (Radiochemical Centre, Amersham, England), fully described elsewhere.^{10,11} In brief, 200 to 300 mL of blood was collected in syringes containing acid-citrate dextrose NIH formula A (ACD). Platelet-rich plasma was prepared by centrifugation and was acidified with ACD to pH 6.2–6.5. The maximum number of platelets was harvested from the blood by washing platelets from the red cell layer four times. Platelets were labeled with ^{111}In -oxine in a saline medium, resuspended in autologous platelet-poor plasma, and reinjected only if they aggregated normally with ADP. Homologous platelets were labeled with ^{51}Cr -disodiumchromate as recommended by the International Committee for Standardization in Hematology.⁶ ^{111}In -AP and ^{51}Cr -HP were injected simultaneously. Contamination of the platelet product with ^{111}In or ^{51}Cr -labeled plasma proteins and red cells was measured.⁶

In vivo distribution of ^{111}In -AP was quantitated with a scintillation camera and a computer-assisted imaging system as previously described.^{8,11} Whole body and region of interest (ROI) ^{111}In -radioactivity was corrected for attenuation by the geometrical mean method, and ROI activity was expressed as a percentage of whole body activity. The initial (equilibrium) radioactivity was derived by back extrapolation of linear least-squares regression analysis of data, and the final organ radioactivity was determined at the patient's own platelet survival time.

Platelet survival was estimated from whole blood specimens collected at 5, 15, 30, 45, 60, 90, and 180 minutes after reinjection of

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Table 1. Patient Data and In Vivo Organ ^{111}In Radioactivity at Zero Time

Patients and Pattern of Platelet Sequestration	Sex/Age (yr)	Number of Pregnancies	Blood Platelet Count ($\times 10^9/\text{L}$)	^{111}In Activity in Spleen (% of Whole Body)	^{111}In Activity in Liver (% of Whole Body)
Splenic					
1	F/16	0	18	32.1	25.9
2	F/34	1	95	29.8	12.5
3	F/68	9	88	22.1	12.9
4	F/16	0	119	46.9	12.3
5	F/25	0	60	33.5	23.9
Mean $\pm$ 1 SD			76 $\pm$ 39	32.8 $\pm$ 9.8	15.8 $\pm$ 6.7
Diffuse Res					
Normal recovery					
6	F/20	0	8	20.6	40.1
7	F/19	0	4	8.8	39.6
8	M/20	—	5	22.2	46.8
Low recovery					
9	M/20	—	5	22.4	47.8
10	F/16	0	7	32.9	38.5
Mean $\pm$ 1 SD	—	—	6 $\pm$ 2	21.4 $\pm$ 8.6	42.6 $\pm$ 4.4
Reference values	—	—	150–400	31.1 $\pm$ 6.1	9.6 $\pm$ 1.2

labeled platelets and thereafter as necessitated by the rate of disappearance of platelets from the circulation.⁶ Blood volume was estimated,¹² and platelets were counted by phase microscopy, or, if more than $30 \times 10^9/\text{L}$, with an electronic particle counter. Mean platelet survival was calculated by a nonlinear, least-squares curve-fitting technique. A computer program fitted the data to the following mathematical models: weighted mean,⁶ multiple hit,¹³ Dornhorst,¹⁴ Meuleman,¹⁵ polynomial,¹⁶ and α order.¹⁷ All data points lying between equilibrium or maximum blood radioactivity, and 20% of this activity, were included. Curve fitting was visually inspected with a graphics display monitor. Goodness of fit of each of the mathematical models to the data was evaluated by calculating the SD of the data points around the regression curve. There was no significant difference in the goodness of fit with these models.¹⁸ Therefore, only the results of the mean platelet survival time estimated with the multiple hit model are presented. In all patients, the clearance of platelets from the circulation was exponential, reflected by removal of platelets after a single "hit."

Platelet recovery in the circulation at equilibrium was derived by back extrapolation of the platelet survival curve to zero time. Platelet turnover was calculated from the blood platelet count and the platelet intravascular life span.¹ The correction factor for the splenic pool was 90% divided by the percentage of ^{111}In -AP quantitated to be present in the spleen at equilibrium.

Platelets from four normal subjects were labeled with ^{111}In and ^{51}Cr as described above. Platelet subpopulations in the platelet-rich plasma were separated by density on discontinuous arabinogalactan (stractan) gradients as described by Corash et al.,¹⁹ and the radioactivity was measured. Radioactivity in the four buoyant density subpopulations was expressed as a percentage of that of the total. Mean platelet volume was measured electronically with a multi-channel analyzer.¹⁹

Elution of Radionuclide From Labeled Platelets

The in vivo release of ^{51}Cr and ^{111}In from platelets was measured by determining the radioactivity present in platelet-poor plasma of all the specimens collected for the estimation of mean platelet life span. This radioactivity, not associated with platelets or other cells, was expressed as a percentage of ^{111}In or ^{51}Cr whole blood radioactivity at equilibrium, normalized to 100%.

Statistics

The normal distribution of the data was assessed with the Shapiro-Wilk test. Paired or unpaired data were evaluated with either the *t*-test for different means or the nonparametric Wilcoxon's rank sum test where appropriate. The relationship between two variables was expressed as the correlation coefficient, *r*, and its significance was evaluated with the *t* test.

The percentage SD of the platelet turnover owing to the propagation of errors introduced by the measurements of the platelet count, the platelet life span, and the estimate of the splenic platelet pool size was computed by addition in quadrature of the fractional SDs.²⁰

RESULTS

Pattern of ^{111}In -Labeled Platelet Destruction

The results of quantification of organ ^{111}In radioactivity at the end of platelet life span are given in Table 2.

The patients were separated into two groups on the basis of major organ or regional ^{111}In -AP radioactivity accumulation: five with a splenic sequestration pattern, and five with a diffuse pattern of platelet destruction in the RES. The distinction was based on the percentage of ^{111}In -AP in the spleen and the liver at the end of the platelet life span. The normal spleen-liver ratio is 1.4 ± 0.6 . Those patients with a spleen-liver ratio ≥ 1.4 were classified as having "splenic sequestration," and those with a lower ratio were classified as having "diffuse RES sequestration." Two patients (9 and 10) of the latter group were segregated as a subgroup because they had exceptionally low recovery values and, as compared with others in this group, had relatively long platelet survival times (Table 3).

In the group with a splenic sequestration pattern, the mean spleen ^{111}In activity was $50.8\% \pm 13.6\%$, and that of the liver was $19.4\% \pm 7.1\%$. Splenic activity was significantly higher ($P < .025$), and that of the liver was significantly lower ($P < .025$), than that of controls (*t* test). In the group with diffuse RES sequestration, the mean hepatic ^{111}In activity of

Table 2. Sequestration of ^{111}In -Labeled Platelets at the End of Platelet Life Span

Patient and Sequestration Pattern	Organ or Region ^{111}In Radioactivity (Whole Body Activity) (%)			Ratio	
	Spleen	Liver	Remainder*	Spleen-Liver Ratio	Spleen-Remainder Ratio
Splenic					
1	33.6	24.4	41.6	1.4	0.8
2	61.9	26.6	11.3	2.3	5.5
3	41.6	12.1	43.6	3.4	1.0
4	66.3	11.4	22.2	5.8	3.0
5	50.5	22.6	27.2	2.2	1.9
Mean $\pm$ 1 SD	50.8 $\pm$ 13.6	19.4 $\pm$ 7.1	29.2 $\pm$ 13.6	3.0 $\pm$ 1.7	2.4 $\pm$ 1.9
Diffuse RES					
Normal recovery					
6	21.0	41.1	37.9	0.5	0.6
7	9.9	40.0	49.9	0.3	0.2
8	21.7	44.8	33.4	0.5	0.6
Low recovery					
9	22.5	47.6	29.7	0.5	0.8
10	35.5	39.1	29.3	0.9	1.2
Mean $\pm$ 1 SD	22.1 $\pm$ 9.1	42.5 $\pm$ 3.6	36.6 $\pm$ 8.5	0.5 $\pm$ 0.2	0.7 $\pm$ 0.4
Reference values (n = 6)	37.2 $\pm$ 8.8	29.2 $\pm$ 9.6	31.7 $\pm$ 8.3	1.4 $\pm$ 0.6	1.3 $\pm$ 0.5

*The remainder is ^{111}In radioactivity located neither in the spleen nor liver. This may be regarded as approximately reflecting bone marrow activity.

42.5% $\pm$ 3.6% was significantly higher ($P < .0005$), and the mean splenic activity of 22.1% $\pm$ 9.1% was significantly lower ($P < .025$), than that of normal subjects (t test).

The ^{111}In radioactivity other than in the liver or the spleen, designated "remainder" (Table 2), varied from patient to patient and did not reflect the pattern of platelet sequestration. Although the spleen-remainder ratio was lower in the diffuse RES sequestration group, this ratio was too variable to distinguish patterns of platelet destruction.

Distribution of the In Vivo ^{111}In Platelet Activity at Equilibrium

In the group with diffuse RES destruction, mean hepatic ^{111}In activity was four times higher ($P < .005$, t test) and that of the spleen was significantly lower ($P < .025$, t test) than normal (Table 1).

Platelet Recovery, Life Span and Platelet Turnover

Blood platelet counts, mean platelet survival, recovery in the circulation, and platelet turnover measurements as determined with ^{111}In -labeled and ^{51}Cr -labeled platelets are given in Table 3.

^{111}In -labeled platelets. Patients were reinjected with a mean of 11.8 MBq $\pm$ 5.3 (319 $\mu\text{Ci} \pm 143$) ^{111}In , labeling $20.7 \times 10^8 \pm 22.1$ platelets. The blood platelet counts were much higher in the splenic sequestration group than in the diffuse RES sequestration group. A greater mean number of labeled platelets and more ^{111}In was thus reinjected per patient in the splenic sequestration group. Mean recovery of platelets in the circulation of all patients was 55% $\pm$ 25%, which was somewhat lower, but not significantly so ($P > .2$), than that of normal. In patients 6, 7, and 8, with diffuse sequestration of platelets, recovery was about normal. Patients 9 and 10 had very low recoveries.

Platelet size distribution in whole blood, platelet-rich

plasma, and the labeled platelet-rich saline eventually injected was determined in three patients. Although there was a loss of approximately one-half of the platelets during the isolation and labeling procedures, mean platelet volume did not change significantly: whole blood 16 ± 2 fL, platelet-rich plasma 17 ± 2 fL, and platelet-rich saline 15.5 ± 2 fL.

The mean platelet life span, range 4.3 to 65.9 hours, of ^{111}In -AP was decreased in all patients and correlated with the platelet count ($r = 0.58$, $P < .05$). The mean platelet life span of the diffuse RES sequestration group was shorter than that of patients with splenic sequestration. The results, however, were variable, and patients 9 and 10 had platelet survival times that were much longer than those of the others in this group.

Mean platelet turnover of all patients was 53×10^9 platelets per liter per day $\pm$ 49 (range 4 to 114). The mean platelet turnover of the splenic sequestration group was significantly higher ($P < .005$, Wilcoxon test) than normal. Platelet turnover in patients with a diffuse RES sequestration pattern was variable and was markedly low in patients 9 and 10.

^{51}Cr -labeled platelets. Patients were reinjected with a mean of 2.8 MBq $\pm$ 0.8 (84 $\mu\text{Ci} \pm 15$) ^{51}Cr , labeling $135.2 \times 10^8 \pm 57.3$ platelets. Mean platelet recovery in the circulation in all patients was 51% $\pm$ 21% and, although lower than normal, was not significant ($P > .5$). The mean recovery in the circulation of the diffuse RES sequestration group was much lower than normal in patients 9 and 10.

The mean life span of ^{51}Cr -HP was shortened to varying degrees (range 1.8 to 39.6 hours), and in the patients with diffuse RES sequestration it was shorter than in patients with splenic sequestration. Patients 9 and 10 were noteworthy exceptions. Mean platelet turnover of the whole patient group was normal, 93×10^9 platelets per liter per day $\pm$ 80 ($P > .1$). There was, however, a significant ($P < .005$, Wil-

Table 3. Isotope Data: Estimates of Platelet Recovery, Life Span, and Turnover

Patient and Sequestration Pattern	Blood Platelet Count ($\times 10^{-9}/L$)	^{111}In -Labeled Autologous Platelets					^{51}Cr -Labeled Homologous Platelets				
		Platelets Injected ($\times 10^{-9}$)	^{111}In Administered MBq(μCi)	Recovery in Circulation (%)	Life Span (h)	Turnover ($10^{-9}\text{pl}/L/\text{day}$)	Platelets Injected ($\times 10^{-9}$)	^{51}Cr -Administered MBq(μCi)	Recovery in Circulation (%)	Life Span (h)	Turnover ($\text{pl}/L/\text{day}$)
Splenic											
1	18	40.0	7.4(200)	51	5.0	115	180.8	3.2(120)	65	3.3	174
2	95	52.0	18.5(500)	65	65.9	44	132.0	4.1(150)	35	28.5	103
3	88	40.0	22.2(600)	76	20.8	117	122.8	3.0(112)	89	11.1	220
4	119	52.0	14.1(380)	58	37.6	129	130.1	4.1(150)	52	24.5	198
5	60	3.4	13.3(360)	79	35.6	55	67.1	2.4(90)	55	23.5	83
			15.1 $\pm$ 5.6					3.4 $\pm$ 0.7			
Mean $\pm$ 1 SD	76 $\pm$ 39	30.3 $\pm$ 24.8	(408 $\pm$ 151)	66 $\pm$ 12	33.0 $\pm$ 22.6	92 $\pm$ 39	126.6 $\pm$ 40.4	(124 $\pm$ 26)	59 $\pm$ 20	18.2 $\pm$ 10.6	156 $\pm$ 60
Diffuse RES											
Normal recovery											
6	8	3.5	9.3(250)	62	12.9	17*	83.7	2.7(100)	59	8.3	26
7	4	2.7	6.7(180)	88	4.3	22	106.1	2.7(100)	53	2.7	35
8	5	4.0	9.3(250)	40	7.2	19	82.0	2.2(80)	61	1.8	77
Low recovery											
9	5	4.5	5.6(150)	15	37.6	4	205.1	1.9(70)	16	24.6	6
10	7	5.2	11.1(300)	16	35.6	6	241.9	1.9(70)	28	39.6	6
Mean $\pm$ 1 SD		5.8 $\pm$ 1.6	8.4 $\pm$ 2.2	44 $\pm$ 31	19.5 $\pm$ 15.9	14 $\pm$ 8	144 $\pm$ 74.6	2.3 $\pm$ 0.4	43 $\pm$ 20	15.4 $\pm$ 16.3	30 $\pm$ 29
Reference values ($\pm$ 1 SD)	150-400	—	—	61 $\pm$ 12	226 $\pm$ 24	38 $\pm$ 6	—	—	68 $\pm$ 13	220 $\pm$ 24	35 $\pm$ 5

Abbreviation: pl, platelets.

*The percentage SD of platelet turnover introduced by the fractional SD of the platelet count (0.5), the platelet life span (0.1), and the estimate of the splenic pool size (0.06) was 26%.

coxon test) difference in the mean platelet turnover of the patients with different sequestration patterns.

Comparison of Mean Platelet Survival and Turnover Measurements With ^{111}In -AP and ^{51}Cr -HP

A mean of $8.0\% \pm 3.1\%$ ^{51}Cr and $7.5\% \pm 6.5\%$ ^{111}In was present in the plasma in which the labeled platelets were suspended. Contamination of labeled platelets with labeled red cells was low; $1.3\% \pm 1.1\%$ and $1.1\% \pm 1.6\%$ for ^{51}Cr and ^{111}In , respectively.

Mean survival of ^{111}In -AP was $26.3 \text{ hours} \pm 19.7$, and that of ^{51}Cr -HP was 16.8 ± 13 hours. This difference was significant ($P < .005$, Wilcoxon test). This difference was evident whether women patients had had previous pregnancies or not. The difference influenced the estimate of mean platelet turnover: with ^{111}In -AP it was 53×10^9 platelets per liter per day ± 49 , which was significantly ($P < .005$, Wilcoxon test) lower than the 93×10^9 platelets per liter per day ± 80 as estimated with ^{51}Cr -HP.

Labeling of Platelet Subpopulations

Labeling efficiencies with ^{111}In and ^{51}Cr of density-dependent platelet subpopulations separated on a stractan gradient, were similar ($P > .2$; t -test) (Table 4).

Plasma Radioactivity

The ^{111}In and ^{51}Cr radioactivity in platelet-poor plasma was measured for the duration of the platelet life span. The changes in radioactivity in plasma during the first nine hours after reinjection of labeled platelets are illustrated in Fig 1. ^{51}Cr activity was higher than that of ^{111}In ($P < .005$, t test) throughout the life span of the platelets, but the difference was more striking in the first few hours after reinfusion of labeled platelets.

DISCUSSION

It has generally been stated that platelet turnover is increased in patients with ITP, and that this increase is especially evident when the blood platelet count is $<50 \times 10^9/\text{L}$,¹⁻⁵ but not all authors agree.^{9,21-24} A major difficulty thwarting the clarification of the problem is the inaccuracy of the measurement of platelet turnover in ITP. In ITP, it is difficult to measure three of the variables used in calculating platelet turnover: mean platelet life span, recovery of platelets in the circulation, and the blood platelet count. We have exploited the superior physical characteristics of ^{111}In and its

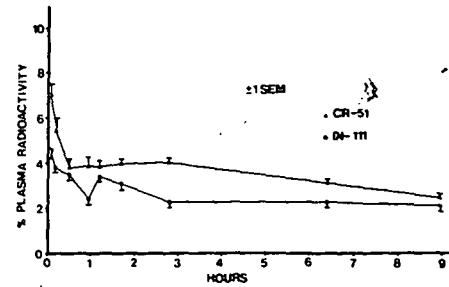


Fig 1. Plasma ^{111}In and ^{51}Cr radioactivity of blood samples taken to measure mean platelet survival time. ^{51}Cr plasma activity was significantly ($P < .005$) higher than that of ^{111}In . This is presumably due to in vivo elution of the label.

high platelet labeling efficiency to minimize these difficulties. This radionuclide makes it possible to determine platelet survival and to image the in vivo distribution of autologous platelets quantitatively even in the presence of severe thrombocytopenia.^{9,10} In addition, the size of the splenic pool may be determined accurately with quantitative imaging.^{11,25}

It is convenient to consider our findings in the context of the in vivo distribution of the labeled platelets. In our previous study, we divided patients with ITP into three groups on the basis of the sites of platelet destruction: splenic, hepatic, and diffuse RES sequestration.⁹ Because this grouping was somewhat arbitrary and there was considerable overlap, we have now grouped the hepatic and diffuse RES sequestration patients together. The division between the two major groups was based on the spleen-liver ratio and was clearcut. Although they had low spleen-liver ratios, patients 9 and 10 had low recovery values and relatively long mean platelet survival times; we have therefore separated them as a subgroup. Most of the platelets not destroyed in the liver or spleen, ie, the remainder, are sequestered in the bone marrow.^{8,9,25} Although the spleen-remainder ratio was lower in patients with diffuse RES platelet sequestration, this ratio neither identified another pattern of sequestration nor improved on the discriminatory value of the spleen-liver ratio. We observed a relationship between the blood platelet count and the sequestration pattern. Splenic sequestration was seen in patients with a relatively high platelet count, whereas diffuse RES sequestration occurred in those with low platelet counts. We therefore agree that the pattern of platelet sequestration is related to the severity of the disease.⁴

Table 4. Percentage of Distribution of Normal Platelets of Different Densities Labeled With ^{111}In and ^{51}Cr

Specimen	Stractan Density (g/mL) 1.084		Stractan Density (g/mL) 1.071		Stractan Density (g/mL) 1.066		Stractan Density (g/mL) 1.062	
	^{111}In	^{51}Cr	^{111}In	^{51}Cr	^{111}In	^{51}Cr	^{111}In	^{51}Cr
1	53.0	51.0	32.0	34.0	10.0	10.0	5.0	5.0
2	48.0	48.6	28.6	31.5	17.1	15.8	6.0	4.0
3	50.0	53.0	24.0	25.0	21.0	19.0	5.0	3.0
4	51.0	50.0	23.0	24.0	14.0	16.0	12.0	10.0
Mean $\pm$ 1 SD	50.5 $\pm$ 2.1	50.7 $\pm$ 1.9	26.9 $\pm$ 4.2	28.6 $\pm$ 4.9	15.5 $\pm$ 4.7	15.2 $\pm$ 3.8	7.0 $\pm$ 3.4	5.5 $\pm$ 3.1

In normal subjects, mean platelet survival estimated with ^{111}In and ^{51}Cr as cell labels is similar.²⁶ We now demonstrate that in ITP the survival of ^{111}In -AP is significantly longer than that determined simultaneously with ^{51}Cr -HP. This agrees with our previous view⁸ and has recently also been found in moderately thrombocytopenic patients with ITP studied with ^{111}In - and ^{51}Cr -labeled autologous platelets.²⁷ It is unlikely that the discrepancy is due to isoimmunization because previous pregnancies or blood transfusions were not relevant in our patients. Neither is varying use of homologous v autologous platelets the explanation.²⁷

It has been shown *in vitro* that small molecules such as ^{51}Cr are more readily lost from platelets than is ^{111}In -binding protein.²⁸ Our results, although not conclusive, suggest that this may occur *in vivo*. Plasma activity of ^{51}Cr was significantly higher than that of ^{111}In , especially during the early period after reinjection of labeled platelets. The *in vitro* contamination of labeled platelets with ^{51}Cr or ^{111}In in plasma was similar; therefore, the infusion of free radionuclide does not explain the difference in platelet survival times with the two isotopes. However, it is possible that the two radionuclides may be bound to different plasma proteins. This would affect the clearance rates of the isotopes from the circulation and is an alternative explanation for the results in Fig 1.

We also investigated the possibility that ^{51}Cr and ^{111}In do not label the circulating platelet population uniformly or to the same extent. Misleading platelet survival information could be obtained if one of the isotopes had a greater affinity for a specific platelet subpopulation. This is unlikely because ^{111}In and ^{51}Cr radioactivities were distributed equally in the platelet subpopulations separated on stractan. In addition, a select population of platelets with a different mean volume from that of whole blood platelets was not lost during harvesting and labeling.

Finally, platelets remaining in the circulation in ITP after a steady state has been reached may be relatively resistant to antibody injury. Autologous labeled platelets may thus have a longer mean survival time in the patient than does the heterogeneous platelet population of a normal donor. This has a precedent in autoimmune hemolytic anemia.²⁹ Our study has not excluded this possibility.

The estimate of the size of the splenic platelet pool will also influence the calculation of platelet turnover. The size of the splenic pool is considered normal in ITP,²⁶ but the estimates of the percentage of recovery of labeled platelets in the circulation vary from low² to near normal.¹ This discrepancy may be owing to the methods used in determining recovery. It is not clear whether it is more appropriate to estimate recovery by extrapolation of blood radioactivity values to zero time (as in ref. 1 and our study) or by measuring blood radioactivity at equilibrium.² Our data do not answer this question, but demonstrate that the estimate of recovery may be influenced by the pattern of platelet sequestration. If one assumes that the only significant exchangeable platelet pool is in the spleen, percentage of platelet recovery in the circulation and splenic radioactivity should total ~100%. In normal subjects, mean platelet

recovery is $61\% \pm 12\%$, and at equilibrium $31.1\% \pm 6.1\%$ of ^{111}In -labeled platelets are present in the spleen.¹¹ This was not so in ITP. In patients with a splenic sequestration pattern, mean recovery was $65.8\% \pm 11.8\%$, and the size of the splenic pool was estimated at $32.8\% \pm 9.8\%$, resembling that of normal subjects. In contrast, the group of patients with diffuse RES platelet sequestration had a mean ^{111}In -AP recovery of $44\% \pm 31\%$ and a mean spleen pool of $21.4\% \pm 8.6\%$. This deficit may readily be accounted for by the very high liver ^{111}In -AP activity at equilibrium. It seems likely that in patients with diffuse RES sequestration of platelets many platelets are destroyed in the liver before attainment of equilibrium between the blood and splenic platelet pools.

All of the above factors should be taken into account when considering platelet turnover in ITP. We estimate that the propagation of errors introduced by the determination of the parameters used to calculate platelet turnover has a percentage SD of 26% in patients with a blood platelet count of $10 \times 10^9/\text{L}$. There was also marked variation from patient to patient as reflected by the large SDs. Therefore, it is clear that the physiological interpretation of results of kinetic studies in these patients should be guarded.

Notwithstanding our reservations, our results indicate that platelet turnover calculated with ^{111}In -labeled autologous platelets identifies a subset of patients with low platelet turnover, and that these patients have more severe disease as reflected by a low platelet count and diffuse RES sequestration of platelets. Platelet turnover measured with ^{111}In -AP was high in all patients with a splenic sequestration pattern. The mean increase was 2.2 times that of normal (range 1.2 to 3.0). In contrast, mean platelet turnover was decreased (mean 0.4 times normal) in all patients with diffuse RES sequestration of ^{111}In -AP, and especially so in patients 9 and 10. Mean platelet turnover estimated with ^{51}Cr -HP was significantly higher in both patient groups than platelet turnover measured with ^{111}In -AP. Our results are thus not in accordance with studies in which platelet turnover was found to be either normal or increased in ITP^{1-4,30} but tend to agree with those of recent studies with ^{51}Cr -labeled platelets^{21,23} and ^{111}In -labeled platelets.^{9,24,27}

These results may be related to other findings difficult to reconcile with the concept that platelet production is always increased in ITP. There is some evidence that antiplatelet antibodies may damage megakaryocytes and impair megakaryocytopoiesis. Rolovic et al³¹ have shown that megakaryocytopoiesis in rats with thrombocytopenia induced with heteroimmune antiplatelet serum is markedly altered. They demonstrate coexistent platelet destruction and defective thrombopoiesis in this experimental model of ITP. It has also been shown that the antiplatelet antibody of ITP binds to both platelets and megakaryocytes^{32,33} and may impair platelet production at the level of the mature megakaryocyte.²³ It therefore seems plausible that the low platelet turnover demonstrated in our patients may be owing to antibody-mediated injury to the megakaryocytes. The more severe impairment of thrombopoiesis seen in patients with diffuse RES sequestration of platelets may be related to the finding that this destruction pattern typical of severe disease is owing

to greater amounts of antibody bound to platelets.³⁴ It seems plausible that megakaryocytes may be damaged in such patients with high antibody levels.

It is evident that the application of the techniques of labeling autologous platelets with ¹¹¹In and the technology of quantification of the *in vivo* distribution of labeled platelets have provided new insights into the pathogenesis of ITP.

However, they have also exposed new problems reflecting the complexities of the disease and the difficulties associated with the interpretation of the data.

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**IN-111-LABELED PLATELET KINETIC STUDIES IDENTIFY
THOSE PATIENTS WITH CHRONIC ITP WHO WILL RESPOND
TO INTRAVENOUS GAMMAGLOBULIN**

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INTRODUCTION

Chronic idiopathic thrombocytopenic purpura (ITP) is an autoimmune disease characterized by accelerated platelet destruction in the presence of antiplatelet antibodies. The platelets, coated with these antibodies, are sequestered by the reticuloendothelial system (RES) and prematurely removed from the circulation, resulting in a shortening of the mean platelet lifespan (MPLS). The main forms of treatment available are corticosteroids and splenectomy, but high doses of intravenous gammaglobulin (IVIgG) has also been shown to be effective therapy for especially acute, but also chronic ITP. The major advantage of IVIgG is that the platelet count rises relatively quickly and this may be of considerable benefit in tiding a patient over an acute hemorrhagic episode.

Although the precise mechanism of action of IVIgG in ITP is unknown, it is generally accepted that non-specific blockade of the Fc receptors in the RES plays an important role. It is therefore evident that a technique that can demonstrate the effect of IVIgG on the pattern of platelet sequestration in ITP, may be useful in eliciting the mechanism of action of this form of therapy.

We have developed reliable methods for studying the *in vivo* distribution of In-111-labeled platelets with computer-assisted image analysis (Heyns et al, 1980). These methods permit the accurate determination of the patterns of platelet sequestration in ITP. In a previous study we recognized two groups of chronic ITP patients: those with predominantly splenic sequestration (spleen:liver ratio > 1.4), and those with diffuse sequestration in the RES (spleen:liver ratio < 1.4) (Heyns et al, 1986). The patients with a diffuse sequestration had more severe ITP reflected by a pronounced thrombocytopenia and decreased platelet turnover. These sequestration patterns not only correlate with the severity of the disease, but can also be used to determine and compare the response to various forms of therapy.

PATIENTS AND METHODS

Patients

Nine patients with chronic ITP, 7 adults and 2 children, and not on any treatment, took part in the study. The patients (or their parents) gave informed, written consent to participate in a study approved by the Ethical Committee of the University of the Orange Free State. All patients had typical chronic ITP, diagnosed on the basis of conventional clinical criteria: adult onset thrombocytopenia of undetermined etiology, or in the case of the children, ITP lasting for longer than 6 months; normal or increased numbers of bone marrow megakaryocytes; and the absence of a demonstrable cause of platelet destruction or utilization such as SLE, drug ingestion or hypersplenism.

Intravenous Gammaglobulin

Sandoglobulin was administered intravenously to all patients in a dosage of 400mg/kg for 5 days (total dose of 2g/kg).

Platelet Studies

Platelets, autologous for the adults and homologous for the children, were labeled with In-111, as previously described (Heyns et al, 1984), and injected into the patients. Platelet survival was estimated from whole blood specimens collected at frequent intervals after injection of the labeled platelets. A least squares computer fit to a gamma function (Murphy and Francis, 1971), was used to calculate the mean platelet lifespan.

In vivo distribution and sites of sequestration of the In-111-labeled platelets were determined with a scintillation camera interfaced with a computer as described elsewhere (Badenhorst and Pieters, 1985). The platelet kinetic studies were performed before and after treatment with IVIgG.

In the adult patients blood was also collected before and after IVIgG therapy for the determination of platelet associated IgG levels.

RESULTS

The results of the platelet counts, MPLS and platelet sequestration patterns for the patients, before and after IVIgG, are given in Table 1.

The patients were separated into responders and non-responders on the basis of disappearance of the bleeding tendency, a clinically meaningful increase in the platelet count and a significant lengthening of the MPLS.

Although the mean platelet counts of all patients increased significantly after IVIgG ($p < 0.05$), only the responders reached clinically safe levels. As a group the responders had significantly higher pre-treatment platelet counts than the non-responders (24 ± 11 vs $14 \pm 9 \times 10^9/l$), but it had no predictive value in the individual patient because the ranges were

TABLE 1. Platelet Counts and Kinetic Data

	Plt count		MPLS		Spleen:Liver	
	(x 10 ⁹ /l)		(hours)		Ratio	
	Pre	Post	Pre	Post	Pre	Post
Responders						
1.	36	188	42	171	4.9	2.2
2.	35	90	2	19	2.8	1.4
3.	18	76	26	54	2.3	0.5
4.	20	230	0.5	18*	5.7	5.3*
5.	10	202	0.3	25	7.6	1.3
Mean	24	157	14	67	4.7	1.4
1SD	±11	±70	±19	±71	±2.2	±0.7
Non-responders						
1.	10	20	8	11	0.3	0.6
2.	9	60	22	23	1.0	1.0
3.	27	30	0.3	0.7	1.6	0.7
4.	10	22	0.2	0.8	1.3	2.5
Mean	14	33	8	9	1.1	1.2
1SD	±9	±19	±10	±11	±0.6	±0.9
Reference	150-400		224 ± 21		1,4 ± 0,6	

* The platelet count started to decrease while the post therapy kinetic study was in progress. These data points were not used for statistical analysis.

equally large in each group ($10 - 36$ vs $9 - 27 \times 10^9/l$). The same applied for the MPLS (14 ± 19 vs 8 ± 10 h). However, the responders could easily be identified by the pattern of platelet destruction. The responders all had a splenic sequestration pattern (spleen:liver ratio 4.7 ± 2.2), which returned to normal after IVIgG (1.4 ± 0.7). On the other hand, the non-responders all had diffuse sequestration patterns (spleen:liver ratio 1.1 ± 0.6), which remained unchanged after IVIgG.

The platelet associated gammaglobulin levels before and after treatment are given in Table 2. There was a significant reduction in platelet associated antibody levels after IVIgG in all patients in which it was measured.

TABLE 2. Platelet Associated IgG Levels (ng/ 10^7 platelets)

	Before Therapy	After Therapy
	58	53
	61	54
	56	43
	40	35
	64	51
Mean	55	47
$\pm 1SD$	11	8
Reference	0 - 35	

DISCUSSION

The spleen plays a key role in ITP: it not only sequesters antibody-coated platelets (Aster and Jandl, 1964), but it is also an important source of antibody production.

(Karpatkin et al, 1972). The spleen removes slightly sensitized platelets particularly effectively, whereas platelets heavily coated with antibody, are preferentially removed by the liver (Aster and Keene, 1969). The results of measurements of the levels of platelet-associated IgG have also confirmed the concept that the sequestration pattern reflects the severity of the disease (Kernoff et al, 1980). More recent studies with autologous, In-111-labeled platelets and accurate computer-assisted quantitative imaging of platelet sequestration in ITP, is also in accord with this view (Heyns, 1985). These studies also clearly demonstrated that platelet sequestration in the more severe forms of ITP is not limited to the liver, but also occurs in the bone marrow.

Although radionuclide-labeled platelet studies reflect the severity of ITP, they were thus far of little benefit in predicting the response to treatment, especially splenectomy (Aster and Keene, 1969; Ries and Price, 1974; Heyns et al, 1982). The fact that the present study could identify those patients with chronic ITP that will respond to IVIgG, is of interest. It confirms the finding of a recent similar study (Kawasugi et al, 1989).

The present study also throws some light on the possible mechanism of action of IVIgG in ITP. Only patients with a splenic sequestration pattern, and representing a less severe form of ITP, responded to IVIgG. This does not fully support the proposal of reticuloendothelial Fc receptor blockade as a mechanism of action. However, the dissociation of antibodies from platelets may be a possibility, since platelet associated IgG levels decreased significantly in all patients in whom it was measured. We could not demonstrate a difference in platelet associated IgG levels between the responders and non-responders, but the technical difficulties encountered when the platelet counts are very low and the lack of specificity of current

antiplatelet antibody determinations make this area difficult to study accurately.

Although this study involved only a few patients, the results clearly show that platelet kinetic studies will identify those patients with ITP who will respond to IVIgG. This is very useful, especially in the light of the high cost of this form of therapy.

It is also evident that a clear understanding of the mechanism of action of IVIgG in ITP will require a much better understanding of immune regulation, macrophage function, and platelet-bound immunoglobulins and their association with platelet antigens.

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Kinetics of indium-111-labelled platelets in HIV-infected patients with and without associated thrombocytopaenia

Van Wyk V, Kotzé HF, Heyns A du P. Kinetics of indium-111-labelled platelets in HIV-infected patients with and without associated thrombocytopaenia.

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Abstract: Seven to 12% of HIV-infected patients have thrombocytopaenia. The pathophysiology of the thrombocytopaenia is not clear. It has been variously suggested that it may be caused by an increased peripheral platelet destruction, a defect in platelet production, or by a combination of these. The aim of the study was to elucidate the pathogenesis of HIV-associated thrombocytopaenia. We determined the mean platelet life span (MPLS) and calculated the turnover of autologous indium-111-labelled platelets in 17 HIV-positive patients, seven with thrombocytopaenia. The sites of sequestration of labelled platelets were quantified. The thrombocytopaenic patients had a very short MPLS (3.0 ± 3.8 h) and a marked increase in platelet production ($18.2 \pm 12.6 \times 10^9/l/h$). The majority of these patients (5 of 7) had excessive sequestration of platelets in the spleen. Five of the patients with a normal blood platelet count had a shortened MPLS (109 ± 23 h) and increased platelet turnover ($3.8 \pm 1.2 \times 10^9/l/h$), i.e. the increased peripheral platelet destruction was compensated for by increased platelet production. The other five patients with a normal platelet count had normal MPLS (195 ± 11 h) and slightly increased platelet production ($2.5 \pm 0.6 \times 10^9/l/h$). We conclude that patients with HIV-associated thrombocytopaenia have increased peripheral platelet destruction. Platelet production is elevated but is insufficient to maintain a normal peripheral platelet count. In these patients platelets are predominantly sequestered in the spleen. Patients with HIV infection and a normal blood platelet count may also have increased platelet production. This may be an early subclinical phase in the development of full-blown HIV-associated thrombocytopaenia.

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Patients infected with HIV/AIDS often have haematological abnormalities such as cytopaenias. In advanced AIDS, pancytopaenia is the rule (1). The pathogenesis of HIV-associated thrombocytopaenia and other cytopaenias differ. The cytopaenia in HIV/AIDS is usually ascribed to bone marrow suppression whereas thrombocytopaenia may be caused by either bone marrow dysfunction or immune destruction of platelets (2).

Seven to 12% of HIV-infected patients present with thrombocytopaenia (1, 2). The etiology of this thrombocytopaenia may also be due to other infections or medication and thus may be difficult to unravel. The mechanism of HIV-associated

thrombocytopaenia *per se*, however, is not clear, and has been attributed to an increase in peripheral platelet destruction, a defect in platelet production or a combination of these (3–7).

The evidence that the pathogenesis of platelet destruction in HIV infection is similar to autoimmune thrombocytopaenic purpura, is persuasive. HIV infected patients with thrombocytopaenia have normal or increased numbers of megakaryocytes in the bone marrow, no splenomegaly and increased amounts of IgG, IgM, C3 and immune complexes associated with their platelets (5, 8–10). Such platelet-associated antibodies may enhance premature sequestration

of platelets primarily in the spleen, but also the liver and bone marrow (11, 12).

Defective platelet production may also play a role in the pathogenesis of the thrombocytopaenia. The megakaryocytes of patients with HIV/AIDS may have abnormal ultrastructural and microscopic features (5), and dysfunctional maturation and defective platelet production may be evident (14–17). The infection of megakaryocytes with HIV may explain why anti-retroviral therapy may alleviate the thrombocytopaenia (18).

The pathogenesis of the platelet destruction may be elucidated by measuring mean platelet life span (MPLS), platelet turnover as a reflection of platelet production (12, 13), and quantifying the importance of various components of the reticuloendothelial system (RES) in the destruction of ^{111}In -labelled platelets.

The aim of this study was to use these methods to clarify the cause of thrombocytopaenia in persons infected with HIV. A control group of patients with HIV infection and normal blood platelet count was included in the study to determine whether the infection had a subclinical effect on platelet kinetics.

Materials and methods

Patients

Seventeen clinically stable patients infected with HIV gave informed consent to participate in a study which was approved by the Ethics Committee for Clinical Studies of the University of the Orange Free State. Their data are summarised in Table 1. Seven of the patients, three male homosexuals and four with heterosexual infection with the virus, had severe to moderate thrombocytopaenia. All the subjects, for at least 10 d prior to and during the study, had not taken drugs that may have influenced platelet function and/or survival. None had splenomegaly or concomitant infection at the time of the investigation.

Platelet kinetics

Autologous platelets were labelled with ^{111}In -tropolone and the MPLS estimated as previously described (19, 20). Radioactivity of blood samples in EDTA was determined in a well-type scintillation counter (Packard Auto-gamma 5650, United Technologies, Packard). In the patients

Table 1. Patient characteristics, platelet count, MPLS, platelet production and sequestration pattern of senescent platelets (* $p < 0.05$)

Patient, sex, age	Platelet count ($\times 10^9/\text{l}$)	MPLS (h)	Platelet turnover ($\times 10^9/\text{l/h}$)	Platelet sequestration (% of whole body)		
				Spleen	Liver	Bone marrow
Thrombocytopenia						
1 M, 38	3	0.6	12.0	64	26	10
2 F, 29	5	0.7	13.1	37	36	27
3 M, 36	19	1.7	27.0	41	55	4
4 M, 31	60	2.2	43.0	50	30	20
5 M, 36	12	2.9	12.0	75	9	16
6 F, 34	54	11.5	6.2	68	9	23
7 M, 30	8	1.2	14.3	68	15	17
Mean $\pm$ 1SD	23 $\pm$ 24*	3.0 $\pm$ 3.8*	18.2 $\pm$ 12.6*	58 $\pm$ 15*	26 $\pm$ 17	17 $\pm$ 8*
Normal Platelet count, shortened platelet life span						
8 F, 33	356	144	5.4	22	31	47
9 F, 25	296	116	3.5	50	19	31
10 M, 33	266	104	4.5	39	26	35
11 F, 22	259	98	2.8	50	20	30
12 F, 40	157	83	2.6	30	37	33
Mean $\pm$ 1SD	267 $\pm$ 72	109 $\pm$ 23*	3.8 $\pm$ 1.2*	38 $\pm$ 12	35 $\pm$ 19	35 $\pm$ 7
Normal platelet count, normal platelet life span						
13 F, 22	296	209	2.4	51	23	26
14 F, 27	186	202	1.6	47	26	27
15 F, 28	345	192	2.9	44	27	29
16 M, 24	238	188	2.4	46	24	30
17 F, 33	369	182	3.4	34	36	30
Mean $\pm$ 1SD	287 $\pm$ 75	195 $\pm$ 11	2.5 $\pm$ 0.6*	44 $\pm$ 6	27 $\pm$ 5	28 $\pm$ 2
Reference value (12, 20)	150–400	224 $\pm$ 23	1.8 $\pm$ 0.4	36 $\pm$ 10	29 $\pm$ 8	30 $\pm$ 8

with thrombocytopenia, blood was collected every 15 min for the first hour after injection of the labelled platelets, every 30 min for the next 4 h and then four times daily. In those patients with normal platelet counts, blood was collected at 15 and 30 min, 1, 2 and 4 h and then twice daily for 7 d. Blood disappearance curves of the ^{111}In -labelled platelets were constructed from the data. MPLS was estimated by fitting a gamma function to the blood clearance curves. In all cases, the radioactivity in the 30-min blood sample and all data points containing $>15\%$ of the radioactivity of the 30-min blood sample were used to calculate MPLS. Recovery of the labelled platelets in the circulation at equilibrium was calculated from the ^{111}In radioactivity injected and by back extrapolation of the survival curve to zero time (21). Platelet turnover was calculated after correcting for the effect of the splenic platelet pool (22). This is an indirect estimate of platelet production.

The *in vivo* distribution and sites of sequestration of labelled platelets was quantified as described in detail (20, 23). Briefly, anterior and posterior images with and without a posterior and anterior placed standard radioactivity source were acquired of the thorax and abdomen using a large field of view scintillation camera interfaced with a data processing system. The images were acquired 90 min after the labelled platelets were injected and then daily for 10 d. Regions of interest were selected for the spleen and liver, and an appropriate background. Organ radioactivity was corrected for attenuation using the depth independent build-up method (23) and expressed as a percentage of injected radioactivity.

Platelet counts were made with a Technicon H*1 blood cell analyser (Bayer Diagnostics, Basingstoke, UK) on blood collected in K_3EDTA .

Results

The results are summarised in Table 1. Patients 1–7 had moderate to severe thrombocytopenia and a significantly shorter MPLS than normal, i.e. outside two standard deviations of the reference value of 224 ± 23 h (20). Their platelet turnover was increased between 6- and 20-fold. The mean sequestration of platelets in the spleen was significantly ($p < 0.05$) higher and that in the bone marrow significantly lower than normal ($p < 0.01$). Sequestration of platelets in the liver was normal.

Patients 8–17 had a normal peripheral blood platelet count. This group was divided into two subgroups on the basis of the results of their MPLS.

The first group, patients 8–12, had a shortened MPLS and a doubling of platelet turnover. The sequestration pattern of senescent platelets in the

spleen, liver and bone marrow in this group was normal.

The second subgroup, comprising patients 13 to 17, had a normal MPLS accompanied by a slight, but statistically significant ($p < 0.05$), higher mean platelet turnover. The platelet sequestration pattern of this group was within normal limits.

Discussion

The results of this study indicate that patients infected with HIV may be divided into those with thrombocytopenia and a markedly shortened MPLS; those with a normal platelet count and a shortened MPLS; and those with a normal platelet count and a normal MPLS.

Patients with HIV-associated thrombocytopenia and marked increase peripheral platelet destruction had increased platelet production. This pattern corresponds to that seen in some patients with idiopathic thrombocytopenic purpura (12, 13).

The second patient group, with moderately shortened MPLS, had a normal blood platelet count. This indicates that their increased platelet production compensated for the platelet destruction, thus maintaining the blood platelet count.

The third patient group, with normal MPLS and a normal platelet count, had a slightly increased platelet turnover. This suggests that there may be a degree of compensated thrombocytolysis in these patients. We have not studied whether this can be ascribed to HIV infection of the megakaryocytes.

Our results do not support the view that thrombocytopenia in patients with HIV can be fully explained by decreased production of platelets by megakaryocytes infected with the virus. The thrombocytopenia in our patients was also not due to a combination of increased peripheral destruction and decreased production of platelets, as has been described elsewhere (17, 18, 24).

The findings of this study, however, are in accord with the notion that the thrombocytopenia is caused primarily by an increased peripheral destruction of platelets (7, 25). There may also be concomitant ineffective thrombocytolysis. Thrombocytopenia develops in those patients who can not compensate for the excessive platelet destruction by an appropriate increase in effective platelet production. We have not investigated the cause of the increased platelet destruction. Therefore, we do not know whether the pattern of platelet sequestration in the reticuloendothelial system could be related to platelet-associated immunoglobulins or complement fractions.

It has been proposed that splenectomy may be a beneficial adjunct to other therapy in patients with HIV-associated thrombocytopaenia (25). Platelet kinetic studies, particularly quantifying the sites of platelet sequestration, may thus be of value to identify those patients who may benefit from the operation (6, 7, 14, 25-27). Five of the 7 patients with HIV-associated thrombocytopaenia in our study had increased splenic platelet sequestration. These patients have not been subjected to splenectomy, and we can therefore not comment on this therapeutic option.

In summary, our results indicate that HIV-associated thrombocytopaenia is mainly due to increased platelet destruction, mainly in the spleen. This platelet destruction may start early in the course of the infection. The effect may be masked by a compensatory increased platelet production that is sufficient to maintain the peripheral blood platelet count within normal limits.

Acknowledgments

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Kinetics and sites of sequestration of indium 111-labeled human platelets during cardiopulmonary bypass

A new approach for the study of the kinetics and quantification of the in vivo and ex vivo sites of sequestration of platelets during cardiopulmonary bypass (CPB) is described. Autologous platelets of four patients were labeled with ^{111}In -oxine and reinfused on the day prior to CPB for coronary artery bypass grafting. Changes in blood ^{111}In -labeled platelet radioactivity and blood platelet counts were monitored during the operation. In vivo ^{111}In -labeled platelet redistribution was quantified with a scintillation camera and a computer-assisted imaging system before and after CPB. Sequestration of ^{111}In -labeled platelets in the bubble oxygenator was measured. ^{111}In -labeled platelet activity in the blood decreased by $46\% \pm 5\%$ within 5 minutes of CPB, but this decrease was mostly due to hemodilution; the true loss of platelets from the circulation was $13\% \pm 4\%$. Intraoperatively, whole body ^{111}In activity decreased by $34.8\% \pm 5.4\%$, and in the 48 ensuing hours a further slight loss of radionuclide occurred. In the pump oxygenator $10.8\% \pm 1.3\%$ of administered platelets were sequestered, especially in the innermost active layers of the defoaming mesh of the bubble oxygenator. Mean survival time of circulating platelets was 58 ± 8 hours and fitted an exponential function best. The bleeding time increased to 40 minutes during operation and returned to normal within 24 hours. During operation ^{111}In -labeled platelets accumulated somewhat in the liver (10.7%) but not in the spleen, thorax, or head. In the 48 hours after operation, platelets were sequestered mainly in the liver. The scintillation camera with computer-assisted imaging allows in vivo quantitative studies of platelet kinetics of a type which has not been possible with previous techniques.

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The widespread use of cardiopulmonary bypass (CPB) makes necessary the understanding of the factors causing the significant risk of hemorrhage during and after this type of operation. The coagulation system, blood platelets, drugs such as heparin and protamine sulfate, design of the extracorporeal oxygenator, and

cyanotic heart disease have all been implicated as causative factors.¹

In this study of four patients we focus on the kinetics, fate, and function of platelets during operations with CPB. Platelets were labeled with ^{111}In indium oxine, a new physiological platelet label.² The physical characteristics of this radionuclide make possible the quantitative studies with a scintillation camera of the in vivo redistribution of labeled cells. We could thus determine the loss of platelets from the circulation and their accumulation in different components of the pump oxygenator, and we could quantitate the in vivo sequestration of labeled platelets in different organs.

Materials and methods

Patients. Four patients, A to D, agreed to participate in a study approved by the Ethical Committee of the Provincial Administration and University of the Orange

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Free State. The patients were white men, aged 37 to 55 years, and had coronary artery bypass grafting with one to three saphenous vein anastomoses. The period of anesthesia lasted from 3 hours, 15 minutes to 5 hours, 10 minutes, and the duration of CPB ranged from 45 to 167 minutes. Just before CPB, they received heparin, 250 U/kg body mass, as a bolus and thereafter 65 U/kg/hr. At the end of CPB, heparin administered during the operation was neutralized with intravenous protamine sulfate in a dose ranging from 325 to 475 mg.

CPB. Patients were perfused with a Sarns Model 5000 pump console equipped with a custom-packed tubing circuit (Bentley Laboratories, Inc., Irvine, Calif.), a Bentley BOS-10 bubble oxygenator, a Bentley 27 μ m cardiomy filter, and a Bentley Q 220 cardiomy reservoir. A microemboli filter was not inserted in the arterial line. A pulmonary artery vent was used only in Case A. The pump was primed with 2,000 to 2,500 ml of electrolyte solution (Plasma-Lyte B, Baxter Travenol Laboratories, Inc., Deerfield, Ill.) containing heparin, 3,000 U/L, cephalosporin, 1 gm, and the autologous red cells recovered from the platelet-labeling procedure. Platelet sequestration in the pump oxygenator was determined postoperatively by draining the apparatus of blood, dismantling it, and counting radioactivity of the components under the scintillation camera. Since the tubing and connections had virtually no radioactivity, imaging was performed only on the oxygenator, cardiomy filter, and reservoir. For purposes of analysis, the components of the oxygenator were separated into the six defoaming layers and the whole of the remainder. ¹¹¹In activity of surgical swabs, waste suction, and chest drainage blood was measured.

Platelet studies. ¹¹¹In-chloride (Radiochemical Centre, Amersham, U. K.) was complexed with 8-hydroxyquinoline² and platelets were labeled with the ¹¹¹In-oxine as previously described.³ Briefly, on the day prior to operation, 425 ml of autologous blood was collected in a double-pack closed-bag system (PA-20, Fenwal Laboratories, Morton Grove, Ill.) containing 75 ml acid-citrate-dextrose NIH formula A (ACD-A) as anticoagulant. The bag was centrifuged at 300 g for 15 minutes and the platelet-rich plasma (PRP) was transferred to polystyrene tubes (Falcon 2070, Oxnard, Calif.). The pH of the PRP was adjusted to 6.2 to 6.5 with ACD-A. Platelets were centrifuged 800 g for 15 minutes, resuspended in physiological saline, incubated for 30 minutes with ¹¹¹In-oxine, washed once with autologous platelet-poor plasma (PPP), and finally resuspended in 2 to 3 ml of PPP. Radioactivity was determined in a Picker radionuclide source calibrator

Table I. Changes in ¹¹¹In-labeled platelet radioactivity in the peripheral blood during cardiopulmonary bypass

	Patient				Mean $\pm$ 1 SD
	A	B	C	D	
Decrease in ¹¹¹ In activity (%)	43	52	48	40	46 $\pm$ 5
Hemodilution (%)	28	43	31	30	33 $\pm$ 7
Loss of ¹¹¹ In activity (%)	15	9	17	10	13 $\pm$ 4

and approximately 14.8 MBq (400 μ Ci) of ¹¹¹In-labeled platelets was administered to the subject. Platelets were counted with a Coulter Model FN electronic particle counter.⁴ Aggregation of labeled platelets was induced with collagen (Hormon-Chemie, München) and assessed in vitro under the phase microscope.³ ¹¹¹In-labeled platelets were not infused unless a normal aggregation response, shown by the presence of aggregates consisting of more than 10 platelets, was observed.

Platelet survival studies were performed as recommended by the International Committee for Standardization in Hematology.⁵ The platelet survival curves were constructed from the data of blood collected twice daily after the operation and back extrapolation to zero time. Mean autologous platelet survival was calculated by computer analysis of a multiple hit gamma function model.⁶ The normal platelet survival determined by this method in our laboratory is 179 $\pm$ 40 hours ($\pm$ 1 SD), and the survival curve fits a linear function best.⁷

In vivo platelet distribution and sites of destruction were determined quantitatively with a scintillation camera and a computer-assisted imaging system, with slight modification of our previously described method.^{7, 8} Quantitation was performed 90 minutes after injection of the ¹¹¹In-labeled platelets (the baseline value) and repeated about 3 hours before and 24 and 48 hours after operation. Imaging was done with a mobile scintillation camera, data being acquired on a magnetic tape and later transferred to a PDP-8 based, computer-assisted imaging system. The camera with a divergent collimator and the pulse-height analyzer set to include the 173 keV photopeak was positioned to visualize the heart, spleen, and liver simultaneously. Geometry in day to day imaging was maintained by using the xiphisternum and anterior iliac spines as points of reference. Areas of interest were selected with the computer and radioactivity in specific organs was determined daily by summation of regional images of the head and neck, thorax, abdomen and pelvis, and lower limbs. Radio-

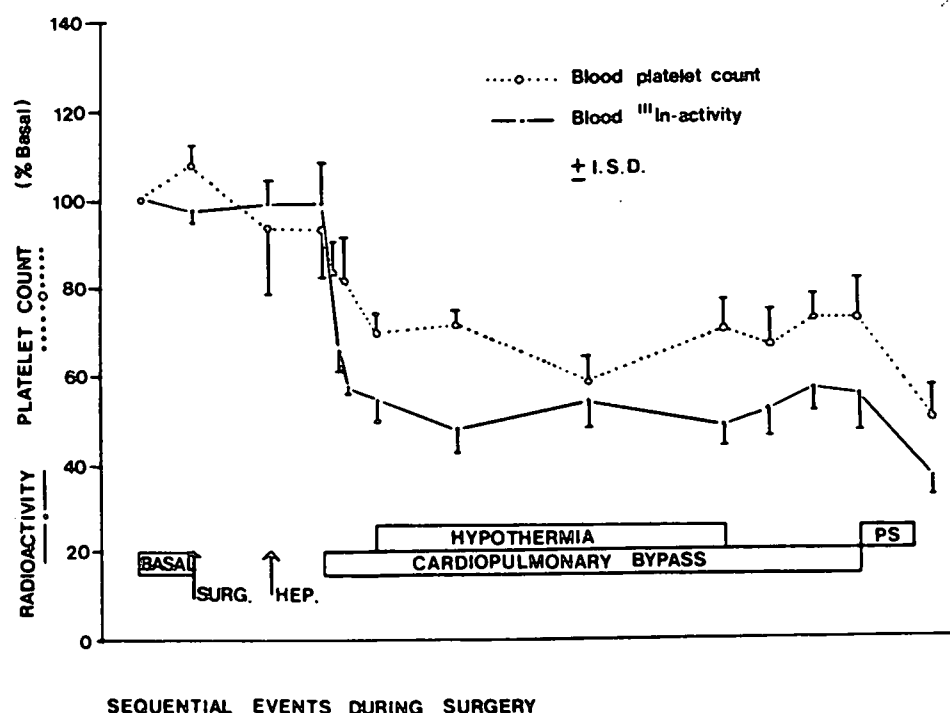


Fig. 1. Percentage changes in blood platelet count and blood ^{111}In -labeled platelet radioactivity during operations performed with cardiopulmonary bypass in four patients. This is a composite figure and results are related to the sequential surgical events. Times when the operation started and when heparin (HEP) and protamine sulfate (PS) were administered are indicated.

activity of organs was expressed as a percentage of whole body radioactivity. For purposes of analysis, preoperative whole body ^{111}In activity was regarded as 100% and all other measurements were expressed relative to this. No attempt was made to correct the relative slight errors of geometry and attenuation inherent in this method of in vivo quantitation of radioactivity.⁸

Alterations in blood volume caused by connection to the CPB system were measured by monitoring changes in blood ^{125}I -albumin radioactivity injected just prior to CPB. Platelet function in vivo was evaluated with the standardized bleeding time.⁹

Results

Platelet loss during cardiopulmonary operations. The loss of ^{111}In -labeled platelets from the circulation during operation was determined by comparing changes in blood ^{111}In concentration and platelet counts before, during, and after operation. Hemodilution resulting from CPB was measured by the decrease in peripheral blood ^{125}I -albumin radioactivity before and after CPB. The results are given in Table I and illustrated in Fig. 1. Blood ^{111}In activity decreased markedly, but two thirds of this decrease was due to hemodilution, which occurred when the patient was put on the CPB circuit. Blood platelet counts changed in parallel with the

changes in blood ^{111}In activity. The true loss of ^{111}In -labeled platelet radioactivity was $13\% \pm 4\%$ of that present in the blood preoperatively. This loss was evident within 5 minutes of CPB.

The loss of ^{111}In activity from the body was also determined by measurement of whole body radioactivity with the scintillation camera before and after operation (Table II). Results are expressed relative to preoperative ^{111}In activity assigned an arbitrary value of 100%. During the operation there was a mean loss of 34.8% of ^{111}In activity, and in the 24 to 48 hours after operation a further slight loss of the radionuclide occurred. We could account for the intraoperative loss of ^{111}In -labeled platelet radioactivity as follows: A mean of $10.8\% \pm 1.3\%$ of administered labeled platelets were sequestered in the different components of the extracorporeal pump oxygenator (Table III). The remainder, determined as $17.6\% \pm 3.6\%$, was located in the blood remaining in the pump oxygenator at the end of the operation, in the surgical swabs, waste suction, and chest drainage blood.

Distribution of platelets in the pump oxygenator. Sequestration of ^{111}In -labeled platelets in the different components of the defoaming mesh layers and the oxygenating column/heat exchanger of the pump oxygenator was quantitated. Results, expressed as a per-

Table II. Loss and redistribution of ¹¹¹In radioactivity before and after cardiopulmonary bypass

Area	Time of ¹¹¹ In quantification			
	Pre-op.	Immediately postop.	24 hr postop.	48 hr postop.
Whole body	100 ± 0	65.2 ± 5.4	57.9 ± 11.0	56.8 ± 5.8
Liver	7.9 ± 1.0	7.0 ± 1.8 (10.7)	8.5 ± 1.8 (14.7)	8.4 ± 1.6 (14.8)
Spleen	14.9 ± 6.0	8.7 ± 3.4 (13.3)	7.8 ± 3.1 (13.5)	7.8 ± 2.4 (13.7)
Thorax (including heart)	30.4 ± 3.2	21.3 ± 1.3 (22.7)	19.3 ± 3.5 (33.3)	19.8 ± 2.4 (34.9)
Lower limbs	5.8 ± 3.2	1.8 ± 1.1 (2.8)	1.6 ± 1.6 (2.8)	1.8 ± 1.0 (3.2)
Head	18.7 ± 6.3	12.2 ± 2.8 (18.7)	12.1 ± 4.3 (20.9)	11.1 ± 1.9 (19.5)
Lower abdomen and pelvis	15.8 ± 1.5	10.6 ± 4.2 (16.3)	6.8 ± 1.1 (11.7)	6.2 ± 0.5 (10.9)

Legend: Quantities are expressed as a mean (± 1 SD) percentage of the preoperative whole body ¹¹¹In activity. The values in parentheses are those of organ radioactivity expressed as a mean percentage of the whole body activity at the time of the ¹¹¹In quantification.

Table III. Sequestration of ¹¹¹In-labeled platelets in components of the extracorporeal pump oxygenator

Component	Patients				Mean ± 1 SD
	A	B	C	D	
M: Gaseous microemboli inhibitor (PUF)	0.2	0.2	0.2	0.2	0.2 ± 0
A: Mesh (PPAC)	1.0	2.6	2.2	3.6	2.3 ± 1
B: Mesh (PPAC)	0.2	0.8	1.0	0.8	0.7 ± 0.35
C: Mesh (PPAC)	0.2	0.3	0.3	0.3	0.28 ± 0.05
D: Mesh (PPAC)	0.2	0.3	0.2	0.3	0.25 ± 0.06
E: Polyurethane foam with anti-foam (20 pores/sq inch)	0.4	0.2	0.2	0.3	0.28 ± 0.10
F: Nylon tricot filter (mesh 150)	0.7	0.5	0.6	0.6	0.6 ± 0.08
Total (M,A,B,C,D,E,F)	2.9	4.9	4.7	6.1	4.65 ± 1.32
Remainder of oxygenator	4.8	2.8	4.5	4.7	4.2 ± 0.9
Cardiotomy reservoir	2.1	0.9	0.7	0.4	1.03 ± 0.75
Cardiotomy filter	2.1	0.6	0.6	0.5	0.95 ± 0.77
Totals	11.9	9.2	10.5	11.7	10.8 ± 1.3

Legend: Results are expressed as a percentage of ¹¹¹In administered to the patient. PUF, Polyurethane foam. PPAC, Polypropylene mesh with antifoam coating. A to D mesh, E foam, and F filter refer to components of the oxygenator illustrated in Fig. 2.

centage of total ¹¹¹In activity administered to the patients, are given in Table III and illustrated in Fig. 2. Most of the platelets were sequestered in the bubble oxygenator, and relatively small quantities of radioactivity accumulated in the cardiotomy reservoir and filter. Since there was minimal radioactivity in the tubing, connectors, and cannulas after they were drained of blood, this ¹¹¹In activity was disregarded. As is evident, the highest concentration of trapped platelets were in the bubble column and the innermost active layers of the defoaming mesh (Fig. 2, A and B, Table III).

Circulating blood platelets. Blood platelets were counted and ¹¹¹In-labeled platelet activity determined at intervals before, during, and after the operation. The changes in the ¹¹¹In-labeled and absolute blood platelet counts, expressed relative to an assumed 100% preoperative value, are illustrated in Fig. 1. The mean basal platelet count was 245,000 ± 28,300/μl pre-operatively and decreased by 29% to a mean of 174,000 ± 58,300/μl at the end of CPB. There was a further temporary decrease of 26% in circulating platelets to

128,000 ± 31,000/μl after intravenous administration of protamine sulfate. The decrease in the ¹¹¹In-labeled platelet activity was consistently of greater magnitude than that of the blood platelet count. Circulating platelet counts and ¹¹¹In activity tended to decrease during hypothermia and to increase during the period of re-warming, but these changes were not statistically significant ($p > 0.1$, one-tailed Student's *t* test for paired data).

Survival of ¹¹¹In-labeled platelets in the circulation was determined. The survival curves fitted an exponential function best. Platelet life-span determined by the gamma function model was 46, 61, 62, and 64 hours for Patients A to D, respectively, with a mean survival of 58 ± 8 hours. Platelet function was assessed in vivo with the standardized bleeding time. During operation, while the patients were on CPB, the bleeding time in all four patients was longer than 40 minutes (normal 3 to 6½ minutes). Twenty-four hours postoperatively the bleeding times had returned to within the normal range.

Distribution of ¹¹¹In-labeled platelets in organs.

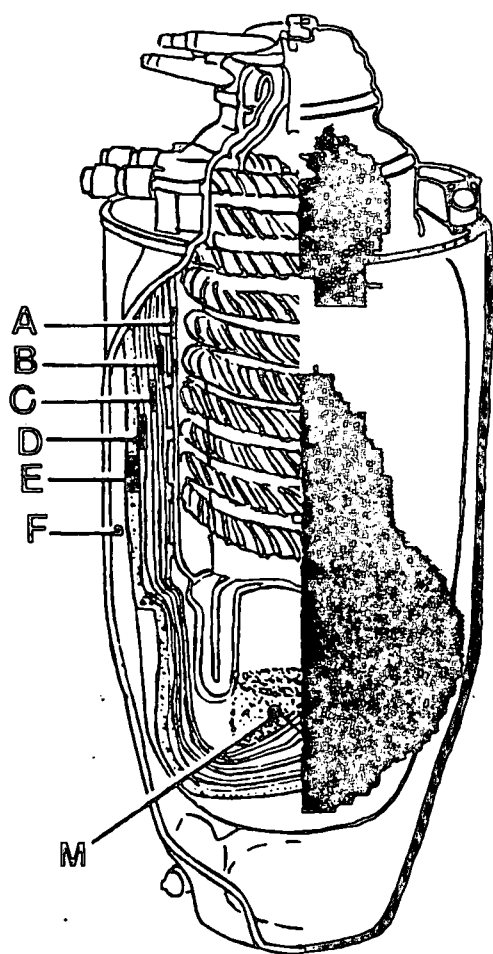


Fig. 2. Sequestration of ^{111}In -labeled platelets in the different components of the pump oxygenator. This scintillation camera image shows that most platelets are trapped in the bubble column and the innermost layers (A and B) of the defoaming mesh. The gaseous microemboli inhibitor is shown (M).

^{111}In -labeled platelet radioactivity in organs was quantitated preoperatively, immediately after the operation, and 24 and 48 hours after the operation. Results are given in Table II. Organ radioactivity is expressed relative to preoperative whole body radioactivity, assigned as 100%, and also is calculated as a percentage of whole body activity at the time of quantification. During operation there was a relative increase in hepatic ^{111}In activity, whereas that of the spleen, lower limbs, thorax, head, and abdomen decreased in parallel with the loss of ^{111}In -labeled platelets from the whole body. In the 24 to 48 hour postoperative period, ^{111}In radioactivity in the liver increased while that in the spleen, thorax, lower limbs, and head remained constant. Radioactivity in the lower abdomen and pelvis decreased.

Discussion

^{111}In is a cyclotron-produced radionuclide, half-life 2.8 days, with 183% gamma emissions of 171 and 245 keV. These physical characteristics permit quantitative scintillation camera imaging of the in vivo distribution of labeled platelets. We have reported on the labeling of a physiologically normal human platelet population with ^{111}In -oxine³ and the study of platelet kinetics and quantitation of the in vivo distribution with a whole body counter and a scintillation camera interfaced with a computer-assisted imaging system in man⁷ and dog.⁸ Quantitation of isotope distribution is somewhat affected by geometry and attenuation.⁸ Radioactivity in the liver and spleen is slightly overestimated, but this does not affect the results materially. The techniques that we have used in our previous studies^{7, 8} had to be modified for the present investigation. The patients could not be transported to a whole body counter and a stationary scintillation camera. Whole body and organ ^{111}In activity was thus determined with a mobile scintillation camera. Results may also have been influenced by the difficulties with postoperative positioning of the patients under the camera. The results of the preoperative quantification of organ ^{111}In activity corresponds reasonably closely to those of our previous study,⁷ except that spleen radioactivity ($11.6\% \pm 3.5\%$) is low compared to the $25.9\% \pm 10.1\%$ of the standard method.

Although operations performed with CPB have become commonplace, they may still be complicated by serious bleeding. Platelets play a key role in hemostasis: Defects in vascular endothelium are breached by platelet adhesion and aggregation, and platelet phospholipid provides a surface on which thrombin generation is accelerated. Activation or sequestration of platelets by the CPB equipment may thus induce a bleeding diathesis.¹⁰ It is generally agreed that CPB is associated with a 30% to 50% decrease in the preoperative blood platelet count.¹¹ The thrombocytopenia has been attributed to various factors such as adhesion of platelets to machine material, consumption of platelets in disseminated intravascular coagulation, platelet aggregation induced by heparin and protamine, mechanical damage to platelets, and hemodilution by pump priming solutions.¹²

The pattern and degree of thrombocytopenia observed in our patients is in agreement with that of the literature.¹³ There was a rapid mean decrease of 29% in the platelet count evident within the first 5 minutes of CPB. The platelet count stabilized during the period of extracorporeal circulation but decreased further after administration of protamine sulfate (Fig. 1). We assessed platelet function in vivo with the standardized

bleeding time,⁹ a suitable screening test for the measurement of the overall hemostatic role of platelets in vivo.¹⁴ During CPB the bleeding time was longer than 40 minutes in all four patients. This degree of prolongation should occur only with a blood platelet count less than 10,000/ μ l if platelet function is normal.¹⁴ We may thus conclude that our patients had an acquired qualitative platelet defect. This finding is in accord with those of previous reports.^{15, 16} The defect may be caused by the release of the platelet granule contents induced by contact with foreign surface or by the thrombin generated during the operation.¹⁷

The in vivo redistribution of ¹¹¹In-labeled platelets was studied to determine the sites of platelet sequestration. Organ ¹¹¹In activity was quantified in vivo with the scintillation camera, and peripheral blood ¹¹¹In activity was determined. ¹¹¹In activity in the pump oxygenator was also measured. The mean intraoperative loss of ¹¹¹In activity, as determined with the scintillation camera, was 38% $\pm$ 4.6%. Some of the intraoperative loss was the result of the operative procedure, with its attendant blood loss, but there was a definite accumulation of ¹¹¹In-labeled platelets in the pump oxygenator. This finding is not in accord with the results of a previous study, which revealed that platelets were not lost in the extracorporeal circuit, but returned to the circulation and were sequestered in the liver after bypass was discontinued.¹⁸ In the pump oxygenator most of the platelets adhered where the bubbles coalesce at the top of the oxygenating column and where the bubbles burst on the first layer of the silicone-treated defoaming mesh. This finding would be consistent with the view that platelet adhesion to biomaterials is favored by the formation of blood-gas interfaces on the surface of the biomaterial.^{19, 20}

The changes in organ ¹¹¹In radioactivity during operation are interesting (Table II). Hepatic ¹¹¹In activity remained constant, whereas mean total body activity decreased by 34.8%. This would suggest that platelet microaggregates or platelets damaged in the oxygenator pump are sequestered in the liver. Microemboli have been shown to be present in cardiectomy return blood in bubble oxygenators and to a lesser extent in membrane oxygenators.²¹ ¹¹¹In activity in the thorax decreased during the operation. The fact that this decrease was of a degree equivalent to the loss of ¹¹¹In activity from the whole body indicates that pulmonary microembolization during CPB was not significant and that ¹¹¹In-labeled platelets in the lung are in equilibrium with those in the blood. Radioactivity in the head decreased by 32% intraoperatively, similarly indicating that significant microembolism to the brain did not occur.

Splenic ¹¹¹In activity decreased during the operation. The decrease (40%) was very similar in magnitude to that of whole body ¹¹¹In loss. This reflects mobilization of platelets from the exchangeable splenic platelet pool²² in response to platelet loss from the peripheral blood.

Platelets pool temporarily in the spleen and liver of dogs during hypothermia.²³ In our study this was observed in only one patient (data not shown), in whom platelet ¹¹¹In activity clearly increased in the liver during cooling and decreased again with warming. The composite data of Fig. 1 show that ¹¹¹In activity in the blood did not change significantly during the period of hypothermia.

This study illustrates the advantages of ¹¹¹In as a platelet label for the study of kinetics, in vivo redistribution and sites of sequestration, and ex vivo aggregation in the pump oxygenator. We were able to measure loss of radionuclide from the body and show accumulation in the CBP oxygenator pump and mobilization of platelets in the liver, but not in the lungs or brain. This type of study is not possible with the other currently available platelet isotope labels.

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Kinetics and In Vivo Distribution of In-111-Labelled Platelets and Platelet Function in Familial Hypercholesterolaemia

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Key words

Hypercholesterolaemia – In-111-labelled platelets – Platelet function – Platelet kinetics – Platelet lifespan

Summary

The kinetics, *in vivo* distribution and sites of sequestration of autologous In-111-labelled platelets and other platelet function parameters were studied in ten patients with type IIa or IIb familial hypercholesterolaemia and thrombotic complications of atherosclerosis. The *in vitro* platelet aggregation response to ADP ($P = 0.50$) and collagen ($P = 0.46$); binding of fibrinogen to platelets ($P = 0.61$); and plasma beta-thromboglobulin levels ($P = 0.42$) of the patients and normal reference subjects did not differ significantly. The *in vivo* distribution of In-111-labelled platelets at equilibrium was within normal limits, and at the end of platelet life-span the sequestration pattern of labelled platelets in the reticuloendothelial system was also normal (spleen $P = 0.31$; liver $P = 0.54$). There was minimal evidence of *in vivo* platelet activation: only mean platelet lifespan (MPLS), 195 ± 57 hours (difference between mean MPLS of patients and controls was 25 hours, with a 95% confidence interval from 23 to 31 hours; $P = 0.02$); mean platelet turnover, 2298 ± 824 platelets/ μ l/hour ($P = 0.005$); plasma platelet factor 4 ($P = 0.02$); and the mean circulating platelet aggregate ratio, 0.8 ± 0.1 ($P = 0.02$); differed significantly from normal. These results suggest that abnormalities of platelet function and kinetics observed in type II hyperlipoproteinaemia cannot be ascribed wholly to the hyperlipidaemia, but may be induced by the associated atherosclerosis.

Introduction

The prevalence of coronary vascular disease in South Africa is the highest in the world (1). The mortality is particularly high amongst the indigenous Afrikaners and Indians, and Jews, whereas the rural Black Africans are not affected. The most important risk factor is the high incidence of familial hyperlipidaemia among the Afrikaners (2).

Long-standing elevation of plasma lipoproteins, particularly low-density lipoprotein and very-low-density lipoprotein, is associated with an increased incidence of atherosclerosis. This may be because elevation of plasma LDL damages the vascular endothelium (3), increases the infiltration of the artery media with lipoprotein, and stimulates the proliferation of the smooth muscle cells (4). Endothelial damage triggers a response which is characterized by complex cellular and biochemical interactions culminating in the secretion of growth factors by monocytes, the

endothelial cells themselves, and platelets. These growth factors induce the proliferation of vascular smooth muscle cells converting fatty streaks into fibrous plaques. This succession, appropriately termed the response-to-injury hypothesis, may eventually be complicated by the occlusion of the vessel or, by thrombosis (5).

It is clear that platelets and lipids may play an important, possibly decisive, interrelated role in the pathogenesis of atherosclerosis and its major complication, thrombosis. In this study we therefore studied the effect of hyperlipidaemia, due to type IIa or IIb familial hypercholesterolaemia, on *in vitro* tests of platelet function and for evidence of *in vivo* platelet activation, focussing on the kinetics of In-111-labelled platelets.

Patients, Materials and Methods

Patients

The patient data are summarized in Table 1. Ten males, aged 28 to 46 years, gave informed consent to participate in the project approved by the Ethical Committee of the Provincial Administration and the University of the Orange Free State. A diagnosis of familial hypercholesterolaemia, of either type IIa or IIb, was made according to standard criteria (6). All were on medication, but therapy was withheld for at least 14 days before commencing the study.

Reference values for the *in vitro* and *in vivo* platelet function tests were determined in our laboratory employing the same standardized methods, reagents and internal and external quality control procedures used in this study. Normal healthy male volunteers, aged 21 to 44 years, with the same socio-economic background as the patient group, and with no clinical evidence or history of cardiovascular disease, participated. These subjects were nonsmokers and had taken no medication known to affect platelet function for at least 2 weeks prior to, and during the study. Since the reference values for kinetics of In-111-labelled platelets were ascertained over a longer period of time than the course of the present study, and to avoid the unnecessary administration of radioactive materials to volunteers, all the reference values were not determined in the same group of subjects. The numbers of subjects used to determine reference values for the different tests are given in the relevant sections reporting the results of this study.

Blood Platelet Studies

Platelet labelling and measurement of mean platelet life-span (MPLS) were done as described in detail elsewhere (7–9). Briefly, autologous platelets were isolated from citrated blood by differential centrifugation and repeated washing of the red cell layer. The platelets were concentrated, resuspended in Plasmalyte B (Baxter, Travenol Laboratories Inc; USA), and In-111-oxine (Radiochemical Centre, Amersham, UK) added. After incubating for 30 minutes at room temperature, the labelled platelets were concentrated and resuspended in platelet poor plasma for reinjection. The ability of the labelled platelets to aggregate in response to ADP was tested on a glass slide (10). The labelled platelets were regarded as damaged by the *in vitro* manipulation, and therefore unsuitable for *in vivo* studies, if they did not respond to ADP by forming aggregates consisting of 10 or more platelets.

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Table 1 Patient clinical data

Patient	Age	S-cholesterol (mmol/l)	S-triglycerides (mmol/l)	S-LDL (mmol/l)	S-HDL (mol/l)	Relevant history
1	41	7.9	0.7	5.9	1.7	Myocardial infarction 1982, 1983
2	45	9.5	2.4	7.5	0.9	Myocardial infarction 1970, 1976 Pulmonary embolism 1976, 1981
3	31	9.3	4.2	6.4	1.0	Clinical stigmata of hyperlipidaemia
4	46	6.3	2.8	4.6	0.4	Clinical stigmata of hyperlipidaemia Peripheral arterial disease
5	31	6.2	0.4	5.0	1.0	Coronary bypass surgery 1981
6	40	11.6	2.2	9.5	1.1	Coronary bypass surgery 1981
7	27	12.0	2.0	9.7	1.4	Clinical stigmata of hyperlipidaemia
8	37	8.8	1.6	7.7	0.4	Myocardial infarction 1982, 1983 Coronary bypass surgery 1982
9	42	9.8	1.4	8.0	1.2	Myocardial infarction 1982
10	28	12.0	1.5	10.8	0.5	Clinical stigmata of hyperlipidaemia
Mean	37	9.3	1.0	7.5	1.0	
SD	7	2.1	1.1	2.1	0.4	
Reference values (n = 100)		3.9-6.5	<1.7	2.2-4.6	0.9-1.5	

MPLS was determined from the rate of disappearance of labelled platelets from the circulation. The disappearance curves were constructed from the radioactivity counts in blood samples collected 15 and 90 minutes after reinjection of the labelled platelets, and then twice daily. The count rate of the blood samples was measured in a well-type scintillation counter. MPLS was estimated by fitting a gamma function (11) to the blood clearance curves. Recovery of the labelled platelets in the circulation at equilibrium was calculated from the radioactivity/ml injected and back extrapolation of the survival curve to zero time.

Quantification of *in vivo* redistribution and sites of sequestration of platelets was done as described fully elsewhere (8, 9). Briefly, the distribution of labelled platelets was imaged with a large field of view scintillation camera and the scintigrams analyzed on an A² MDS data processing system. Whole body and region of interest (ROI) In-111-radioactivity was corrected for attenuation by the geometric mean method, and ROI activity expressed as a percentage of whole body activity. That radioactivity not in the liver or spleen, is referred to as the "remainder". At the end of platelet lifespan this radioactivity is situated mainly in the bone-marrow (12). The initial (equilibrium) radioactivity was derived by back extrapolation of linear least-square regression analysis of data, and the final organ radioactivity was determined at the subject's own mean platelet survival time.

Platelets were counted with an electronic particle counter (Coulter Model S-Plus; Coulter Electronics, Florida, USA). Platelet turnover was calculated from the MPLS and the platelet count, correcting for the splenic platelet pool (13). The size of the splenic pool was determined with quantitative imaging.

The *in vitro* platelet aggregation response to adenosine diphosphate (ADP, Sigma), and collagen (soluble collagen Behringwerke) was measured turbidimetrically in an aggregometer (Aggregometer PA3210, Kyoto, Japan). The platelet count in the aggregometer was adjusted to 300×10^9 l. The maximum change in optical density induced by the aggregating agent was related to the difference in optical density between platelet rich and platelet poor plasma and expressed as percentage aggregation. Concentrations of agonists are final cuvette concentrations.

The platelet aggregation response to different concentrations of ADP was determined, and a log-log dose-response curve reflecting the relationship between ADP concentration and percentage platelet aggregation, plotted. A linear regression function was fitted to the data points and the concentration of ADP required to aggregate 50% of the platelets (ADP₅₀) determined. The threshold value, that is the minimum concentration ADP needed to induce the secondary aggregation response, was also determined (14).

Platelet aggregation induced by a single concentration of collagen, 1 µg/ml, was determined. The aggregation index, i.e. the maximum

percentage aggregation observed, divided by the time needed to attain maximum aggregation, was also measured (14).

Beta-thromboglobulin (BTG) and platelet factor 4 (PF4) were measured in blood samples collected with no venous stasis. The first 5 ml of blood was discarded and the blood collected directly into Thrombotect tubes (Abbott Laboratories, North Chicago, USA). The specimens were handled exactly as recommended by the manufacturers and BTG and PF4 assayed with commercial kits for PF4 (Abbott Laboratories, North Chicago, USA) and BTG (Radiochemical Centre, Amersham, UK) respectively.

Circulating platelet aggregates, expressed as an aggregate ratio, were determined according to Wu and Hoak (15). Blood is collected into either formalin, which fixes aggregates, or ethylenediaminetetraacetic acid, which disperses them.

Binding of I-125-fibrinogen to platelets was measured (16).

Statistical Methods

Data were assessed for normal distribution with the Shapiro-Wilk test. Unpaired data were evaluated with the t-test for different means, or the non-parametric Wilcoxon's rank sum test, where appropriate. The difference between sample means was compared by determining the 95% confidence intervals (17).

Results

Platelet Labelling

The results are summarized in Table 2. Virtually all platelets were isolated from the whole blood by the repeated washing procedure. Platelets were labelled with a high specific activity. The contamination of the labelled platelets with plasma protein was $2.8 \pm 1.2\%$ and with red cells $0.4 \pm 0.8\%$.

The labelled platelet population was considered viable because *in vitro* their aggregation response to ADP was normal, and the *in vivo* recovery of labelled platelets in the circulation was normal.

Platelet Kinetics

The difference between the sample mean MPLS in family hypercholesterolaemia and normal subjects was 25 hours, with 95% confidence interval (CI) from 23 to 31 hours; and $P = 0.001$. The MPLS of three of the patients (no. 3, 8 and 10; Table 2) w

Table 2 Platelet labelling data

Patient	Blood platelet count ($\times 10^9/l$)	Platelets isolated from blood (%)	Mean platelet lifespan (hours)	Hits	Recovery (%)	Platelet turnover ($\mu l/hour$)	Labelling efficiency (%)	In-111 administered (MBq)
1	301	100	258	20	57	1835	84	17
2	373	100	218	17	55	2794	88	17
3	155	96	102	6	57	2710	78	15
4	222	93	210	30	47	1649	74	16
5	271	98	200	30	57	2218	76	17
6	206	94	221	30	62	1472	84	17
7	323	98	240	30	55	2007	83	17
8	278	100	99	1	83	4262	82	16
9	279	100	244	13	60	1647	78	15
10	221	100	155	3	67	2382	78	17
Mean	272	98	195	18	60	2298	81	16
SD	63	3	57	12	10	824	4	1
Reference values (n = 40)	150-400	98 $\pm$ 3	221 $\pm$ 21	22 $\pm$ 14	61 $\pm$ 10	1797 $\pm$ 350	86.4 $\pm$ 6.8	-

shorter than 179 hours: i.e. outside the 95% (2 SD) limits delineated by the reference population.

Mean platelet turnover in the patients was significantly higher ($P = 0.005$) than normal (Table 2).

The results of the measurement of the *in vivo* distribution of In-111-labelled platelets are summarized in Table 3.

At equilibrium, platelets were apportioned mainly to the spleen and the blood. The difference between the mean size of the splenic platelet pool in hypercholesterolaemia and normal subjects was 3.7%, with a 95% CI from -0.6% to 8%; and $P = 0.09$. The difference in mean hepatic radioactivity of the patients and the reference group at equilibrium was 1.6% with the 95% CI from -0.8% to 2.8%; and $P = 0.18$. Hepatic radioactivity of patients no. 4, 5, and 10 was high and outside the 95% limits of the normal population.

At the end of mean platelet life-span the radioactivities in the spleen and liver had increased significantly compared to that at equilibrium ($P = 0.0004$), whereas the radioactivity located in the remainder of the tissues (i.e. non-hepatic, non-splenic activity) decreased significantly with time ($P = 0.0001$). The difference in mean final hepatic radioactivity of the patients and the reference group at equilibrium was 1.7% with a 95% CI from -0.4% to

11.4%; and $P = 0.54$. The difference in mean final splenic radioactivity was 2.9% with a 95% CI from -2.8% to 11.4%; and $P = 0.31$. Also, the difference in mean final radioactivity in the remainder of the body was 0.7% with a 95% CI from -0.45% to 5.9%; and $P = 0.79$.

Tests for in vivo platelet activation: The results are summarized in Table 4.

In vitro platelet aggregation. The platelets of the patients, compared to those of the reference group, were not more sensitive to ADP since the ADP_{50} ($P = 0.50$) and the threshold response to ADP ($P = 0.98$) did not differ significantly. Percentage platelet aggregation induced by collagen ($P = 0.46$) and the collagen-induced aggregation index ($P = 0.07$) of the platelets of the patients, also did not vary significantly from normal.

Binding of fibrinogen to platelets. The platelets of the patients and controls bound I-125-fibrinogen to the same extent ($P = 0.61$).

Circulating platelet aggregates. The platelet aggregate ratios were slightly, but significantly lower (indicating that there are more aggregates in the circulation) in the patients with familial hypercholesterolaemia than in the normal control subjects: the

Table 3 In vivo distribution of In-111-labelled platelets

Patient	Organ or whole body radioactivity (%)			At end of platelet lifespan		
	Equilibrium Liver	Spleen	Remainder	Liver	Spleen	Remainder
1	8.4	32.8	58.7	26.0	50.4	23.8
2	10.0	34.8	55.2	30.9	52.9	16.1
3	11.6	39.5	50.0	20.0	49.8	30.3
4	18.9	32.2	48.9	27.0	44.3	28.7
5	18.9	35.0	46.1	27.2	47.1	25.8
6	9.6	33.1	57.3	25.1	43.5	31.3
7	7.7	29.6	62.7	17.7	54.1	28.3
8	13.0	30.4	56.6	21.2	39.5	39.3
9	15.0	27.4	57.5	45.5	29.8	24.7
10	17.3	36.0	46.8	25.8	40.2	34.0
Mean	13.0	33.1	54.0	26.6	45.2	28.2
SD	4.3	3.3	5.3	7.3	7.0	6.0
Reference values (n = 17)	11.4	29.4	59.1	28.3	42.3	28.9
SD	1.9	6.1	6.0	6.7	7.0	6.6

Table 4 In vitro platelet function tests

Patient	1	2	3	4	5	6	7	8	Mean $\pm$ SD	Reference values (n = 20)
Platelet aggregation										
ADP threshold (μ M)	4	1	2	2	4	3	4	2	2.8 ± 1.2	2.8 ± 0.9
ADP (50) (μ M)	11.5	3.6	4.4	4.6	7.3	6.7	8.3	4.6	6.4 ± 2.6	7.0 ± 1.9
Collagen										
aggregation (%)	76	69	73	69	73	71	64	75	71.3 ± 3.9	74.6 ± 12.2
aggregation index	7.4	6.3	4.9	5.1	7.8	8.9	5.6	7.2	6.7 ± 1.4	8.4 ± 2.4
125 I-fibrinogen binding (μ g/ 10^6 platelets)	5.2	8.2	ND*	4.4	10	5.2	4.4	9.0	6.6 ± 2.4	6.9 ± 0.7
Circulating platelet aggregates (ratio)	0.9	0.7	1.0	0.8	0.6	0.7	0.9	0.7	0.8 ± 0.1	0.9 ± 0.1
Plasma BTG (μ g/l)	24	47	8	45	60	43	48	35	38.8 ± 16.2	32.6 ± 18.5
Plasma PF4 (μ g/l)	6	4	6	2	8	5	2	4	4.6 ± 2.1	1.6 ± 3.0

* ND = not done

difference in the ratio was 0.1, with a 95% CI ranging from 0.01 to 0.19, and an associated $P = 0.02$.

Plasma BTG and PF4. The differences in mean plasma BTG of the patients and the reference group did not differ significantly ($P = 0.42$) but mean plasma PF4 levels were slightly higher in the patients compared to controls ($P = 0.02$).

Discussion

Although platelet aggregation, measured in an aggregometer, is the most common platelet function test employed, several factors, difficult to standardize, complicate the interpretation of the results. In this study technical variations were minimized and the results of aggregation tests made more sensitive by constructing a dose-response curve and determining the minimum concentration of ADP needed to induce the secondary aggregation response and the collagen aggregation index. Our results indicate that the aggregation response of platelets to ADP and collagen is normal in types IIa and IIb familial hypercholesterolaemia. In our study, the binding of fibrinogen to the platelet membrane in patients and controls also did not differ significantly, supporting the notion that platelet aggregation in response to ADP and collagen is normal in this disease. This association between fibrinogen binding and the results of *in vitro* platelet aggregation tests, is in accord with the belief of Plow (16). Our results of the platelet aggregatory response are in agreement with those of several other workers (18–21), but are contrary to other reports indicating that platelet aggregation is significantly increased in type II hyperlipidaemias (22–26). The view that platelet hyperreactivity may not be an important facet of type II hypercholesterolaemia, is further supported by the findings that hypolipaeic drug therapy does alter platelet aggregation in such patients (18, 21). It should however be noted that we have measured the response of the platelets to ADP and collagen only. Evidently differences in platelet reactivity, not detected by studying the responses to only these two agonists, may exist. Thus, the platelets of rats (27) and rabbits (28) with diet-induced hyperlipidaemia are hypersensitive to thrombin-induced aggregation. Our results may therefore be ascribed to the fact that the mechanism whereby thrombin aggregates platelets, differs from that of collagen and ADP (29).

BTG and PF4, platelet specific proteins stored in the alpha granules of platelets, are released when platelets are activated. Elevation of the plasma levels of these two proteins may therefore reflect *in vivo* platelet activation. However, the half-life of PF4 in plasma is extremely short (30) because it binds to the vascular endothelial cells almost immediately (31). Elevation of the plasma PF4 levels probably therefore only reflect *in vitro* platelet

activation in response to venesection and manipulation of the platelets. To avoid this artefact, it is thus necessary to control all the procedures involved in the measurement of these proteins rigidly. That the precautions taken in our study were adequate, is supported by the finding that PF4 levels were low in the reference group.

In our study mean plasma BTG levels in the patients with hyperlipidaemia were within normal limits. Our results are similar to those reported by Zucker (21) and Nordoy et al. (32). However, conflicting results have been reported in the literature. Similar to our results, several studies have found increased levels of plasma PF4 in hyperlipidaemia (19, 21, 33, 34). Elevation of BTG as well as PF4 has also been reported (33, 35, 36). Although these results suggest that BTG and PF4 levels may be elevated in familial hyperlipidaemia, it should be noted that the differences are small, and that the concomitant increase in PF4 may reflect artefactual *in vitro* platelet activation despite taking precautions to avoid this.

Our patients did have some evidence of *in vivo* platelet activation. Our finding that circulating platelet aggregates were elevated, is in accord with those of others (33, 37). It is however not clear what the significance of this finding is and why the results of this test differed from the other *in vitro* tests for platelet function. It is also notable that the difference between the mean aggregate ratio of normal subjects and the patients was very small, although statistically significant. This test is therefore likely to be of little, or no, clinical value in the assessment of individual patient.

We also investigated *in vivo* platelet function by the quantitative measurement of platelet kinetics. In-111-oxine is now firmly established as the platelet label of choice, especially because the physical characteristics of this radionuclide permits computer assisted image analysis of the *in vivo* distribution and sites of sequestration of labelled platelets. The methods of platelet labelling have been refined in this laboratory. Techniques have been developed to isolate and label a fully representative platelet population and the labelled platelets are viable (8, 9). The methods of quantifying the platelet redistribution and sites of sequestration with the geometric mean method, have also been validated (12).

The measurement of MPLS is widely regarded as the most sensitive and most specific test of *in vivo* platelet function. Random removal of platelets from the circulation shortens MPLS and alters the shape of the platelet survival curve. The resultant increased platelet utilization will also be manifested as an increase in platelet turnover. Our results suggest that this may indeed be so in some of the patients with hypercholesterolaemia.

The MPLS was shortened in 30% of our patients. Although the means of the MPLS of patients and the reference group differed significantly ($P = 0.02$), the 95% confidence interval from 4 to 48 hours between the means of the two groups, suggests that it may be difficult to interpret the results of this measurement in clinical studies. As expected, mean platelet turnover of the patients was elevated, compensating for the increased utilization of platelets; their blood platelet counts therefore remained normal.

There is evidence that hyperlipidaemia does shorten platelet survival in man (38), monkeys (3, 39) and rabbits (40, 41). However, this has not been found in all studies: Zucker et al. (21) and Harker and Hazzard (18), described normal MPLS in type IIa heterozygous hyperlipidaemia. Age may also be an important determinant, since in elderly, and not the younger patients with heterozygous familial hypercholesterolaemia, platelet lifespan is shortened (19). However, our patients with shortened MPLS were not older than the others. It should also be noted that our patients, despite their relative young age, had evidence of complications of atherosclerosis. Although vessel injury by itself does not necessarily shorten platelet survival (42), there is evidence that atherosclerosis *per se* may, because of endothelial damage, cause increased platelet utilization and by this mechanism shorten the MPLS (18). Furthermore, the MPLS may also be influenced by the presence or absence of an active thromboembolic process at the time of the study. Since such processes are episodic, results of estimates of the MPLS will probably vary in the same patient from time to time. Considering these factors and the results of the above mentioned and our studies, it seems likely that the extent of concomitant degenerative vascular disease in patients with hyperlipidaemia may be the most important cause of the shortened MPLS in this complex situation. This view is strengthened by the finding in rabbits (40) and in pigtail monkeys (3) that platelets from hyperlipidaemic animals survive normally in normal animals, and that platelets from normal animals have a shortened MPLS in hyperlipidaemic animals. This suggests that the shortened MPLS is due to an altered vascular environment and does not primarily reflect functionally changed platelets. The adherence of platelets to the exposed vascular subendothelium may however be facilitated by the exposure of platelets to chronically elevated lipid levels. This alters the cholesterol: phospholipid ratio of the platelet membrane (23) making the platelets hyperreactive (43).

We have also investigated the *in vivo* distribution of In-111-labelled platelets at equilibrium, and their sites of sequestration at the end of the MPLS. Our results suggest that the platelets of these patients may be more susceptible to the "collection injury" induced by the isolation and labelling of platelets. In three of the patients hepatic activity at equilibrium was higher than normal. The accumulation of the platelets in this organ is apparently transient, because recovery of labelled platelets in the circulation was normal. Our results indicate that the alteration of the platelet membrane by chronic exposure to hypercholesterolaemia has no measurable influence on the size of the splenic platelet pool.

We determined the sites of sequestration of the In-111-labelled platelets in the patients, testing the hypothesis that the platelet abnormality associated with hyperlipidaemia may be reflected by an altered platelet sequestration pattern. Because the incorporation of excess cholesterol into the platelet membrane increases platelet microviscosity (43), it has been suggested that the rigid platelets may be easily sequestered in the spleen and susceptible to phagocytosis (41).

However, the pattern of the sites of sequestration of In-111-labelled platelets was normal in this patient group: at the end of platelet life-span, spleen and hepatic radioactivities of all patients, including those with shortened MPLS, were within normal limits.

The results of our study therefore indicate that the determination of the sites of platelet sequestration is not likely to be of value for the assessment of *in vivo* platelet activation. This finding was not surprising, since we have previously demonstrated that platelets interacting with extensive arterial lesions, such as aortic aneurysms, are also eventually sequestered normally in the reticuloendothelial system (44). This, and the result of the present study, indicate that the interaction of platelets with a diseased arterial wall is reversible and transient.

Although there is some evidence of *in vivo* platelet activation in this disease, the precise effect of hypercholesterolaemia on platelet function and the interaction of platelets with the vessel wall, remains to be established. It seems likely that many, if not all, of the changes in platelet function may be due to the activation of the platelets when they come into contact with an atherosclerotic vessel wall, rather than attributable to elevated blood cholesterol and other lipids. In any event, the effects of atherosclerosis and hyperlipidaemia on platelet function and kinetics seem to be closely interrelated in a complex fashion that may be difficult, if not impossible to separate by techniques presently available.

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Platelet Survival in Patients With Beta-Thalassemia

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Thromboembolic events associated with significant morbidity and mortality have been observed in patients with beta-thalassemia major (TM). These include arterial as well as venous thrombosis and the development of early arteriosclerosis. To elucidate the possibility that TM patients may develop a hypercoagulable state we carried out a study of platelet kinetics on ten patients with TM and four patients with thalassemia intermedia (TI). Autologous platelets were labeled with indium-111-oxine, and the platelet lifespan (PLS) was determined. A significant shortening of PLS was observed in 13 out of 14 patients examined. The mean PLS (± 1 SD) in ten patients (8 TM, 2 TI) who underwent splenectomy was 107 ± 36 hr (control splenectomized 248 ± 51 hr) ($P < .001$) and in four nonsplenectomized patients (2 TM, 2 TI) was 102 ± 64 hr (control 224 ± 23 hr) ($P < .01$). The short PLS in addition to reported findings of increased circulating platelet aggregates and the decreased response of TM platelets to aggregating agents suggests in vivo platelet activation in thalassemic patients.

Key words: thalassemia, thrombosis, arteriosclerosis

INTRODUCTION

Hemostatic disorders have been observed in patients with beta-thalassemia major. A mild hemorrhagic tendency, which includes tendency to bruise easily and frequent epistaxis, has been reported by Hilgartner et al. [1] and by us [2,3]. More severe cerebral hemorrhages were also reported especially following multiple blood transfusions; however, their frequency has not been determined [4,5]. Thromboembolic events, which are associated with significant morbidity and mortality, also occur in beta-thalassemia major patients. These include recurrent and transient ischemic cerebral manifestations and stroke [6-8], and a high frequency of thrombotic lesions in the pulmonary arteries has been observed at autopsy [9].

Several groups have investigated different aspects of the hemostatic system in beta-thalassemia. The reported minor abnormalities in the prothrombin time and the plasma levels of the clotting factors I, II, V, VII, IX, and X probably result from parenchymatous liver damage [1]. We and others have noticed a significant decrease in platelet aggregation responses to various agonists [2,3,10]. While these platelet function anomalies could be the cause of the mild hemorrhagic tendency observed

in thalassemia, they could also result from a hypercoagulable state and could reflect an acquired functional defect caused by "exhaustion" of the platelets following their in vivo activation (e.g., by ADP from lysed erythrocytes) [11]. Evidence for this assumption is provided by the significant increase in the numbers of circulating platelet aggregates, particularly in splenectomized thalassemic patients with high platelet counts [12]. The beneficial effect of aspirin therapy in splenectomized thalassemic patients who suffer from hypoxemia without any evidence of heart failure or pulmonary infections [13], lends additional support to this hypothesis.

To elucidate further the possibility of in vivo platelet activation in beta-thalassemia major, we have studied platelet kinetics in 14 patients. The shortening of the mean platelet lifespan that was found is another sensitive indicator of increased platelet consumption in vivo.

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TABLE I. Patients' Data

Patients	Sex/age (yr)	Origin ^a	Diagnosis ^b	Synthetic ratio of globin chains (β/α)	No. of transfusions per year
Splenectomized					
1. M.B.	M 23	K.J.	TM	0.01	16
2. Z.O.	F 22	K.J.	TM	0.19	17
3. N.S.	F 17	K.J.	TM	0.29	12
4. A.T.N.	M 29	A	TI	0.43	—
5. G.L.	F 24	K.J.	TM	0.18	14
6. S.I.	F 30	A	TM	0.06	5
7. S.Y.	F 26	A	TM	0.05	7
8. I.D.	M 17	K.J.	TM	0.01	16
9. D.S.	F 18	A	TM	0.04	11
10. V.A.	M 25	A	TI	0.1	4
Nonsplenectomized					
11. S.D.	M 16	A	TI	0.41	1
12. A.J.	F 19	A	TM	0.31	11
13. A.S.	M 13	A	TI	0.19	2
14. L.A.	F 10	A	TM	0.07	12

^aOrigin: K.J., Kurdish-Jewish; A, Arab.

^bDiagnosis: TM, thalassemia major; TI, thalassemia intermedia.

MATERIALS AND METHODS

Patients

Fourteen patients with homozygous beta thalassemia, eight females and six males, aged 10–30 years, were investigated. The patients received no treatment at the time of the study, and their last blood transfusion was at least 4 weeks prior to the study. The patients consented to participate in the study, which was approved by the Human Experimentation Committee of the Hadassah University Hospital. Thalassemia major was diagnosed as previously described [14].

Platelet Kinetics

Platelets were labeled, and the mean platelet lifespan (MPLS) was estimated as described in detail elsewhere [15–19]. After discarding the first 2 ml, 42.5 ml of blood was collected into a syringe containing 7.5 ml acid-citrate dextrose (ACD), NIH formula A (Fenwal, Baxter-Travenol Laboratories Inc., IL). The blood was transferred to a 50 ml sterile, conical polystyrene tube (Falcon Labware, Oxnard, CA) and platelet rich plasma (PRP) was prepared by centrifuging at 180g maximum. After acidifying the PRP to 6.5 ± 0.2 , by adding 0.5 ml ACD-A for each 10 ml PRP, the platelets were concentrated by centrifuging the PRP at 750g maximum for 3 min. The supernatant was removed, and platelets were resuspended in 3–4 ml physiological saline. The appropriate volume of In-111-oxine (Radiochemical Centre, Amersham, UK) containing 1.25 times the required In-111 radioactivity was added to the PRP and incubated for 30 min at room temperature. The unbound radioactivity was removed by centrifuging the platelets to a pellet for 10

min at 1,200g maximum, decanting the supernatant, and resuspending the platelets in platelet-poor plasma. Labeled platelets were only used if their aggregation response to ADP was normal [15–18]. The platelet suspension was reinjected after allowing it to stand at room temperature for 1 hr.

The MPLS was determined from the rate of disappearance of labeled platelets from the circulation. The disappearance curves were constructed from the radioactivity counts in the blood samples collected 30 min after reinjection of the labeled platelets and then daily. MPLS was estimated by fitting a gamma function to the blood radioactivity data [20]. This model assumes that platelets are removed from the circulation after sustaining a number of insults or "hits." If platelets are removed after a single hit, it reflects random removal, and this results in an exponential curve. Removal after an infinite number of hits will theoretically result in linear survival, reflecting age-dependent platelet destruction. The number of hits will therefore give a clue to the mechanism of platelet destruction and will reflect the shape of the survival curve.

STATISTICS

The mean results of platelet lifespan of the normal subjects and the patient groups were evaluated with the t-test for unpaired data [19].

RESULTS

The patients' relevant clinical and laboratory details are presented in Table I. Ten patients had beta thalassemia major, as judged by the characteristic clinical

TABLE II. Platelet Kinetics

Patient	In-111 administrated (μCi)	Platelet count ($\times 10^{-9}/\text{L}$)	MPLS (hr)	"Hits"
Splenectomized				
1. M.B.	15.6	562	76	1
2. Z.O.	18.7	440	73	1
3. N.S.	17.4	355	137	3
4. A.T.N.	5.8	700	87	1
5. G.L.	9.1	463	84	1
6. S.I.	21.3	744	155	3
7. S.4.	6.6	652	164	1
8. I.D.	7.4	638	59	1
9. D.S.	12.0	750	113	2
10. V.A.	7.3	1093	96	1
Mean ± 1 SD	12.1 ± 5.7	594 ± 257	107 ± 36	1.5 ± 0.8
Reference (n = 4)*	13.2 ± 3.4	290 ± 89	248 ± 51	59 ± 27
Nonsplenectomized				
11. S.D.	15.3	419	192	1
12. A.J.	18.1	206	96	1
13. A.S.	13.7	280	44	1
14. L.A.	9.6	199	74	1
Mean ± 1 SD	14.2 ± 3.6	276 ± 102	102 ± 64	1.0 ± 0.0
Reference (n = 27)*	9.1 ± 4.4	274 ± 59	224 ± 23	40 ± 29

*Reference: data from patients without hemoglobinopathy.

cal features, transfusion requirements, and the β/α synthetic ratio. Four patients had beta thalassemia intermedia. The hemoglobin levels on which the patients were maintained were 8–9 g/dL. All patients except one had very high plasma ferritin levels (2,500–7,000 $\mu\text{g/L}$), while patient S.D. had a level of 390 $\mu\text{g/L}$ (normal levels 50–300 $\mu\text{g/L}$). Ten patients underwent splenectomy between 3 and 18 years prior to the study. All patients had enlarged liver with different degree of impaired liver function tests. None of the patients had esophageal varices, impaired coagulation tests (prothrombin time, activated partial thromboplastin time), or thrombocytopenia. Some of the patients received desferrioxamine therapy, which was discontinued 14 days prior to the platelet kinetic studies.

Platelet Survival

The relevant subject data, estimate of the MPLS, and the number of "hits" given by the multiple hit model are summarized in Table II. The time-radioactivity curves illustrating the mean rate of disappearance of labeled platelets in normal splenectomized subjects and the splenectomized patients with thalassemia are illustrated in Figure 1.

The MPLS of the splenectomized thalassemics was 107 ± 36 hr (± 1 SD); this is significantly shorter than that of the splenectomized normal subjects with no hemoglobinopathy ($P = .0001$) investigated with the same techniques and reported previously [19]. The nonsplenecto-

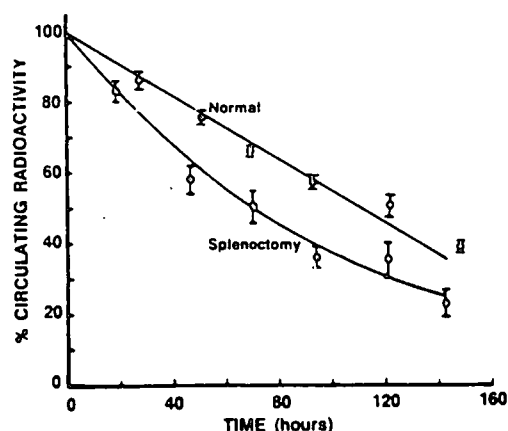


Fig. 1. The mean platelet survival curves (± 1 SEM) in normal splenectomized subjects and the splenectomized patients with thalassemia. The curves were generated by computer fitting of the data to the multiple hit model. Whereas the curve of normal subjects is linear (reflecting a mean platelet lifespan of 228 ± 51 hr [SD]), that of patients with thalassemia is exponential (mean platelet lifespan 107 ± 36 hr [SD]). The curve of the nonsplenectomized thalassemia patients (mean platelet lifespan 102 ± 64 hr [SD]) is not shown; it closely resembles that of the splenectomized patients with thalassemia.

mized patients with thalassemia also had a significantly ($P = .0001$) shortened MPLS (102 ± 64 hr [± 1 SD]) compared to normal. The MPLS of the splenectomized

and nonsplenectomized thalassemia patients did not differ significantly ($P = .94$).

The number of "hits" required to remove the platelets from the circulation indicates that in thalassemia, platelets are removed from the circulation randomly, and not in an age-dependent manner. Thus, in splenectomized patients, platelets were removed after 1.5 ± 0.8 "hits" and in those with intact spleens after 1.0 "hits." Both these values are significantly ($P = .0001$) less than the reference values of the normal subjects with or without a spleen that we have studied.

DISCUSSION

Several methods to measure the *in vivo* processes of platelet activation have evolved in recent years. The measurement of the mean platelet survival time is now regarded as more sensitive and specific than platelet imaging, the measurement of beta-thromboglobulin and platelet factor 4 in the plasma, the detection of circulating platelet aggregates, and the measurement of plasma thromboxane A₂ levels [21]. A representative population of indium-111-labeled platelets disappear from the circulation in an age-dependent manner in healthy human subjects, with or without a spleen [15,19]. The multiple hit model assumes that in this instance an infinite number of insults or "hits" are required to effect destruction of an individual platelet. The resultant platelet survival curve is almost linear [22].

Our data show that the mean survival of platelets is decreased in adult patients with beta-thalassemia major or intermedia with or without prior splenectomy. The shortened mean platelet survival time is associated with an exponential curve and removal of platelets from the circulation after one to three hits. This indicates random destruction of the circulating platelets in thalassemia, which may be due to *in vivo* platelet activation. This hypothesis is supported by the results of several studies. For instance, in thalassemia, there is an increase in the number of platelet aggregates in the circulation [12], malonyldialdehyde (MDA) production is enhanced [23], and the platelets are hyporeactive to aggregating agents [1,3,10]. The latter finding, first thought to be responsible for the mild hemorrhagic tendency manifested by many of the patients [1,3], does not negate the view that platelets are activated *in vivo* in thalassemia. Indeed, platelets of patients with a thrombotic tendency may show a decreased response to aggregating agents [11]. This may be related to a "refractory state" induced by preexposure and preactivation of the platelets by agonists such as ADP [24,25]. In thalassemia it may be caused by the release of ADP and other thromboplastic materials from the hemolyzing red blood cells. Hypoxia caused by

chronic anemia may also be involved in platelet activation, since it has been shown that chronic hypoxia may damage the vascular endothelium [36]. Endothelial injury initiates the interaction of circulating platelets with the vessel wall. The association between intravascular hemolytic anemia and predisposition to hypercoagulable state has been suggested by Rabiner and Rosenfeld [26]. Findings suggestive of hypercoagulable state and *in vivo* platelet activation were also observed in sickle cell disease (SCD), which is also a chronic hemolytic disorder. These findings included impaired platelet aggregation [27-29], the presence of increased number of circulating platelet aggregates [30], elevated concentration of plasma beta-thromboglobulin, a platelet protein released during platelet activation [31], and a significantly shortened MPLS during sickle cell crisis [32]. These findings support the view that platelet activation may contribute to the vasoocclusive problems associated with SCD. Consequently, in one study a beneficial effect in preventing SCD pain crisis has been demonstrated by the platelet-inhibitor drugs aspirin and dipyridamole [33].

Vasoocclusive crises do not occur in thalassemia; however, there is accumulating evidence that patients with thalassemia major may develop a hypercoagulable state. In a survey of 138 patients with thalassemia, 27 patients suffered from ischemic cerebral manifestations, including transient ischemic attacks and strokes [6-8]. Furthermore, thrombosis in the pulmonary arterial tree may be more important in thalassemia than is generally recognized. Wasi et al., from Thailand, have reported that pulmonary arterial thrombotic lesions are very prevalent at autopsy in patients with beta-thalassemia major or hemoglobin E disease [9]. In 19 of 43 (44%) of their patients there were obstructive lesions in the pulmonary artery. These consisted of organized, recanalized thromboemboli, partly or completely occluding the lumen of the arteries. Some large arteries showed changes indistinguishable from primary pulmonary arteriosclerosis. In 13 patients there was hypertrophy of the right ventricle consistent with longstanding pulmonary vascular embarrassment, which culminated in right heart failure. The authors noted that this complication was associated with splenectomy (17/19), and that 18 of the patients were older than 20 years. These findings imply that many of these patients suffered repeated "silent" pulmonary microembolization culminating in cor pulmonale. Extending these findings, the group from Thailand later reported the high prevalence of hypoxemia especially in patients who have been splenectomized. These patients responded to treatment with aspirin and dipyridamole with a rise in their arterial oxygen content [13].

Our experience supports the view that there may be a thrombotic tendency in thalassemia. Four of our adult patients (not included in the present study) developed

major thrombotic events. One patient had peripheral arterial thrombosis, the second had cerebral thrombosis, the third had two documented events of massive pulmonary embolism, and the fourth had two events of deep venous thrombosis of his legs demonstrated by venography.

Several additional factors may predispose a thrombotic tendency in thalassemia. Splenectomy in patients with erythroid hyperplasia caused by hemolytic anemia is associated with a persistent thrombocytosis, which may lead to venous thrombosis and pulmonary embolism [34]. Splenectomy may also predispose to arterial thrombosis; young healthy individuals previously splenectomized have an increased incidence of myocardial infarction [35].

In conclusion, in this study we have shown that the mean platelet lifespan is shortened in thalassemia. This can be related to platelet activation secondary to injury of the vascular endothelium caused by anemia and hypoxia and/or to the chronic hemolysis with continuous release of ADP and other thromboplastic materials from the hemolyzing erythrocytes. Although the risk for thrombosis seems to increase after splenectomy, our results show that the MPLS is shortened even in those patients with intact spleen. The present findings indicate that it may be appropriate to administer platelet-inhibitor drugs to these patients. Prolonged antithrombotic therapy is definitely indicated in thalassemia patients who have had a thrombotic event since such events may be regarded as a complication of the underlying hemolytic process.

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In Vivo Inhibition of Acute Platelet-Dependent Thrombosis in a Baboon Model by BAY U3405, a Thromboxane A₂-Receptor Antagonist S-43

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Summary

Bay U3405 is a thromboxane A₂ (TxA₂)-receptor antagonist that inhibits the binding of TxA₂ to its target cells. The aim of this study was to determine if Bay U3405 could be used to inhibit arterial thrombosis. A thrombogenic device, consisting of uncrimped Dacron vascular graft material (0.5 cm²) built into the wall of silicone rubber tubing with 4 mm inside diameter, was exposed to native flowing blood under arterial blood flow conditions (100–140 ml/min) by interposing the devices as extension segments into permanent femoral arteriovenous shunts implanted in baboons. Thrombus formation was quantified in vivo by measuring the deposition of ¹¹¹In-labelled platelets onto the graft material with a scintillation camera. In six baboons, a bolus injection of Bay U3405, calculated to attain an initial plasma concentration of 300 ng/ml, reduced the maximum thrombus formation measured over a 2 h study period. Platelet deposition was reduced by 33 ± 14% (SD) at 2 h as compared to control studies done in the same baboons. The accumulation of additional platelets onto a thrombus that was allowed to form for 1 h, was reduced by 58 ± 28% at 2 h. Ex vivo platelet aggregation in response to ADP, activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT) were not affected by the treatment. Ex vivo platelet aggregation in response to collagen was markedly inhibited for 2 h after treatment. The results demonstrated that selective blocking of the TxA₂-receptor on platelets reduced platelet-dependent thrombus formation and the accumulation of additional platelets in a freshly formed thrombus. This may provide a viable approach for preventing excessive thrombus formation in patients undergoing arterial reconstructive surgery.

Introduction

Recognition of the role of thromboxane A₂ (TxA₂) as a physiological modulator of thrombosis, vasospasm and broncho-spasm (1–3) provided a strong incentive for developing TxA₂ antagonizing drugs. Two approaches have been followed: the first involved the development of TxA₂-synthetase inhibitors that suppress the conversion of prostaglandin H₂ to TxA₂ (2), and the second the development of TxA₂-antagonists that block the effect of TxA₂ on its target cells (3). Bay U3405 [(3R)-3-(4-fluorophenyl-sulfonamido) 1,2,3,4-tetrahydro-9-carbazolepropanoic acid, Bayer AG, Germany] is a TxA₂-receptor antagonist that effectively blocks the effects of TxA₂ on platelets and smooth muscle cells in vitro and ex vivo (4, 5). Studies in animal models have shown that treatment with Bay U3405 reduces in vivo coronary artery thrombosis (6), protects against myocardial damage following coronary artery occlusion and reperfusion (7), is effective in the prevention of TxA₂ mediated thromboembolic disorders and

sudden death (8, 9) and inhibits platelet aggregate formation on deendothelialized blood vessels (10). Comparative studies further suggest that Bay U3405 may be a more effective antithrombotic drug than aspirin (10, 11).

The thrombotic nature of prosthetic graft material and its interaction with platelets may be one of the most important contributing factors in early thrombosis and thromboembolic complications following arterial reconstructive surgery (12). This study was therefore designed to determine the antithrombotic effects of systemic treatment with Bay U3405 on graft-induced thrombus formation. We used an in vivo baboon model, where acute arterial thrombosis was initiated by exposure of flowing blood to Dacron vascular graft material. We also determined if Bay U3405 could reduce the accumulation of additional platelets onto a preformed thrombus. The results showed that administration of a single dose of Bay U3405, calculated to attain an initial plasma concentration of 300 ng/ml, reduced both platelet deposition on a vascular graft as well as the accumulation of additional platelets in a fresh thrombus. There was no measurable effect on coagulation or the ex vivo aggregation response of platelets to ADP.

Methods

Animals

Six normal male baboons (*Papio ursinus*) weighing between 9 and 13 kg were studied. The project was approved by the Ethics Committee for Animal Experimentation of the Provincial Administration and University of the Orange Free State. The animals were apparently disease free and did not receive drugs that may affect platelet function or coagulation for at least 2 weeks before the study. Permanent exteriorized silicone rubber arteriovenous shunts (AV-shunts) had been implanted between the femoral artery and vein as described (13). These shunts neither shorten platelet survival time, nor produce activation of platelets or coagulation (14).

In each experiment a thrombogenic device, prefilled with isotonic saline to avoid a blood-air interface, was incorporated as an extension segment into the permanent AV-shunt by means of teflon connectors (13). Blood flow rate through the device was 100–140 ml/min, producing a wall shear rate similar to that found in medium- to large-sized arteries (15). In the treatment studies, Bay U3405 was injected as a bolus into the permanent AV-shunt at least 15 min before the start of a study. The dose was calculated to achieve an initial plasma concentration of 300 ng/ml. Bay U3405 was dissolved in pure ethanol (stock concentration = 5 mg/ml) and diluted in saline for injection. The total injected dose was determined by calculating the plasma volume from the hematocrit, measured before the study, and by assuming a blood volume of 65 ml blood/kg (16). We excluded possible residual drug effect on the control studies by doing the control studies the day before the treatment studies. It was shown that previous, short-term placement of grafts does not affect platelet deposition on days subsequent to graft placement (13).

Thrombogenic Device

We used uncrimped, knitted Dacron vascular graft material (0.5 cm²) as a thrombogenic surface. It was built into the wall of silicone rubber

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tubing (4 mm inside diameter). Half the circumference of the tubing wall (1.5 cm long) was carefully cut from the tubing. A template, cut from a teflon rod, was used to ensure that the pieces which were removed from different devices were identical. A teflon rod (4 mm diameter) was inserted into the tubing, and the graft material (1.5 cm long) positioned to fit snugly over the cutting. The piece of tubing that was removed was fastened over the Dacron graft and the edges were covered with Silastic medical adhesive (Type A, Dow Corning Incorporated). To ensure that the device was impervious to blood leakage, heat shrink teflon was shrunk over the fitted piece. After the polymer was allowed to cure for 24 h, the teflon rod was carefully removed and the device washed with a mild soap solution, then rinsed with ethanol and finally with distilled water.

Graft Imaging and Quantification of Graft Platelet Deposition

Autologous platelets were labelled with ^{111}In -tropone (16, 17). Imaging and quantification of the deposition of the labelled platelets on the graft material were done as previously described (13, 18, 19). Briefly, the deposition of labelled platelets on the graft surface was imaged with a Searle Pho scintillation camera fitted with a high resolution collimator. Good image resolution was obtained by imaging the low energy peak (172 keV) of ^{111}In with a 5% window. Imaging of the graft segments, including proximal and distal silicone tubing segments, was started simultaneously with blood flow through the extension segment of the AV-shunt. The images (128 × 128 byte mode) were continuously acquired for 2 h, with data storage every 5 min on an A² MDS data processing system. An image of a blood standard (3 ml) was also acquired to determine circulating ^{111}In -radioactivity per ml of blood. A region of interest (1 × 1.5 cm) of the graft segment was selected to determine deposited and circulating radioactivity in each of the images of the dynamic study. Radioactivity in a region of similar size of circulating radioactivity in the proximal segment of the extension tubing was determined and subtracted from the radioactivity in the graft region of interest in order to calculate deposited ^{111}In -platelet radioactivity only. The ratio between deposited radioactivity and circulating radioactivity in 1 ml blood (from the blood standard) was calculated. The latter was appropriately corrected for plasma radioactivity (13). The number of platelets deposited on the graft was calculated by multiplying the ratio with the number of platelets per ml blood.

We have measured the effect of systemic treatment with Bay U3405 on deposition of platelets on a native graft and the effect on the accumulation of additional platelets in a fresh thrombus. To measure the latter, a thrombogenic device was incorporated as an extension segment in a permanent AV-shunt of a baboon whose platelets were not labelled with ^{111}In . After the "cold" thrombus was allowed to form for 1 h, the device was removed, carefully washed with saline and incorporated into the AV-shunt of an animal whose platelets were labelled. Imaging of the deposition of labelled platelets on the fresh thrombus was done for 2 h and platelet deposition quantified.

Laboratory Procedures

In each experiment, blood samples were collected before and after exposure of the blood to the thrombogenic device. The platelet count and hematocrit were determined with a Technicon H2 blood cell analyser on blood collected in EDTA. The same sample was used to correct whole blood radioactivity for plasma radioactivity in order to calculate platelet radioactivity (13). Blood was collected in 3.8% sodium citrate (9 volumes of blood to 1 volume citrate) for the determination of the APTT, PT, TT, plasma fibrinogen concentration, antithrombin III (AT III), activated protein C (APC) and D-Dimer. APTT and PT (Lennon Diagnostics) and TT (Roche Diagnostica) were measured on a fibrinometer (Clotex II, Hyland), fibrinogen was indirectly determined with an Automated Coagulation Laboratory (Instrumentation Laboratories), AT-III was measured using a chromogenic substrate (Instrumentation Laboratories, Viole Monza, Italy) and APC and D-Dimer were determined using commercially available assay kit systems (Behringwerke).

The *in vitro* aggregation response of platelets, before, 10 min and 130 min after treatment with Bay U3405, was determined turbidimetrically in an Monitor IV Plus aggregometer (Helena Laboratories). The method has been described fully (20). Briefly, blood was collected in 3.8% sodium citrate (9:1), platelet rich plasma prepared by differential centrifugation, and the platelet count adjusted to $200 \times 10^9/\text{l}$ with platelet

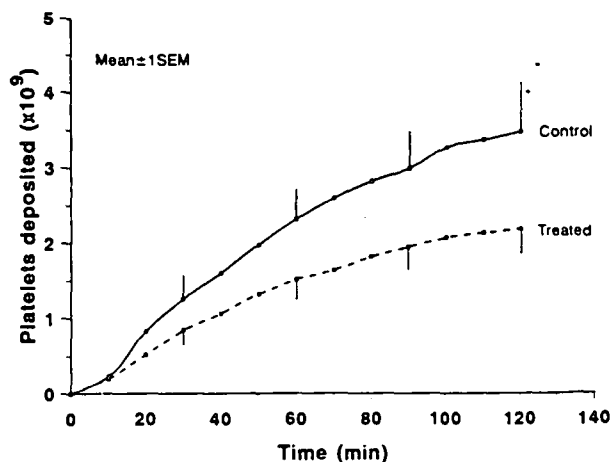


Fig. 1 Platelet deposition onto a native Dacron vascular graft

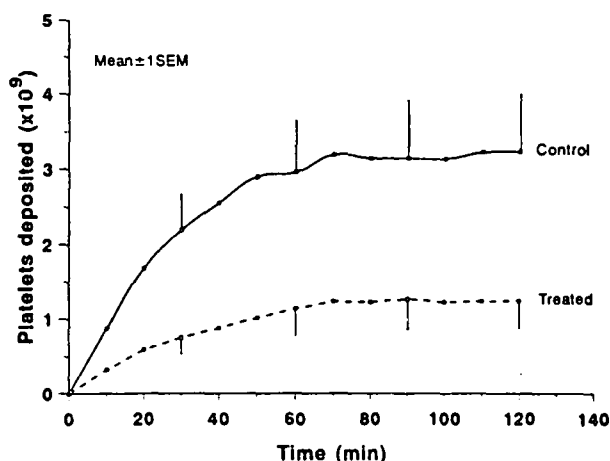


Fig. 2 Accumulation of additional platelets onto a non-radioactive thrombus that was allowed to form for 1 h before it was inserted in the permanent AV shunt of a baboon whose platelets had been labelled with ^{111}In

poor plasma. The aggregation response, measured at 4 min follow addition of ADP (20, 10, 2 and 1.5 μM) or collagen (0.05 g/l), was related to the difference in light transmission between platelet rich and platelet poor plasma and expressed as a percentage. Dose response curves were constructed to calculate the effective dose of ADP that will cause 50% aggregation (ED_{50}).

Statistical Analyses

Student's *t*-test for paired data was used to test for differences between means. Data in the text are given as a mean $\pm$ 1 SD.

Results

Platelet Deposition on Fresh Grafts

Exposure of flowing blood to the thrombogenic surface for 2 h reduced the mean platelet count by approximately 17%, approximately 13% decrease in ^{111}In -platelet radioactivity was significantly different (Table 1). In the treatment studies mean platelet count decreased by approximately 4% and platelet radioactivity by approximately 11%. The 17% decrease in platelet count observed in the control studies was significantly larger than the 4% measured in the treatment studies ($t = 2.2$).

Table 1 Antithrombotic and anticoagulating effects of treatment with Bay U3405 (300 ng/ml plasma)

	Control studies		Treatment studies	
	Baseline	At 2 h	Baseline	At 2 h
Platelet count ($\times 10^9/l$)	337 $\pm$ 91	281 $\pm$ 97	283 $\pm$ 103	271 $\pm$ 84
% Decrease	—	17 $\pm$ 10	—	4 $\pm$ 4
% Platelet				
In-111-activity	100 $\pm$ 0	87 $\pm$ 6	100 $\pm$ 0	89 $\pm$ 7
Fibrinogen (g/l)	5.1 $\pm$ 1.1	5.6 $\pm$ 1.0	5.9 $\pm$ 1.4	6.2 $\pm$ 1.5
APTT (s)	42 $\pm$ 6	43 $\pm$ 3	44 $\pm$ 3	41 $\pm$ 2
PT-(s)	15 $\pm$ 1	14 $\pm$ 1	15 $\pm$ 1	14 $\pm$ 1
TT (s)	27 $\pm$ 3	26 $\pm$ 2	27 $\pm$ 3	27 $\pm$ 3
AT III (%)	75 $\pm$ 11	82 $\pm$ 10	78 $\pm$ 6	81 $\pm$ 11
APC (%)	78 $\pm$ 16	69 $\pm$ 30	69 $\pm$ 8	63 $\pm$ 16
D-Dimer (μ g/ml)	<0.5	<0.5	<0.5	<0.5

Table 2 The ex vivo aggregation response of platelets before, 10 min and 130 min after treatment of the baboons with Bay U3405 (300 ng/ml plasma)

Agonist	Concentration	Before treatment	10 min after treatment	130 min after treatment
ADP	20 (μ M)	100 $\pm$ 0	96 $\pm$ 8	
	10 (μ M)	87 $\pm$ 6	84 $\pm$ 12	
	2 (μ M)	15 $\pm$ 11	21 $\pm$ 11	
	1.5 (μ M)	5 $\pm$ 8	6 $\pm$ 7	
Collagen	0.05 g/l	80 $\pm$ 5	14 $\pm$ 5	19 $\pm$ 9

$p < 0.05$, 95% confidence interval from 2 to 24%), suggesting that less platelets were consumed as a result of treatment.

Since the decrease in platelet count was similar to the decrease in ^{111}In -platelet radioactivity, it is reasonable to assume that the interaction of labelled platelets with the thrombogenic device is comparable to that of unlabelled platelets. It is therefore valid to calculate the number of platelets deposited from the ratio of deposited labelled platelet radioactivity to circulating labelled platelet radioactivity.

Measurements of acute deposition of platelets on grafts are shown in Fig. 1. In the control studies the deposition curves were generally sigmoidal, almost reaching a plateau by 120 min. At this point a mean of $3.48 \pm 1.58 \times 10^9$ platelets were deposited. In the treatment studies the shapes of the deposition curves were similar. The plateau in deposition was reached after approximately 100 min, at which time $2.08 \pm 0.85 \times 10^9$ platelets were deposited. After 2 h of blood exposure a mean of $2.18 \pm 0.77 \times 10^9$ platelets were deposited. This represents a reduction of $33 \pm 14\%$ in deposition when compared to control studies ($t = 5.7281$, $p < 0.001$, 95% confidence interval from 18 to 48%).

Accumulation of Additional Platelets onto a Preformed Thrombus

This is shown in Fig. 2. A plateau in accumulation was reached after approximately 70 min in the control and treatment studies. At 2 h a mean of $3.23 \pm 1.96 \times 10^9$ platelets were deposited in the control studies and $1.25 \pm 0.92 \times 10^9$ platelets in the treatment studies. Treatment reduced the mean by $58 \pm 28\%$ ($t = 4.9744$, $p < 0.01$, 95% confidence interval from 28 to 88%).

In Vitro Platelet Aggregation

The results are summarized in Table 2. We regarded the aggregation response to 20 μ M ADP before treatment as 100%,

and expressed all other aggregation responses relative to this. Treatment with Bay U3405 had no measurable effect on the platelet aggregation response. The ED_{50} was not significantly different, i. e. $4.9 \pm 1.2 \mu\text{M}$ before treatment versus $4.5 \pm 1.1 \mu\text{M}$ 2 h after treatment ($r = 0.8201$, $p > 0.05$, 95% confidence interval from -0.7 to $1.7 \mu\text{M}$). The aggregation response to collagen was markedly inhibited 10 min after treatment with Bay U3405, and this inhibition was maintained for 2 h.

Coagulation and Fibrinolysis

The results are summarized in Table 1. In both the control and treatment studies, two hours of exposure of the thrombogenic devices to flowing blood had no measurable effect on coagulation and fibrinolysis.

Discussion

The inhibitory effects of Bay U3405 on the action of TxA_2 and TxA_2 -mimetics lasts for 3–8 h (5, 6). Although we did not determine the plasma concentrations of Bay U3405 during the study period, collagen-induced platelet aggregation was still markedly inhibited 2 h after treatment (Table 2). The bolus injection was therefore sufficient to study the effects of Bay U3405 on acute arterial thrombosis during the 2 h of exposure of the thrombogenic device to native flowing blood.

A bolus injection with Bay U3405, which would result in a calculated initial concentration of 300 ng/ml plasma, inhibited platelet-dependent thrombus formation induced by the vascular grafts (Fig. 1). Both the rate and the extent of platelet deposition were reduced. Treatment also reduced the rate and extent of accumulation of additional platelets in a fresh thrombus (Fig. 2). In this model, the blood platelet count is an important determinant of the number of platelets that will be deposited; more platelets are deposited if the peripheral blood platelet count is higher (18, 23). It is therefore possible that some of the reduction in platelet deposition in the treatment studies can partially be attributed to the lower platelet count (Table 1). However, the difference in platelet count between the control and treatment studies was small, and we believe that this difference cannot fully explain the observed reduction in platelet deposition and platelet turnover in the thrombus.

A significant inhibition of acute arterial thrombus formation was achieved by blocking the TxA_2 -receptor. Aspirin, which acetylates cyclo-oxygenase and so prevents TxA_2 synthesis by platelets (21), has no measurable effect on platelet deposition in a similar model of acute arterial thrombosis (22). Our results, and those of others using different approaches (10, 11, 24, 25) strongly suggest that receptor blockade may be a more effective strategy of treating arterial thrombosis than inhibition of TxA_2 -synthesis. This assumption is further supported by the variable success that was achieved with TxA_2 -synthetase antagonists (2, 25). Aspirin prevents the synthesis of both the prostaglandin derived agonists (TxA_2 , cyclic peroxides) and antagonists (prostaglandin D_2) by platelets (24, 26). When the TxA_2 -blockers are used, the pro-aggregatory effect of TxA_2 and the cyclic peroxides on platelets is blocked, while inhibition of platelet function by prostaglandin D_2 (and prostacyclin) is not affected (25). Our results therefore imply that the inhibitory role of the prostaglandin derived antagonists is important in thrombogenesis. The fact that their function is not impaired by Bay U3405 may therefore explain why the TxA_2 -receptor blocker may be a better approach to treat arterial thrombosis.

The ex vivo aggregation response of platelets to ADP was not measurably affected by treatment with Bay U3405 (Table 2), a finding which contrasts with that in other studies (5, 10).

However, there are differences between the studies that may explain our results. It is possible that the plasma concentration of 300 ng/ml of Bay U3405 was too low to inhibit the effects of ADP on platelets. In *in vitro* studies where platelets were incubated with Bay U3405, a concentration of 355 ng/ml was needed to decrease platelet aggregation by 50% (5). In another, similar study, a concentration of 4.2 µg/ml had no effect on ADP-induced aggregation (9). On the other hand, when the drug was infused, a concentration in excess of 500 ng/ml was required to "significantly inhibit ADP and collagen aggregation responses" (10). It therefore seems that higher plasma concentrations are needed to inhibit *ex vivo* platelet aggregation. Our results nevertheless compare favourably with the results obtained in studies where other TxA_2 -antagonists were used. These antagonists inhibited platelet deposition on Dacron vascular grafts in humans (24), and improved graft patency in dogs (25) without inhibiting *ex vivo* platelet aggregation induced by ADP.

Treatment with Bay U3405 did not completely inhibit platelet deposition onto the vascular graft, as is found when potent antithrombins (19, 27), or monoclonal antibodies and peptides, that block the binding of fibrinogen to its glycoprotein IIb/IIIa receptor on the platelet membrane (22, 28) are used. It is possible that higher concentrations of Bay U3405 may cause more inhibition of thrombus formation. At the dosages that we have used, neither coagulation (Table 1) nor the *ex vivo* platelet aggregation response to ADP (Table 2) were affected. The lower dosage which was used may explain why platelet deposition and accumulation of additional platelets onto the preformed thrombus were only partially inhibited, since both thrombin formation and the effect of ADP on platelets were not affected. Also bear in mind that thrombin and ADP activate and aggregate platelets via non-thromboxane pathways (19, 27).

In conclusion, we have demonstrated that a single dosage of Bay U3405 effectively inhibited acute platelet-dependent thrombus formation induced by a vascular graft. It also inhibited the accumulation of additional platelets in a freshly formed thrombus. These effects were seen despite the findings that the ADP-induced platelet aggregation response and coagulation were not measurably altered. Bay U3405 can therefore be used to prevent excessive thrombus formation in patients undergoing arterial reconstructive surgery, an effect that may improve graft performance over the long term.

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Prolonged Inhibition of Acute Arterial Thrombosis by High Dosing of a Monoclonal Anti-platelet Glycoprotein IIb/IIIa Antibody in a Baboon Model

S-44

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Summary

The *in vivo* activity of MA-16N7C2, the first monoclonal antibody that contains an echistatin-like RGD-sequence and inhibits platelet glycoprotein (GP)IIb/IIIa function, was determined in baboons. A dose-finding study assessing haemostatic variables such as bleeding time and *ex vivo* platelet aggregation showed that doses of as low as 0.2-0.3 mg/kg resulted in a pronounced effect. The effects were dose-dependent and lasted for several days, implying that MA-16N7C2 is a potent and long-acting GPIIb/IIIa inhibitor. Following the initial studies, the antithrombotic effect of 0.1 and 0.3 mg/kg of the antibody, given as a bolus, was determined in a baboon model of platelet-dependent, arterial-type thrombus formation. In these studies, a thrombogenic device consisting of Dacron vascular graft material was inserted as extension segments into a permanent arteriovenous shunt. The results confirmed the potent and long-lasting antithrombotic effect of MA-16N7C2. Surprisingly, the antithrombotic effect was stronger 48 h after a dose of 0.3 mg/kg administration than on the day of treatment with 0.1 mg/kg, despite the fact that comparable numbers of GPIIb/IIIa receptors were occupied on resting platelets. We postulate that with the high dose of MA-16N7C2 and after an extended period, occupied GPIIb/IIIa may be internalised by the platelets. Upon platelet activation, these receptors become reexposed but are unable to participate in thrombus formation. This is in contrast to unoccupied internal GPIIb/IIIa receptors early after a low dose of MA-16N7C2.

Introduction

Blood platelets are the first line of host defence when normal blood vessels are injured. Platelet adhesion, aggregation and further platelet recruitment culminate in haemostatic plug formation to prevent excessive bleeding. This is accompanied by the consolidating effect of fibrin(ogen) binding to glycoprotein (GP) IIb/IIIa, the fibrinogen receptor on the platelet membrane. Endothelial cells in the proximity possess thromboregulators that limit the size of the haemostatic plug. However, induction of excessive platelet activation by biomaterials and structures such as an ulcer or fissure in the fibrous cap of atheroma overcomes the normal thromboregulatory mechanisms. The platelets then become a major prothrombotic offender predisposing to arterial vaso-occlusive disease (1-6). Inhibition of platelet function is therefore a major avenue of search for potent antithrombotic agents. The present mainstay of this

approach is to develop monoclonal antibodies, peptides and peptidomimetics that can prevent the binding of fibrinogen, through the carboxy terminal dodecapeptide (7) or the Arg-Gly-Asp (RGD) recognition sequence of fibrinogen to GPIIb/IIIa (8-15). Such agents prevent *in vitro* and *ex vivo* platelet aggregation and *in vivo* thrombosis in experimental models and patients (13-17). The major differences amongst the agents are mainly in their affinity for GPIIb/IIIa and in their plasma half-life, i.e. the time they can act as inhibitors of platelet function *in vivo* (8-16). An intriguing further possibility is that platelets can become loaded with the inhibitor: indeed, evidence has been provided that GPIIb/IIIa is a cycling receptor (18, 19) and as such can internalize not only fibrinogen but also anti-GPIIb/IIIa products into the platelet α -granules (20, 21). Such process would be favored when products are used with a low off-rate, such as high affinity antibodies.

In this study we report on the antithrombotic effects of a murine monoclonal antibody, MA-16N7C2, in a baboon model of platelet-dependent arterial type thrombus formation. MA-16N7C2 inhibits GPIIb/IIIa function and is the first antibody described with an echistatin-like RGD-containing sequence in the CDR3-region of its heavy chain, which however is not fully responsible for the inhibitory effect (22). The antibody furthermore recognises the Ca^{2+} -dependent GPIIb/IIIa complex also on resting platelets, but binding is accelerated and with higher affinity upon platelet activation.

Materials and Methods

Antibody Preparation

Antibody MA-16N7C2 was purified from ascitic fluid from BALB/c mice by protein A Sepharose 4B affinity chromatography in 0.1 M Tris-HCl, pH 8.3. After elution at low pH (0.1 M glycine, pH 2.8), the antibody was dialyzed against PBS and purity was analyzed with SDS-PAGE. Protein content was determined using the Bio-Rad protein assay (BioRad Laboratories, Brussels, Belgium) with bovine gamma globulin as standard.

Species Specificity

Blood from dogs, cats, rabbits, pigs and baboons was anticoagulated with 0.12 vol 145 mM trisodium citrate, whereas for rat, hamster and guinea pig blood, an additional 1 U/ml heparin (Liquemine; Roche, Basel, Switzerland) was added.

Platelet-rich plasma was obtained after centrifugation of the blood for 10 min at 150 $\times$ g. Platelet aggregation was performed on PRP with ADP (Sigma Chemicals Co., St. Louis, MO) or collagen (Hormon Chemie, München, Germany) as inducers using the optical method. Gel-filtered platelets were prepared from PRP by chromatography on a 20 $\times$ 2.5 cm Sepharose 2B column (Pharmacia, Uppsala, Sweden) using 5 mM Hepes, 0.34 mM Na_2PO_4 , 12 mM NaHCO₃, 134 mM NaCl, 2.9 mM KCl, 10% ACD, pH 5.5.

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Platelets were counted in a Platelet counter (Cell-Dyn 610; Sequoia-Turner Co., Mountain View, CA) and 200 μ l of $150\text{--}200 \times 10^3/\mu$ l gel-filtered platelets was put into the wells of microtiter plates which had been pretreated with 10 μ g/ml poly-L-lysine (100 μ l/well). The plates were centrifuged at 4°C for 15 min at 150 g. After removal of the supernatant, the platelets were fixed for 15 min at 25°C with 0.5% (v/v) formalin (50 μ l/well). After washing with PBS containing 0.002% (w/v) Tween 80, plates were blocked with 1% bovine serum albumin and next incubated with MA-16N7C2 for 1 h at room temperature. After another washing step, the bound material was detected with rabbit anti-mouse IgG (H + L) coupled to horseradish peroxidase (Nordic Immunology, Tilburg, The Netherlands) diluted 1/3000 in PBS, 0.1% BSA, 0.002% Tween for 1 h. Following a final wash, colour development was done using 160 μ l of a solution containing 0.4 mg/ml 1,2-orthophenylene-diamine (Fluka Chemie, Buchs, Switzerland) in 17 mM citric acid, 65 mM Na₂HPO₄ and 0.003% H₂O₂. The reaction was stopped with 50 μ l of 4 M H₂SO₄ and absorption was measured at 442 nm in an ELISA-reader (EAR 40; AT, SLT-Lab instruments, Austria).

Animal Studies

1. Dose-finding studies. Baboons (*Papio hamadryas*) of either sex, weighing 7–12 kg, were anaesthetized with 10 mg/kg ketamine hydrochloride (Imalgene 500; Sanofi, Toulouse, France) and 0.06 mg/kg atropine i.m. During the first experimental day anaesthesia was maintained with 30 mg pentobarbital i.v. (Nembutal; Abbott Laboratories, North Chicago, IL) at hourly intervals.

Antibody used for i.v. injection was in PBS, 1 mM CaCl₂ and appropriately diluted with saline in order to have an injection volume of 1 ml. The preparation contained less than 29 EU/ml endotoxin (KabiVitrum, Stockholm, Sweden). Samples were taken before and 1 h after injection until a dose was obtained giving a prolongation of the bleeding time of at least 30 min. Subsequent determination of bleeding times and blood collection of samples were carried out until the bleeding time was back into the normal range (≤ 10 min).

Bleeding times were performed on the shaved volar surface of the arm using an automated template device (Simplat II; General Diagnostics, Morris Plains, N.J.). No pressure cuff was applied. Blood was collected for determination of haematocrit, platelet count, ADP- and collagen induced platelet aggregation, determination of remaining binding sites for MA-16N7C2 and determination of plasma levels of murine monoclonal antibodies.

Determination of remaining binding sites. ¹²⁵I-labeling of antibody was done using Iodogen-reagent (Pierce Chemical Company, Rockford, IL) as described (22, 23). Remaining binding sites on platelets from baboons treated with antibody, were determined in duplicate by incubating PRP, with platelet counts adjusted to 200,000 μ l/ μ l with autologous plasma, with a saturating concentration (2 μ g/ml) of ¹²⁵I-MA-16N7C2 for 15 min at room temperature. 80 μ l of this was then carefully layered over either 200 μ l of an oil-layer consisting of 3/4 vol silicone oil (Jansen Chimica, Beerse, Belgium) and 1/4 vol paraffine oil (VEL, Haasrode, Belgium) or over 200 μ l 15% sucrose in Eppendorf tubes and centrifuged at 12,000 g for 4 min. The tips, containing the platelet pellet, were cut and platelet-associated radioactivity was measured with a gamma-counter (Packard, Meriden, CT).

Plasma levels. Plasma levels of murine monoclonal antibodies were measured in an ELISA using plates coated with 4 μ g/ml affinity-purified (AP)-goat anti-mouse IgG (H + L) antibodies (Jackson Immuno Research, West Grove, PA) and developed with AP-goat anti-mouse IgG coupled to horseradish peroxidase (Jackson Immuno Research, West Grove, PA). Standard curves were constructed by adding known amounts of MA-16N7C2 to baboon plasma.

2. Thrombosis studies. Thirteen normal male baboons (*Papio ursinus*) were used. The animals weighed between 10 and 15 kg and were disease-free for at least 6 weeks prior to use. All procedures were approved by the Institutional Ethics Committee for Animal Experimentation in accordance with Federal Guidelines (Guide for the Care and Use of Laboratory Animals, Public Health Service National Institute of Health publication number 85-23, 1985). The peripheral blood platelet counts, $282 \pm 62 \times 10^9/l$, were normal. The baboons

supported permanent Teflon-Silastic arteriovenous (AV) shunts implanted in the femoral vessels (24). These shunts do not detectably shorten platelet survival or produce measurable platelet activation (24–26).

Study protocol. Platelet dependent, arterial type thrombus formation was induced by Dacron vascular graft material (0.5 cm²) that was built into the wall of silicone rubber tubing (inside diameter = 4 mm). The method of preparation of the thrombogenic device is described in detail (27).

In each experiment, a thrombogenic device, prefilled with saline to avoid a blood-air interface, was incorporated as an extension segment into the permanent AV-shunt by means of teflon connectors (24). The devices were kept in place for 2 h whereafter they were removed and blood flow through the permanent shunt was reestablished. On the day before the treatment studies (day 0), control studies were done in all baboons. On day 1, the baboons were treated with a bolus injection of 0.3 mg/kg ($n = 7$) or 0.1 mg/kg ($n = 6$) of MA-16N7C2 15 min before the devices were placed. Devices were again placed on day 2 and 3. Blood flow rate through the device was 100–140 ml/min, producing wall shear rates similar to that found in medium to large sized arteries (25). The antibody was diluted in saline for injection.

Graft imaging and quantification of platelet deposition. Autologous blood platelets were labeled with ¹¹¹In-chloride as previously described (28) and reinjected at least one hour before thrombogenic devices were placed on the day the control studies were done. Imaging and quantification of the deposition of ¹¹¹In-platelets were done as described in detail (24). Image acquisition of the grafts, including proximal and distal silastic segments, was done with a Searle Pho scintillation camera fitted with a high resolution collimator. The images were stored on and analyzed with a Medical Data Systems A³ computer (Medtronic, Ann Arbor, MI) interfaced with the camera. Dynamic image acquisition, 5 min images (128 $\times$ 128 byte mode) for 2 h, was started simultaneously with the start of blood flow through the devices. A five minute image (128 $\times$ 128 byte mode) of a 3 ml autologous blood sample was also acquired each time that the grafts were imaged to determine circulating blood radioactivity (blood standard). A region of interest (1 $\times$ 1.5 cm) of the graft segment was selected to determine the deposited and circulating radioactivity in the proximal segment of the extension tubing. This activity was subtracted from the radioactivity in the graft region to calculate deposited radioactivity. The ratio between deposited ¹¹¹In-platelet radioactivity and circulating radioactivity in one ml blood (from the blood standard) was calculated. The latter was appropriately corrected for plasma ¹¹¹In-radioactivity (24). The number of platelets deposited on the graft was calculated by multiplying the ratio with the number of platelets per ml of blood (27).

Laboratory measurements. In each experiment, blood samples were collected before and after exposure of the blood to the thrombogenic device. The platelet count and haematocrit were determined in blood collected in 2 mg/ml disodium EDTA using a Technicon H2 blood cell analyser (Bayer Diagnostics). This sample was also used to correct whole blood radioactivity for plasma radioactivity in order to calculate platelet radioactivity (24).

Blood from MA-16N7C2-treated baboons without implants was furthermore collected in 3.8% sodium citrate (9 volumes blood to 1 volume citrate) to determine the activated partial thromboplastin time (APTT) and the plasma fibrinogen concentration. APTT (Dade) was measured on a fibrinometer (Clotex I Hyland) and fibrinogen was indirectly determined with an Automated Coagulation Laboratory (Instrumentation Laboratories). The template bleeding time was measured as described (25).

The in vitro aggregation response to platelets before, 10 min and 24 and 48 after treatment with MA-16N7C2 was measured turbidimetrically in a Monit IV Plus aggregometer (Helena Laboratories). The method has been fully described elsewhere (29). The aggregation response, measured at 4 min following addition of ADP (20 μ M) or collagen (0.05 g/l), was related to the difference light transmission between platelet-rich and platelet-poor plasma and expressed as a percentage.

The number of GPIIb/IIIa receptors occupied by MA-16N7C2 was calculated as described above. Platelet-rich plasma was incubated with near saturating levels of ¹²⁵I-MA-16N7C2 and the number of radiolabelled molecules bound per platelet determined.

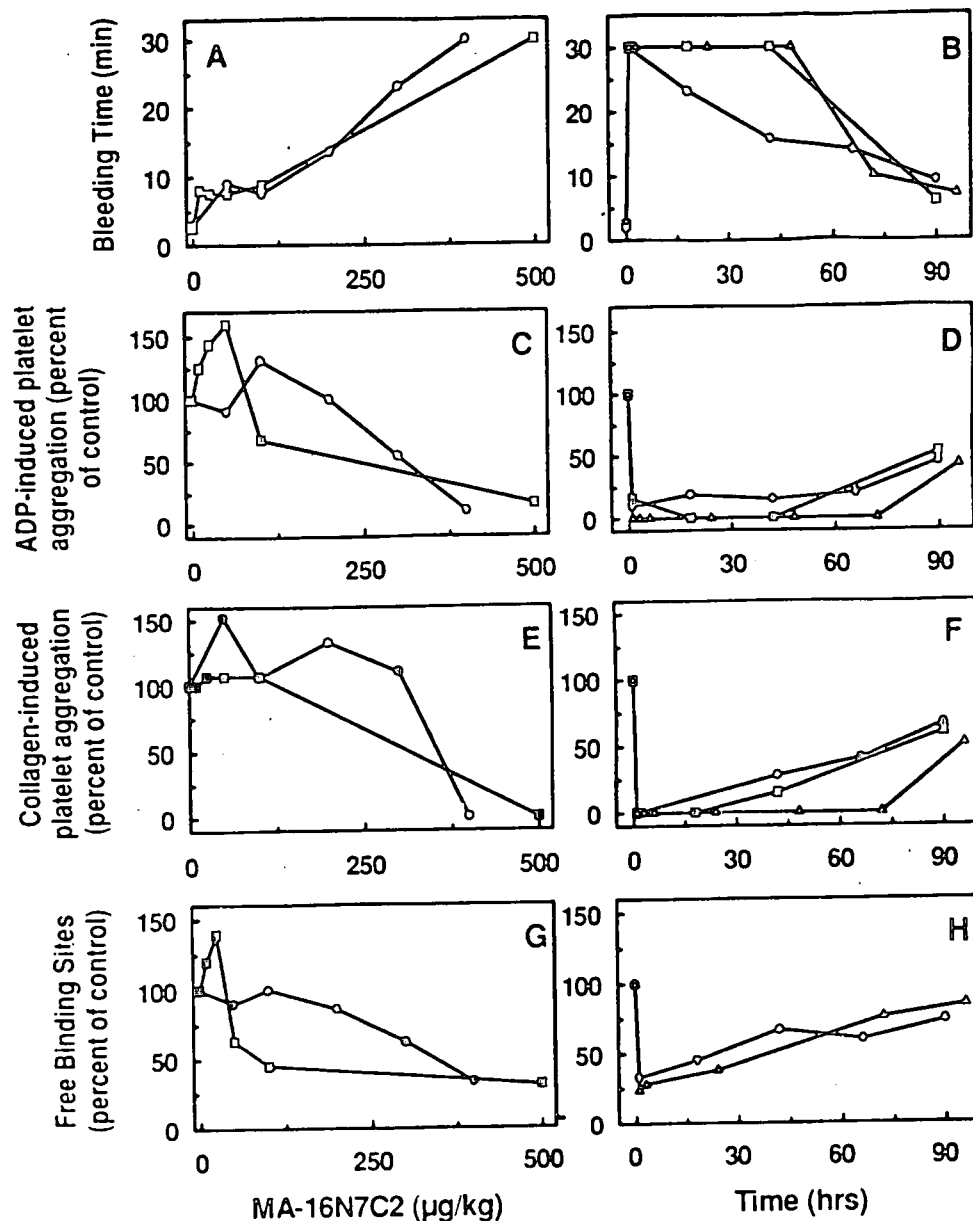


Fig. 1 Bleeding times (A, B), ADP- (C, D) and collagen- (E, F) induced platelet aggregation, and remaining binding sites for ^{125}I -MA-16N7C2 (G, H), as a function of cumulative doses of MA-16N7C2 (left) resulting in a final total dose of 0.4 (●) or 0.5 (■) mg/kg, and as a function of time after administration of a total dose of 1 (△), 0.5 (■) or 0.4 (●) mg/kg MA-16N7C2 (right) to baboons

The number of receptors occupied by the monoclonal antibody was finally obtained by subtracting the number of labelled molecules bound to the platelets after infusion of antibody from that obtained before infusion

Statistical analysis. Student's test (two-tailed) was used to test for differences when the data were normally distributed. Data in the text are given as the mean $\pm$ SD.

Results

1. Dose-finding Studies

Preliminary studies indicated that MA-16N7C2 did not bind to platelets or inhibit platelet aggregation when platelets from dogs, cats, pigs, rabbits, rats, hamsters, mice or guinea pigs were used. Platelets from baboons on the other hand clearly did bind and were inhibited by the antibody. We therefore used this species to evaluate the in vivo potential of the antibody.

First a dose finding study was done, the results of which are represented in Fig. 1. In the initial experiment a bolus injection of 1 mg/kg MA-16N7C2 was given, in the second and third, consecutive doses at one hour intervals were administered. Following these injections, the studies on the baboons were continued until bleeding times returned into the normal range.

I.v. administration of MA-16N7C2 had profound, dose-dependent and long-lasting effects on bleeding times and platelet aggregability. Full recovery from the total dosage of 0.4 mg/kg, 0.5 mg/kg and 1 mg/kg required about 90 h, with some dose-dependent variation. Platelet counts never dropped below $150,000/\mu\text{l}$. An overall significant correlation was found between the number of remaining binding sites and the extent of aggregation induced by ADP ($r = 0.85$; $p < 0.001$), collagen ($r = 0.74$; $p < 0.001$) and the bleeding times ($r = -0.83$; $p < 0.001$). However, normalization of bleeding times and collagen-induced aggregation was faster following 0.4 mg/kg than following 1 mg/kg MA-16N7C2 administration, despite parallel recovery of remaining binding sites.

Based on estimations of 65 ml blood volume/kg, 15% of the platelets being sequestered in the spleen (29) and the determinations of platelet count and number of remaining binding sites in pre- and 1 h post-injection samples, one can calculate that 77, 75, 63, 66, 69, 40 and 47% of

injected antibody became bound to the platelets when 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 1.0 mg/kg MA-16N7C2 was administered respectively. This apparent saturation of platelet receptors was reflected in the peak plasma levels of murine antibodies measured 1 h after injection: low to undetectable plasma levels of murine antibody persisted until platelet binding sites were nearly saturated, at which point the plasma levels increased significantly.

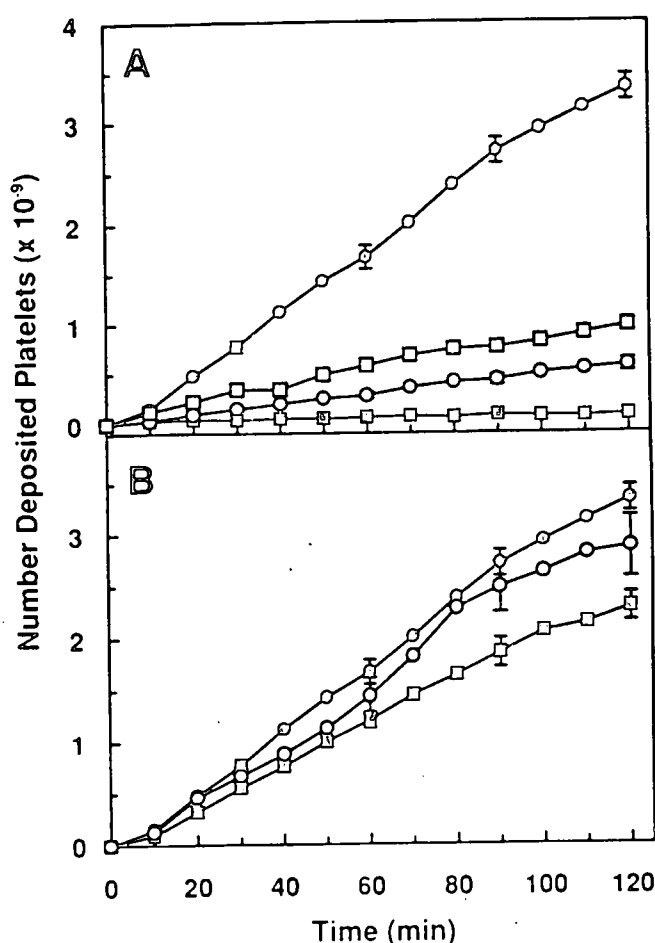


Fig. 2 Platelet depositions over a 2 h period onto Dacron vascular grafts incorporated into a permanent AV-shunt in baboons before (○), 15 min (□), 24 h (○) and 48 h (□) after IV-injection of 0.3 mg/kg (A) or 0.1 mg/kg (B) MA-16N7C2

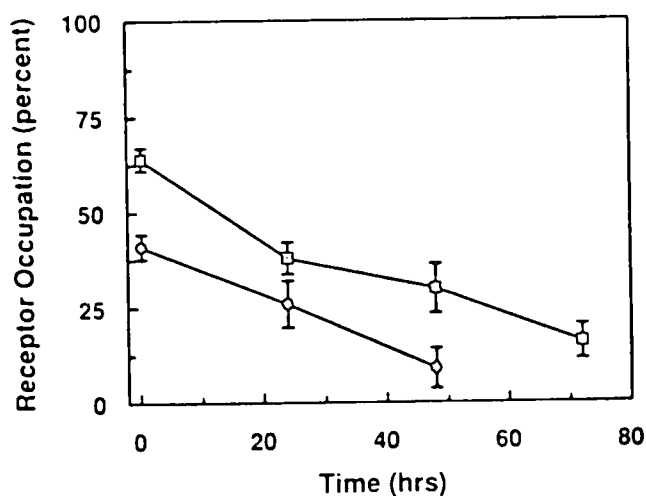


Fig. 3 Percentage occupied binding sites for ^{125}I -MA-16N7C2 on baboon platelets as a function of time following IV-administration of 0.3 mg/kg (□) or 0.1 mg/kg (○) of MA-16N7C2

2. Thrombosis Studies

Platelet deposition. Platelet deposition onto the thrombogenic device is shown in Fig. 2. In the control studies, platelets were rapidly deposited. After two hours of exposure of the flowing blood to the thrombogenic surface, $3.34 \pm 0.34 \cdot 10^9$ platelets were deposited. Treatment with 0.3 mg/kg MA-16N7C2 virtually abolished deposition on the day of treatment and the antibody still resulted in marked inhibition after 24 and 48 h (Fig. 2A). After 24 h, deposition was still inhibited by approximately 83% vs control after two hours of placement of the thrombogenic device. The corresponding inhibition after 48 h was 71%. Treatment with 0.1 mg/kg antibody was not as effective as treatment with 0.3 mg/kg (Fig. 2B). On the day of treatment, deposition was inhibited by approximately 31% 2 h following placement of the device. The corresponding inhibition after 24 h was approximately 14%.

Platelet receptor occupation. Ten minutes following a bolus infusion of 0.3 mg/kg of MA-16N7C2, approximately 64% of the GPIIb/IIIa receptors were occupied by the antibody (Fig. 3). Occupation decreased almost linearly, reaching a value of approximately 16% 72 h after treatment. Extrapolation of the decrease of receptor occupation suggested that the antibody remained platelet bound for up to 61 h after injection. Treatment with 0.1 mg/kg MA-16N7C2 blocked approximately 41% of the GPIIb/IIIa receptors 10 min after injection. The decrease that followed also fitted a linear function best, and the estimate of antibody bound to the platelet was approximately 41 h.

Bleeding time and ex vivo platelet aggregation. The bleeding time was lengthened ten minutes following injection of 0.3 mg/kg MA-16N7C2, but returned to normal values 24 h later (Table 1).

MA-16N7C2 inhibited the ex vivo aggregation of platelets in response to ADP and collagen and these effects were dose-dependent (Table 1). The inhibitory effect also lasted for some time following injection, i.e. in the case of the high dose, normal aggregation was obtained approximately 72 h following treatment. In the case of the low dose, the aggregation response returned to normal 24 h following treat-

Table 1 Bleeding time and the ex vivo platelet aggregation response before and following treatment with 16N7C2 (ND = not done)

Measurement	Dose	Time				
		Pre	15 min	24 hours	48 hours	72 hours
Bleeding time (min)	0.3 mg/kg	2 $\pm$ 0.2	20 $\pm$ 7	5 $\pm$ 1	4 $\pm$ 1	3 $\pm$ 0.5
Ex vivo platelet aggregation response						
ADP (20 μM)	0.3 mg/kg	100	0	52 $\pm$ 10	83 $\pm$ 17	100
	0.1 mg/kg	100	75 $\pm$ 16	100	ND	ND
Collagen (0.05 g/l)	0.3 mg/kg	100	21 $\pm$ 0.2	52 $\pm$ 11	74 $\pm$ 17	100
	0.1 mg/kg	100	68 $\pm$ 19	100	ND	ND

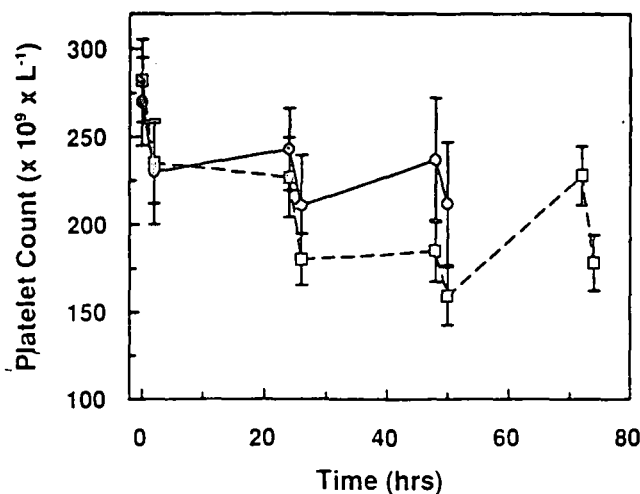


Fig. 4 Change in peripheral platelet counts in baboons treated with 0.3 mg/kg (■) or 0.1 mg/kg (●) of MA-16N7C2 as a function of time before and after placement of the thrombogenic device at 0, 24, 48 and 72 h

ment. The estimated half-life of inhibition of ADP-induced aggregation by 0.3 mg/kg antibody was approximately 12 h. The corresponding half-life of inhibition of collagen-induced aggregation was approximately 10 h.

Platelet count. The changes in platelet count as a result of the placement of thrombogenic devices and between placements are given in Fig. 4. In all studies, the platelet count decreased as a result of placement of devices irrespective of the presence of the antibody. The decrease ranged from 11 to 22%. The platelet count between placements of devices dropped reaching a minimum 48 h after antibody administration.

Coagulation measurements. Neither treatment with MA-16N7C2 nor placement of the thrombogenic devices affected the APTT or plasma concentration of fibrinogen markedly.

Discussion

In this study, we assessed the *in vivo* activity of the first described anti-GPIIb/IIIa monoclonal antibody with an echistatin-like RGD-peptide containing sequence (22) in baboons. The initial part of the study was done to determine the effects of different intravenous doses of MA-16N7C2 on GPIIb/IIIa-receptor occupation, bleeding time and the *ex vivo* aggregation of platelets in response to ADP and collagen. In the second part, the antithrombotic potential of MA-16N7C2 was assessed in a baboon model of platelet-dependent arterial-type thrombus formation.

Intravenous infusion of MA-16N7C2 caused a dose-dependent lengthening of the bleeding time and inhibition of *ex vivo* platelet aggregation, which took several days to recover. Plasma levels of murine monoclonal antibodies only started to increase when 60% to 70% of available GPIIb/IIIa-receptors were occupied, confirming a high affinity of the antibody for its target (22). In addition, the disappearance of MA-16N7C2 from the plasma preceded normalisation of the bleeding time, return of platelet aggregability and recovery of the number of binding sites on the platelet membrane.

The relationship between the number of GPIIb/IIIa receptors available after treatment with MA-16N7C2 and the inhibition of *ex vivo* platelet aggregation is in agreement with results obtained upon administration of 7E3, another anti-GPIIb/IIIa antibody, to humans (16):

when approximately 70% of receptors are occupied, *ex vivo* platelet aggregation in response to ADP is abolished. 30–40% receptor occupancy results in an attenuated aggregation, whereas blocking 15–25% of the receptors did not affect aggregation.

As was observed with other anti-GPIIb/IIIa antibodies, such as 7E3, LJ-CP-8 and AP-2 when infused in humans or baboons (12, 14, 31), we also here could observe that bleeding times normalised before ADP-induced platelet aggregation. This implies that detection of an abnormal *ex vivo* aggregation response not necessarily predicts abnormal platelet haemostatic function *in vivo* (12).

A bolus infusion of MA-16N7C2 inhibited platelet-dependent thrombus formation in a dose- and time-dependent manner. Maximum inhibition was observed on the day of injection of 0.3 mg/kg MA-16N7C2. Following this dose, platelet deposition was still markedly inhibited 48 h later. Similarly, when LJ-CP-8 and AP-2 were studied in a comparable model, platelet deposition was markedly inhibited shortly after treatment and returned progressively to control values over the next 48 h (12, 27, 31). However, in the case of these antibodies, much larger doses were required to obtain equivalent effects. The effect of MA-16N7C2 therefore seems to be more powerful and longer lasting than that of the above mentioned antibodies.

In the dose-finding studies, administration of MA-16N7C2 caused a decrease of about 60% in the blood platelet count, which reached a nadir after approximately 48 h. The decrease in platelet number due to the placement of the thrombogenic device in untreated animals, was also found in the animals that received MA-16N7C2. This is in contrast with results obtained when baboons were treated with a thromboxane receptor blocker (BAY U3405, 27), a thrombin inhibitor (D-Phe-Pro-Arg chloromethylketone, 32, 33), a factor Xa inhibitor (rTAP, 34) and more particularly with other monoclonal anti-GPIIb/IIIa antibodies (12). The reason for this discrepancy is at present unclear. The decrease in the platelet number strongly suggests that platelets were consumed by the thrombogenic surface. However, in view of the relatively few platelets that were deposited, especially on the day of infusion of 0.3 mg/kg, one must conclude that the turnover rate of deposited platelets was high.

In contrast to other studies using anti-GPIIb/IIIa antibodies, we here could relate differences in platelet function to receptor occupation. The decrease in receptor occupation following the bolus injection fitted a linear function best, and extrapolation of these curves suggest that MA-16N7C2 remained platelet bound for between 41 and 61 h. Furthermore, the differences in inhibition of platelet deposition following 0.3 and 0.1 mg/kg administration in relation to GPIIb/IIIa receptor occupation are noteworthy. For example, on the day of injection of 0.1 mg/kg approximately 41% of receptors were occupied with MA-16N7C2, which resulted in about 31% inhibition of platelet deposition. In contrast, 48 h following injection of 0.3 mg/kg, receptor occupation was approximately 31%, while platelet deposition was inhibited by about 71%.

The reason for this difference in inhibition at similar levels of receptor occupation is not clear. A possible explanation may well be the interchange between extra- (50–80%) and intraplatelet (40–20%) pools of GPIIb/IIIa (18,19) and uptake of anti-GPIIb/IIIa antibodies and desintegrins into the intraplatelet pool (20, 21). With the high dose of MA-16N7C2, initially up to 65% of the external receptors were occupied. Over an extended time period, e.g. 48 h, these receptors can interchange with unoccupied internal receptors. Since receptor occupation was measured on unstimulated platelets, only the surface exposed GPIIb/IIIa are detected. However, when platelets become activated during thrombus formation, the intracellular pool of GPIIb/IIIa is

expressed on the surface (35, 36). In the case of a high dose of antibody and after an extended period, the GPIIb/IIIa receptors are to some extent blocked and thus unable to contribute to platelet deposition.

When on the other hand only 0.1 mg/kg MA-16N7C2 was given and thrombus formation is measured shortly thereafter, i.e. before significant GPIIb/IIIa cycling can occur, an unaffected, functionally active pool of internal receptors became exposed. As a result, a larger thrombus formed. This assumption is further strengthened by the difference in recovery patterns obtained following infusion of 1 and 0.4 mg/kg MA-16N7C2: the number of free available GPIIb/IIIa-receptors recovered in parallel, whereas normalisation of the bleeding time, collagen-induced aggregation, and to a lesser extent ADP-induced aggregation was faster following a dose of 0.4 mg/kg than after a dose of 1 mg/kg.

An alternative hypothesis is that platelet α -granules may become loaded with antibody, which then would be secreted upon platelet activation, in a manner comparable to the normal mechanism of fibrinogen uptake and secretion. This hypothesis is strengthened by observation that transfusion of platelet concentrates from dogs 48 h after treatment with an i.v. bolus injection of 0.8 mg/kg 7E3-Fab, to untreated dogs, still prevented occlusion of an everted (inside out) carotid arterial segment that had been inserted in a transected femoral artery (37).

In conclusion, our results confirmed the antithrombotic action of an anti-GPIIb/IIIa monoclonal antibody. We could also show that a putative "loading" of internal GPIIb/IIIa receptors can result in a higher than expected antithrombotic action. Whether the same holds true for smaller GPIIb/IIIa antagonists awaits further study.

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Antithrombotic Effect of Platelet Glycoprotein Ib-Blocking Monoclonal Antibody Fab Fragments in Nonhuman Primates

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Abstract—Platelet adhesion in arterial blood flow is mainly supported by the platelet receptor glycoprotein (GP) Ib, which interacts with von Willebrand factor (vWF) that is bound to collagen at the site of vessel wall injury. Antibody 6B4 is a monoclonal antibody (MoAb) raised against purified human GPIb. MoAb 6B4 inhibits both ristocetin- and botrocetin-induced, vWF-dependent human platelet agglutination. MoAb 6B4 furthermore blocks shear-induced adhesion of human platelets to collagen I. We studied the antithrombotic effect of this inhibitory murine MoAb 6B4 in a baboon model of arterial thrombosis. When injected into baboons, intact IgG and its F(ab')₂ fragments caused almost immediate thrombocytopenia, whereas injection of the Fab fragments alone did not. Fab fragments were subsequently used to investigate their in vivo effect on platelet deposition onto a thrombogenic device, consisting of collagen-rich, glutaraldehyde-fixed bovine pericardium (0.6 cm²), at a wall shear rate ranging from 700 to 1000 s⁻¹. Baboons were either pretreated with Fabs to study the effect of inhibition on platelet adhesion or treated 6 minutes after placement of the thrombogenic device to investigate the effect on interplatelet cohesion. Pretreatment of the animals with bolus doses ranging from 80 to 640 µg/kg Fab fragments significantly reduced ¹¹¹In-labeled platelet deposition onto the collagen surface by ≈43% to 65%. Only the highest dose caused a significant prolongation (doubling) of the bleeding time. Ex vivo ristocetin-induced platelet agglutination was equally reduced. Treatment with a bolus of 110 µg/kg Fab fragments after a thrombus was allowed to form for 6 minutes had no effect on further platelet deposition. We therefore conclude that Fab fragments or derivatives of inhibitory anti-GPIb antibodies may be useful compounds to prevent thrombosis. (*Arterioscler Thromb Vasc Biol.* 2000;20:1347-1353.)

Key Words: platelet adhesion □ platelet aggregation □ thrombosis □ glycoprotein Ib □ monoclonal antibodies

Blood platelets, through the processes of adhesion, activation, shape change, the release reaction, and aggregation, form the first line of defense when blood vessels are damaged. Platelets form a hemostatic plug at the site of injury to prevent excessive blood loss. Extensive platelet activation, however, may overcome the normal thromboregulatory mechanisms that limit the size of the hemostatic plug. The platelets then become major prothrombotic offenders predisposing to vaso-occlusive disease.^{1,2}

Platelet adhesion is regarded as the trigger for hemostasis and thrombosis. When subendothelial collagen is exposed at the site of vessel injury, circulating von Willebrand factor (vWF) binds to it and, under the influence of arterial blood flow, undergoes a conformational change enabling it to bind to its receptor, glycoprotein (GP) Ib, on the platelet membrane.³ As a result, motion of the platelets on the collagen surface is decreased, which may then be followed by firm adhesion mediated by other collagen receptors.⁴ After the

initial adhesion, platelets will aggregate, an interaction that is assured through the binding of GPIIb-IIIa complexes with multivalent ligands, in particular, vWF and fibrinogen.⁵ The platelet GPIb receptor is thus important for initiating a thrombus at the site of the exposed lesion. Abnormalities in GPIb, as seen in Bernard-Soulier syndrome, result in deficient platelet attachment to a site of vascular injury and a predisposition to clinical bleeding.⁶

Lately, much effort has been directed to develop antibodies and peptides that can block the binding of the adhesive proteins to GPIIb-IIIa, and many of these are being tested in clinical trials.⁷⁻⁹ On the other hand, the development of compounds that interfere with the vWF-GPIb axis has lagged behind. Only a few in vivo studies that investigated the effects of inhibition of platelet adhesion on thrombogenesis are described. They include the use of anti-vWF monoclonal antibodies (MoAbs),¹⁰⁻¹² GPIb-binding snake venom proteins like echicetin¹³ and crotalin,¹⁴ aurintricarboxylic acid

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that binds to vWF,¹⁵ and recombinant vWF fragments like VCL,¹⁶⁻¹⁸ all of which inhibit vWF-GPIIb interaction. All of these studies showed the potential feasibility of antithrombotic therapy based on inhibition of the GPIIb-vWF axis.

A number of potent, inhibitory anti-GPIIb antibodies have been produced and were extensively tested with respect to their in vitro effect under both static (platelet agglutination, vWF binding) and flow conditions.¹⁹ We are aware of only 2 successful in vivo studies on guinea pigs, in which F(ab')₂ fragments of PG-1, a monoclonal anti-guinea pig GPIIb antibody, were used.^{20,21} These fragments were shown to significantly prolong the time to arterial microvascular graft thrombosis without prolonging the bleeding time.²¹ In another guinea pig model, the fragments could effectively reduce thrombus formation on a laser-induced injury.²⁰ However this PG-1 antibody is specific for guinea pig platelets and does not cross-react with human platelets. Part of this rather surprising lack of in vivo studies is due to the low cross-reactivity of the anti-human GPIIb MoAbs with platelets from commonly used laboratory animals. This situation necessitates the use of nonhuman primates as experimental animals. However, even then, attempts to perform in vivo studies are hampered because injection of the anti-GPIIb MoAbs, as well as the snake venom protein echicetin that reacts with GPIIb, invariably causes severe thrombocytopenia.^{10,13,22-23}

We have studied the antithrombotic efficacy of a novel murine MoAb, 6B4 (IgG1), raised against human platelet GPIIb, in in vitro and in vivo studies. In vitro, 6B4 and its F(ab')₂ and Fab fragments all potently inhibited the binding of vWF to human GPIIb under both static and flow conditions, and they also bound to baboon platelets. In the in vivo studies in which 6B4 was injected into baboons, the intact MoAb caused immediate and severe thrombocytopenia; injection of its F(ab')₂ fragments resulted in a moderate decrease in platelet count, whereas the Fab fragments did not have a major effect on platelet count. Furthermore, the Fab fragments were studied in a baboon model of platelet-dependent arterial thrombosis. Platelet deposition onto collagen-rich bovine pericardium was inhibited when the fragments were injected into the baboons before a thrombus was generated. On the other hand, when the Fab fragments were injected after a thrombus was allowed to form, no inhibition of further thrombosis was observed.

Methods

Preparation and Purification of Intact MoAb 6B4, F(ab')₂, and Fab Fragments

The antibody used in these studies, 6B4 (subtype IgG1), is a murine MoAb raised against purified human GPIIb. GPIIb was purified from outdated platelet concentrates collected by the Belgian Red Cross in Leuven, Belgium, according to the method described by Wicki and Clemetson.²⁴ BALB/c mice were immunized by intraperitoneal injection with the purified GPIIb. Murine antibodies were prepared by conventional hybridoma technology as previously described,²⁴ and generated hybridoma cells were screened for antibody production in an ELISA by using purified GPIIb. Hybridoma cells producing anti-GPIIb MoAbs were grown and subsequently injected into pristane-primed BALB/c mice. After 10 days ascites fluid was collected. The IgG was extracted from the ascites by using protein A-Sepharose CL-4B (Pharmacia). These antibodies were screened for inhibition of ristocetin-induced human platelet aggregation as described below. MoAb 6B4 IgG totally abolishes both the ristocetin- and the botrocetin-induced aggregation of human platelet-rich plasma (PRP).

To prepare F(ab')₂ fragments, MoAb 6B4 was dialyzed overnight against a 0.1 mol/L citrate buffer (pH 3.5). The antibody was digested by incubation with pepsin (Sigma Chemical Co; 1 part pepsin to 200 parts MoAb) for 1 hour at 37°C. Digestion was stopped by adding 1 volume of a 1 mol/L Tris-HCl buffer (pH 9) to 10 volumes of antibody solution.

Monovalent Fab fragments were prepared by papain digestion. A 1:10 (vol/vol) solution of 1 mol/L phosphate buffer (pH 7.3) was added to the antibody. Papain (Sigma) was added at a ratio of 1 volume papain to 25 volumes of the phosphate buffer containing the MoAb, 10 mmol/L L-cysteine-HCl (Sigma), and 15 mmol/L EDTA. After incubation for 3 hours at 37°C, digestion was stopped by adding freshly prepared iodoacetamide solution (Sigma) to a final concentration of 30 mmol/L, which was then kept in the dark at room temperature for 30 minutes.

Both F(ab')₂ and Fab fragments were further purified from contaminating intact IgG and Fc fragments by using protein A-Sepharose. The purified fragments were finally dialyzed against PBS. The purity of the fragments was determined by SDS-polyacrylamide gel electrophoresis, and the protein concentration was measured by using bicinchoninic acid protein assay reagent A (Pierce Chemical Co).

Further Antibody Characterization

MoAb 6B4 binds to a (His1-Val289) recombinant GPIIb α (rGPIIb α) fragment expressed by Chinese hamster ovary cells,²⁶ indicating that its epitope is localized within the amino-terminal region of GPIIb α . MoAb 6B4 Fabs were further tested for inhibition of ristocetin- and botrocetin-induced binding of vWF to the rGPIIb α fragment by ELISA.²⁷ Microtiter plates were coated with 5 μ g/mL MoAb 2D4 for 48 hours at 4°C. MoAb 2D4, an anti-GPIIb MoAb that we produced, binds to the rGPIIb α fragment but does not block vWF binding. Nonadsorbed sites were blocked with 3% skimmed milk, whereafter the plates were washed with Tris-buffered saline containing 0.1% Tween 20. Purified rGPIIb α fragments were immobilized on MoAb 2D4 by incubating 2 μ g/mL rGPIIb α for 2 hours at 37°C. After being washed with Tris-buffered saline-Tween 20, increasing concentrations of 6B4 Fab fragments diluted in Tris-buffered saline-Tween 20 were added, followed by 1.25 or 0.6 μ g/mL purified human vWF, respectively, when ristocetin (300 μ g/mL) or botrocetin (0.5 μ g/mL) was used as a modulator. Binding of vWF was determined by incubating the resulting mixture for 1 hour with horseradish peroxidase-conjugated polyclonal anti-vWF antibody (Dako), diluted 1/3000 in Tris-buffered saline-Tween 20. The color reaction, stopped by addition of 4 mol/L H₂SO₄, was generated with *o*-phenylenediamine (Sigma). Ristocetin was supplied by abp, and purified vWF was purchased from the Red Cross, Belgium. The purification of botrocetin from crude *Bothrops jararaca* venom (Sigma) was performed as previously described.²⁸

The effect of 6B4 Fab on shear-induced platelet adhesion to collagen was tested in a Sakariassen-type parallel-plate flow chamber at shear rates of 650, 1300, and 2600 s⁻¹ as previously described.²⁹ Human collagen type I (Sigma) was dissolved in 50 mmol/L acetic acid (1 mg/mL), dialyzed for 48 hours against PBS, subsequently sprayed onto plastic Thermanox coverslips, and stored at room temperature overnight before use. Twelve milliliters of blood anticoagulated with low-molecular-weight heparin (25 U/mL; Clexane, Rhône-Poulenc Rorer) was preincubated with 6B4 Fab fragments at 37°C for 5 minutes. This was then used to perfuse the collagen-coated coverslips. After 5 minutes of perfusion, the platelets were fixed with methanol and the coverslips stained with May-Grünwald/Giemsa. Platelet adhesion (percent of total surface covered with platelets) was evaluated with a light microscope connected to an image analyzer. An average of 30 fields per coverslip were analyzed. Platelet adhesion was expressed as percent maximal platelet adhesion obtained in the absence of inhibitor.

Animal Studies

Normal male baboons (*Papio ursinus*) were used. The animals weighed between 10 and 15 kg and were disease-free for at least 6 weeks before the experiments. All procedures were approved by the Ethics Committee for Animal Experimentation of the University of the Free State in accordance with the National Code for Animal Use

in Research, Education, Diagnosis and Testing of Drugs and Related Substances in South Africa.

The baboons had permanent polytetrafluoroethylene (Teflon)-silicone rubber (Silastic) arteriovenous shunts implanted in the femoral vessels.³⁰ Blood flow through the shunts varied between 100 and 120 mL/min, resulting in wall shear rates between 700 and 1000 s⁻¹, which compares well with the shear rates found in medium-size arteries.³⁰ Handling of the baboons was achieved through anesthesia with ketamine hydrochloride (≈ 10 mg/kg IM; Anakot-V, Centaur Laboratory).

Study Protocol

To test the effect of the MoAb on platelet count, 6B4, its F(ab')₂ fragments, or its Fab fragments were administered to 3 different baboons. The plasma volume was calculated by assuming a blood volume of 65 mL/kg body mass and correcting for the hematocrit.³¹ Platelet-dependent arterial thrombus formation was induced by using bovine pericardium (0.6 cm² fixed in buffered glutaraldehyde).³² The pericardium was built into the wall of Silastic tubing (3-mm inside diameter). The method of preparation of the thrombogenic device has been described in detail,³¹ except that fixed bovine pericardium was used instead of Dacron vascular graft material. In each experiment, a thrombogenic device prefilled with saline to avoid a blood-air interface was incorporated as an extension segment into the permanent arteriovenous shunt by means of Teflon connectors.³⁰ Two approaches were followed to determine the effect of 6B4 Fab fragments on platelet adhesion and interplatelet cohesion onto the collagen of the bovine pericardium.

Dose-Response Effect of 6B4 Fab Fragments on Platelet Adhesion and Deposition

Seven baboons were used in the first studies; in total 13 perfusion experiments were performed. In the first 5 experiments (3 baboons), a thrombogenic device was placed to determine the deposition of platelets (see the section below on graft imaging and quantification of platelet deposition). After 30 minutes, the device was removed and blood flow through the permanent arteriovenous shunt reestablished. Fifteen minutes after removal of the device, each baboon was treated with a bolus of 80 μ g/kg 6B4 Fab fragments (in 2 mL saline), and 15 minutes after treatment, a second thrombogenic device was placed for 30 minutes to determine the effect of the Fab fragments on thrombogenesis. The device was again removed and blood flow through the permanent shunt established. This step was followed by a second bolus injection of Fab fragments (80 μ g/kg) to attain a cumulative dose of 160 μ g/kg. After 15 minutes, a third thrombogenic device was placed for 30 minutes and platelet deposition was measured. In 4 other experiments (2 baboons), the same study protocol was used, except 2 doses of 320 μ g/kg were administered. Sham studies were done in 4 other experiments (2 baboons). In these studies, the same protocol of placement of thrombogenic devices was followed, but the baboons were not treated with Fab fragments.

Effect of Anti-GPIIb 6B4 Fragments on Interplatelet Cohesion

In a second series of 12 experiments, 6 baboons were used. In all baboons, a thrombogenic device was placed for 24 minutes. In 6 experiments (3 baboons), the baboons received a bolus injection of Fab fragments of 110 μ g/kg. The fragments were injected 6 minutes after placement of the thrombogenic device to allow enough platelets to be deposited to cover the collagen surface. In the other 6 experiments (3 baboons), the baboons did not receive any Fab fragments.

In both approaches, blood was collected at different time points to determine platelet count and hematocrit (EDTA), circulating and platelet-associated radioactivity, the ex vivo aggregation of platelets in response to ristocetin, and the plasma concentrations of Fab fragments (see the section on laboratory measurements). The time points at which the blood was collected are given in the figures.

Graft Imaging and Quantification of Platelet Deposition

Autologous blood platelets were labeled with ¹¹¹In-tropolone,³³ and imaging and quantification of the deposition of the ¹¹¹In-labeled

platelets were done as described in detail.^{30,31} In brief, image acquisition of the grafts, including proximal and distal Silastic segments, was done with a large-field-of-view scintillation camera fitted with a high-resolution collimator. The images were stored on and analyzed with a Medical Data Systems A³ computer (Medtronic) interfaced with the scintillation camera. Dynamic 2-minute image acquisition was started simultaneously with the start of blood flow through the devices. A 2-minute image (128 $\times$ 128-byte mode) of a 3-mL autologous blood sample collected in EDTA was also acquired each time that the grafts were imaged to determine circulating blood radioactivity (blood standard). A region of interest of the graft segment was selected to determine the deposited and circulating radioactivity in each of the dynamic images. Radioactivity in a region of similar size in the proximal segment of the extension tubing was determined and subtracted from the radioactivity in the graft region to calculate deposited radioactivity. Platelet deposition was expressed as the total number of platelets deposited. The method to calculate this has been described in detail.³⁰

Laboratory Measurements

Receptor Binding Studies

6B4, its F(ab')₂ fragments, or Fab fragments were labeled with Na¹²⁵I (Amersham) by using the Iodo-Gen method.³⁴ Iodo-Gen was purchased from Pierce. Baboon PRP, adjusted with autologous plasma to a count of 100 000 platelets/ μ L, was incubated with different concentrations of iodinated 6B4, F(ab')₂ fragments, or Fab fragments for 15 minutes at room temperature. The mixture was layered onto 20% sucrose buffer (wt/vol) containing 0.1% (wt/vol) BSA and centrifuged for 4 minutes at 10 000g in Eppendorf tubes. The top fluid, including the plasma, was removed and the pellets were counted in a gamma counter. Binding studies were performed in duplicate on the PRP of 2 baboons.

In Vitro and Ex Vivo Platelet Aggregation

The aggregation of platelets in response to ristocetin (ABP) was done on 10 mL of blood collected in 1 mL of 3.2% trisodium citrate. PRP was prepared by differential centrifugation³⁵ and the platelet count adjusted to 200 000 platelets/ μ L with autologous plasma. The aggregation response was measured in a Monitor IV Plus aggregometer (Helena Laboratories) and recorded for 5 minutes. The percent aggregation at 5 minutes was calculated as the difference in light transmission between PRP and platelet-poor plasma.

In in vitro studies, the PRP was preincubated for 5 minutes with serial dilutions of intact IgG 6B4, F(ab')₂ fragments, or Fab fragments before aggregation was initiated. Inhibition of aggregation was calculated from the difference in the aggregation response of platelets without and with antibody or fragments. In the ex vivo determinations, inhibition was calculated from the difference in the aggregation response of platelets before and after treatment of the baboons.

Plasma levels of 6B4 Fab fragments were measured with a sandwich ELISA. In brief, microtiter plates were coated overnight at 4°C with 5 μ g/mL polyclonal goat anti-mouse IgG (Sigma). After unoccupied binding sites were blocked with BSA, serial dilutions of baboon plasma were added to the wells and incubated for 2 hours. Bound 6B4 Fab fragments were detected by using goat anti-mouse IgG (Fab-specific) conjugated to peroxidase (Sigma) and developed by using *o*-phenylenediamine as described above. Standard curves were constructed by adding known amounts of 6B4 Fab fragments to baboon plasma.

Bleeding Time

The bleeding time was determined by using the Simplate II device (Organon Teknika) according to the instructions of the manufacturer. The volar surface of the forearm of the baboons was shaved, and a pressure cuff was applied and inflated to 40 mm Hg.

Statistical Analysis

Student's *t* test for paired data was used to test for statistically significant differences. Data given in the text are mean $\pm$ SE. Probability values <0.05 were considered statistically significant.

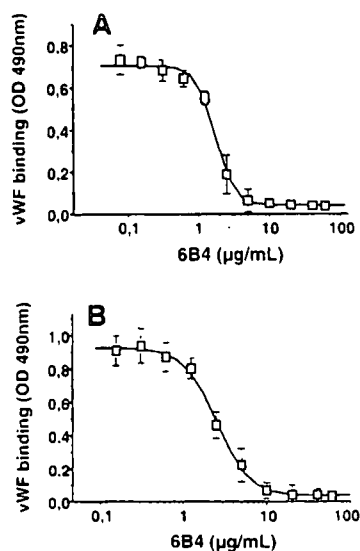


Figure 1. Semilog plots showing inhibitory effect of anti-GPIb 6B4 Fab fragments on ristocetin- (0.3 mg/mL, A) or botrocetin- (0.5 μ g/mL, B) induced vWF binding to an rGPIb α (His1-Val289)-fragment. The rGPIb α fragment (2 μ g/mL) was immobilized on MoAb 2D4 (5 μ g/mL) coated onto microtiter plates. vWF (1.25 or 0.6 μ g/mL, respectively), when binding was induced by ristocetin or botrocetin) was added together with increasing concentrations of 6B4 Fab fragments. vWF binding was detected. Data are mean $\pm$ SD (n=3). OD indicates optical density.

Results

In Vitro Effect of 6B4 Fab Fragments on Human Platelets

MoAb 6B4 Fab fragments blocked the ristocetin- (1 mg/mL) and botrocetin- (0.5 μ g/mL) induced human platelet agglutination, with an IC_{50} of 1.2 ± 0.3 μ g/mL (24 ± 6 nmol/L) and 2.0 ± 0.5 μ g/mL (40 ± 10 nmol/L), respectively. For the intact 6B4 IgG, these values were 0.3 μ g/mL (2.0 nmol/L) and 0.8 μ g/mL (5.3 nmol/L), respectively. 6B4 binds to an epitope localized on the amino-terminal part (His1-Val289) of GPIb α . The inability of 6B4 to bind to denatured GPIb during Western blotting (not shown) strongly suggests that this antibody binds to a conformation-sensitive epitope on GPIb α . The 6B4 Fab fragments dose-dependently inhibited both the ristocetin- (300 μ g/mL) and botrocetin- (0.5 μ g/mL) induced binding of vWF to the (1-289) rGPIb α fragment (Figure 1).

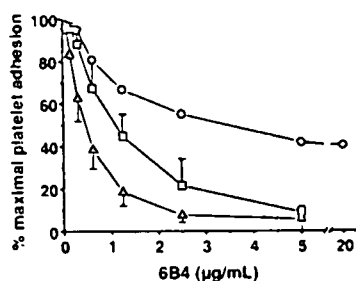


Figure 2. Effect of anti-GPIb 6B4 Fab fragments on shear-induced adhesion of human platelets to human collagen I-coated coverslips. Blood was perfused at a constant shear rate of 650 s^{-1} (O, n=2), 1300 s^{-1} (□, n=3), and 2600 s^{-1} (Δ, n=3) for 5 minutes. Data are mean $\pm$ SD and are expressed as percent of maximal platelet adhesion relative to control value in the absence of 6B4 Fab (35%, 33%, and 29%, respectively, at 650, 1300 and 2600 s^{-1}).

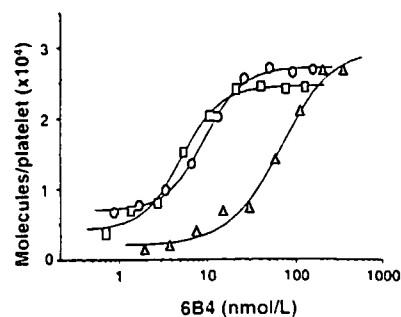


Figure 3. Semilog binding curves of anti-GPIb ^{125}I -6B4 IgG (□), ^{125}I -F(ab') $_2$ (O), and ^{125}I -Fab fragments (Δ) to baboon platelets in plasma. Data are mean of duplicate measurements in PRP from 2 baboons.

with an IC_{50} of 1.8 μ g/mL (36 nmol/L) and 2.5 μ g/mL (50 nmol/L), respectively.

The 6B4 Fab fragments inhibited platelet adhesion to human collagen type 1 in a concentration-dependent manner at shear rates of 650, 1300, and 2600 s^{-1} (Figure 2), with a 50% reduction of surface coverage obtained at a concentration of 3.5 μ g/mL (70 nmol/L), 1.1 μ g/mL (22 nmol/L), and 0.5 μ g/mL (10 nmol/L), respectively.

In Vitro Effect of MoAb 6B4 and Its F(ab') $_2$ and Fab Fragments on Baboon Platelets

Binding of the antibody and its fragments to baboon platelets was dose dependent and saturable. Half saturation (K_{D50}) was obtained with 4.7 nmol/L for 6B4 IgG, 6.4 nmol/L for F(ab') $_2$, and 49.2 nmol/L for Fab (Figure 3). At saturating concentrations, ristocetin-induced aggregation was completely abolished (Figure 4). The IC_{50} of platelet aggregation was obtained at 4.5 nmol/L, 7.7 nmol/L, and 40 nmol/L for 6B4 IgG, F(ab') $_2$, and Fab fragments, respectively.

Effect of Injection of MoAb 6B4, F(ab') $_2$, and Fab Fragments on Peripheral Platelet Count in Baboons

In 1 baboon, 100 μ g/kg intact antibody caused a profound decrease in the blood platelet count ($<30 \times 10^9/L$) within 10 minutes after injection. After 48 hours, the platelet count was still $<100 \times 10^9/L$. When 100 μ g/kg 6B4 F(ab') $_2$ fragments was injected into 2 baboons, the platelet count decreased rapidly to between 120 and $150 \times 10^9/L$, ie, by $\approx 60\%$, but then reached preinfusion values within 24 hours. Finally, when 80 to 320 μ g/kg of the monovalent 6B4 Fab fragments was injected, the platelet count (45 minutes after injection) decreased by only $\approx 10\%$ to 20% and by only 26% when 640

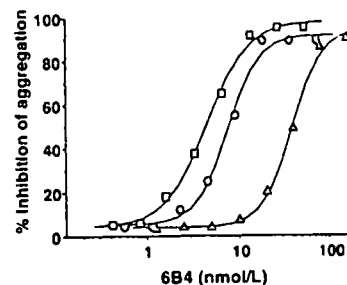


Figure 4. Semilog plot showing effect of anti-GPIb 6B4 IgG (□), F(ab') $_2$ (O), and Fab fragments (Δ) on ristocetin-induced baboon platelet aggregation. Data, expressed as percent inhibition, are mean of duplicate measurements in PRP from 2 baboons.

Platelet Counts, Plasma Levels of 6B4 Fab Fragments, Ex Vivo Ristocetin-Induced Platelet Agglutination, and Bleeding Times After 6B4 Fab Administration

Dose, $\mu\text{g/kg}$	Time, min	Platelet Counts		Plasma Levels, $\mu\text{g/mL}$	% Inhibition of Ex Vivo Ristocetin-Induced (1.5 mg/mL) Platelet Agglutination	Bleeding Times, s
		$\times 10^3/\mu\text{L}$	% Decrease			
n = 5						
0	Pre	307 $\pm$ 32	0	0.07 $\pm$ 0.03	0	190 $\pm$ 20
80	90	272 $\pm$ 22	11	1.72 $\pm$ 0.14	26 $\pm$ 9	160 $\pm$ 33
160	150	248 $\pm$ 19	19	4.84 $\pm$ 0.56	47 $\pm$ 12*	250 $\pm$ 45
	270	315 $\pm$ 31		0.45 $\pm$ 0.09	8 $\pm$ 3	ND
n = 4						
0	Pre	283 $\pm$ 23	0	0.02 $\pm$ 0.01	0	232 $\pm$ 42
320	90	219 $\pm$ 10	23	9.13 $\pm$ 0.48	25 $\pm$ 21	340 $\pm$ 63
640	150	210 $\pm$ 13	26	15.35 $\pm$ 1.38	80 $\pm$ 9*	405 $\pm$ 45*
	270	238 $\pm$ 20	16	1.19 $\pm$ 0.09	15 $\pm$ 9	ND
	24 h	236 $\pm$ 13	17	0.04 $\pm$ 0.01	7 $\pm$ 3	ND

ND indicates not determined. Administration of 80–640 $\mu\text{g/kg}$ 6B4 Fab fragments to baboons was performed. Values are given as mean $\pm$ SE.

Statistical comparisons were made by Student's *t* test for paired sample groups: **P* < 0.05.

$\mu\text{g/kg}$ was injected (the Table). On the basis of this result, the 6B4 Fab fragments were used for further studies.

Effect of Different Doses of MoAb 6B4 Fab Fragments on Platelet Deposition

Platelet adhesion and deposition onto thrombogenic devices sequentially placed 30 minutes apart are summarized in Figure 5. In the sham studies (Figure 5A), placement of the previous graft had no significant effect on platelet deposition that formed on subsequent grafts.

In the treatment studies (Figure 5B), dosages of 80 and 160 $\mu\text{g/kg}$ (2×80 $\mu\text{g/kg}$ 6B4 Fab) significantly inhibited platelet deposition in comparison with control by $\approx 43\%$ and 53%,

respectively. Doses of 320 and 640 $\mu\text{g/kg}$ (2×320 $\mu\text{g/kg}$ 6B4 Fab) significantly reduced platelet deposition by 56% and 65%, respectively.

Plasma levels of 6B4 Fab fragments and inhibition of ex vivo agglutination determined on samples obtained 45 minutes or 2 hours after administration changed in both a dose- and time-dependent manner (the Table). Ex vivo ristocetin-induced platelet aggregation was significantly inhibited at doses of 160 and 640 $\mu\text{g/kg}$. Both ristocetin-induced platelet aggregation and plasma values returned to baseline within 3 hours after antibody injection.

Bleeding times determined in the treatment studies before and 45 minutes after injection of 80 to 320 $\mu\text{g/kg}$ 6B4 Fab fragments were not significantly prolonged. Only a dose of 640 $\mu\text{g/kg}$ significantly prolonged the bleeding time.

Effect of 6B4 Fab Fragments on Interplatelet Cohesion

Treatment of the baboons with 110 $\mu\text{g/kg}$ 6B4 Fab did not affect platelet deposition when the animals were injected after a thrombus was allowed to form for an initial 6 minutes (Figure 6).

Discussion

The initial step in platelet adhesion consists of vWF binding to GPIb. We investigated the effect of inhibiting this interaction on platelet function both in vitro and in vivo. A murine MoAb, 6B4, that binds to a conformational epitope in the amino-terminal part of GPIb α was used. The antibody and its fragments potentially inhibited the binding of vWF to an rGPIb α fragment (His1–Val289) and dose-dependently inhibited vWF-dependent human platelet agglutination. The intact antibody and its fragments also dose-dependently inhibited human platelet adhesion to type I collagen in a flow chamber at wall shear rates of 650, 1300, and 2600 s^{-1} . This inhibition was shear dependent, ie, more pronounced at higher shear.

Of interest was the finding that 6B4 did not react with platelets from dogs, hamsters, pigs, or guinea pigs but did

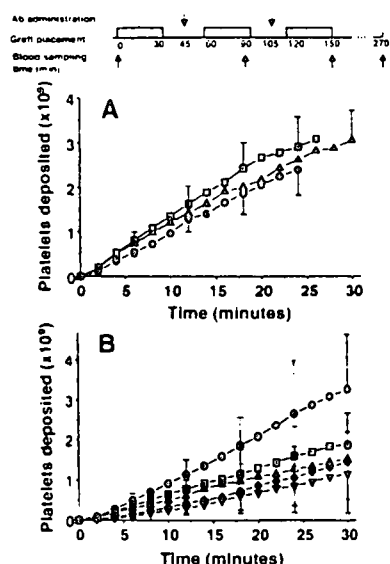


Figure 5. Platelet deposition onto 3 thrombogenic devices containing bovine pericardium placed consecutively at times 0 (●), 60 (■, ◆), and 120 (▲, ▼) minutes for 30 minutes (top shaded bars). A, Sham experiments (*n* = 4). B, After injection of 0 (●) (*n* = 9), 80 (■, ◆), and 160 (▲, ▼) $\mu\text{g/kg}$ 6B4 Fab fragments (*n* = 5) or 320 (◆) and 640 (▼) $\mu\text{g/kg}$ (*n* = 4). Values are given as mean $\pm$ SE.

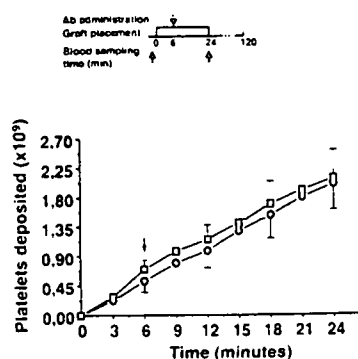


Figure 6. Influence of late treatment of baboons with 6B4 Fab fragments on platelet deposition. The thrombogenic device was placed at time 0 and platelet deposition determined for 24 minutes (top shaded bar). After 6 minutes (arrow), animals were either left untreated ($\square$) or treated with a bolus of 110 ($\circ$) $\mu\text{g/kg}$ 6B4 Fab fragments ($n=6$). Values are given as mean $\pm$ SE.

bind to baboon platelets with much the same characteristics as to human platelets. As a result, baboons were used for in vivo and ex vivo studies. Both the intact antibody as well as its F(ab')₂ fragments caused immediate thrombocytopenia, similar to what was seen when other anti-GPIb MoAbs were injected into different experimental animals.^{10,22,23} On the other hand, the Fab fractions had only a moderate effect on the blood platelet count, and we therefore decided to use the Fab fractions to assess the antithrombotic effect of 6B4 in a baboon model of arterial thrombosis.³⁰ The glutaraldehyde-fixed bovine pericardium was highly thrombogenic: after 30 minutes of exposure to native flowing blood, $\approx 3 \times 10^9$ platelets deposited on the area of 0.6 cm². In similar studies, only $\approx 0.7 \times 10^9$ platelets accumulated in 30 minutes on Dacron vascular graft material (0.9 cm²).³¹ It is therefore also not surprising that a number of control thrombogenic devices became occluded before 30 minutes of exposure to flowing blood.

Treatment of baboons with 6B4 Fab fragments inhibited platelet deposition on the thrombogenic devices by 43% to 65%. The observed effect must be ascribed to the effect of the antibody, because sequential placement of thrombogenic devices in untreated baboons caused no decreased deposition. No complete inhibition of platelet deposition was observed, even at high doses.

It is possible that this incomplete inhibition was due to the medium shear rates (700 to 1000 s⁻¹) used in this study. In general, in flow chambers a more effective inhibition with vWF-GPIb inhibitors is obtained at higher shear rates,^{11,12,36} which we also demonstrated here by finding a more pronounced effect of 6B4 on platelet adhesion to collagen when a higher shear was applied in a flow chamber; also, in vivo an arterial thrombus was more readily prevented than a venous one.¹¹ This implies that inhibition of GPIb would result especially in arterial effects, which in addition could result in less bleeding risk.

The doses used indeed caused a significant reduction in thrombus size without apparently increasing the risk of bleeding since bleeding time was only mildly prolonged at the highest dose. This finding supports data obtained with other GPIb-vWF-blocking agents (VCL, anti-vWF MoAb AJvW-2) that lengthened the bleeding time only moderately.^{11,12,17,18} This finding is important and might provide a major advantage in the development of antithrombotic agents

compared with GPIIb-IIIa antagonists, like ReoPro.³⁷ On the other hand, the GPIb-vWF interaction in contrast to the GPIIb-IIIa-fibrinogen interaction is the ultimate first step in platelet adhesion under fast blood flow. Because binding of vWF to GPIb also activates platelets,^{38,39} it is reasonable to assume that by inhibiting vWF-GPIb binding, fewer platelets will be activated. The smaller thrombus that finally forms may therefore be a consequence of both fewer platelets that adhere to collagen and less platelet aggregation. Thus, where GPIIb-IIIa blockers mainly prevent platelet aggregation, interruption at an earlier stage by a GPIb blocker is expected not only to limit the platelet plug that is formed but also to reduce additional platelet-dependent effects, such as granule release, thought to play a role in the development of arteriosclerosis and restenosis.^{40,41}

There are also indications that the GPIb-IX-V complex is involved in platelet-platelet interactions. Ruggeri et al.⁴² recently reported that blocking the GPIb-vWF interaction, after platelets from PPACK-anticoagulated blood had adhered to bovine collagen in vitro for 100 seconds at 1500 s⁻¹, prevented further thrombus growth measured after another 740 seconds, even at low shear rates that do not normally initiate vWF-dependent platelet adhesion. To test this concept in vivo, we performed a second series of studies to investigate the role of GPIb in platelet-platelet interactions at intermediate shear rates. A thrombogenic device was placed as an extension segment in the permanent arteriovenous shunt and exposed to native flowing blood. After 6 minutes the baboons were treated with the Fab fractions of 6B4. We postulated that a 6-minute exposure (number of platelets deposited was $\approx 0.6 \times 10^9$) was sufficient to allow ample coverage of the pericardium with adhering platelets. Inhibition of platelet deposition due to treatment, when compared with sham studies, would therefore reflect inhibition of platelet-platelet interactions. Because no such effect was seen, it strongly suggested that GPIb does not play a major role in in vivo platelet-platelet interactions under the conditions used in this study.

In conclusion, we have reported on the first anti-human GPIb antibody that can be used successfully to prevent platelet adhesion and thrombus formation in vivo, thereby confirming the predominant role of GPIb in platelet adhesion in vivo. Our studies, however, do not support the hypothesis that GPIb also plays a part in platelet-platelet interactions in vivo. On the basis of our results on bleeding times and the inhibition of thrombogenesis, we propose that the Fab fragments or derivatives of the anti-GPIb MoAb 6B4 may be useful compounds in preventing arterial thrombosis in those patients in whom thrombosis is expected, ie, after vascular engraftment, endarterectomy, or balloon catheterization.

Acknowledgments

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DOSE-DEPENDENT INHIBITION OF ACUTE ARTERIAL THROMBOSIS BY MONOCLONAL ANTIBODY (16N7C2) IN A BABOON MODEL

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INTRODUCTION

The pivotal role that blood platelets play in haemostasis and thrombosis caused a keen interest in the development of specific inhibitors of platelet function. The platelet glycoprotein (GP)IIb/IIIa receptor complex for fibrinogen is a particularly attractive target for therapeutic intervention, since it is the exclusive mediator of platelet aggregation. There are about 50,000 GPIIb/IIIa receptors on the surface of normal platelets. When they are reduced to about 10,000 there is a marked decrease in platelet aggregation, a mildly prolonged bleeding time and abolition of in vivo thrombus formation¹. 16N7C2, a murine monoclonal antibody against human platelets, was developed at the Center for Thrombosis and Vascular Research at the University of Leuven. Preliminary studies showed that it blocks the GPIIb/IIIa receptors of human and baboon platelets, but that it has no effect on cat, dog, pig, rabbit, rat, hamster, mouse and guinea-pig platelets. We therefore decided to evaluate the in vivo potential of this monoclonal antibody in baboons.

METHODS

Thrombosis Model

We measured ¹¹¹In-platelet deposition onto vascular grafts in a model of acute arterial thrombosis in baboons as previously described². A piece of Dacron vascular graft material (0.5 cm²) was inserted as an extension segment in a permanent femoral arterio-venous shunt in a baboon and platelet accumulation dynamically imaged with a scintillation camera.

Experimental Protocol

On the first day of the experiment the baboon's platelet were labelled with ^{111}In -tropolone and reinjected as described³. The thrombogenic device was inserted and platelet deposition imaged for two hours. The thrombogenic device was then removed and blood-flow re-established. On the second day the monoclonal antibody was injected, the thrombogenic device inserted 15 minutes later and imaged for two hours after which it was removed and blood-flow re-established. The latter procedure was repeated for the next two days.

A dose of 0.3 mg/Kg of antibody was administered to seven baboons and a dose of 0.1 mg/Kg to six baboons.

Quantification of GP IIb/IIIa Receptor Occupation

The number of receptors occupied by the monoclonal antibody was estimated as described⁴. Platelet-rich plasma was incubated with a near saturating dose of 125I-16N7C2 and the number of radiolabelled molecules bound per platelet determined. The number of receptors occupied by the monoclonal antibody was estimated by subtracting the number of molecules of 125I-16N7C2 bound to the platelets after 16N7C2 infusion from that obtained before 16N7C2 infusion.

Other Tests

The following routine coagulation tests were performed: repeated platelet counts; a template bleeding time; platelet aggregation studies with ADP and collagen; a prothrombin time and an activated partial thromboplastin time.

RESULTS

Platelet Deposition on the Thrombogenic Surface

A bolus injection of 0.3 mg/Kg of 16N7C2 virtually abolished platelet deposition on the thrombogenic surface on the first day. Platelet deposition was still inhibited by $\pm 79\%$ after 24 hours and $\pm 65\%$ after 48 hours (Fig. 1a). Injection of 0.1 mg/Kg of the antibody inhibited platelet deposition by $\pm 42\%$ on the day of injection and by $\pm 28\%$ after 24 hours (Fig. 1b).

Receptor Occupation

Injection of 0.3 mg/Kg of 16N7C2 blocked $\pm 64\%$ of the GPIIb/IIIa receptors 10 minutes after injection (Fig. 2). Receptor occupation then decreased in a linear fashion over the next few days. With the 0.1 mg/Kg dosage, $\pm 42\%$ of the receptors were blocked after 10 minutes, 28 % after 24 hours and 10 % after 48 hours.

Platelet Aggregation

16N7C2 also affected the platelet aggregation response to ADP and collagen. Injection of 0.3 mg/Kg totally inhibited platelet aggregation induced by ADP. Thereafter there was a steady increase in aggregation that returned to normal by the third day. Collagen induced aggregation was markedly decreased but not totally abolished on the first day. Otherwise the pattern was similar to that found with the

ADP induced aggregation. The injection of 0.1 mg/Kg of the monoclonal antibody only marginally inhibited platelet aggregation (Fig. 3b)

Coagulation Tests

The bleeding time was only prolonged (20 minutes) on the first day after injection of 0.3 mg/Kg 16N7C2, but returned to normal levels (5 min) by the second day.

The platelet count did not drop following the injection of 16N7C2, nor did it have any effect on the routine coagulation tests.

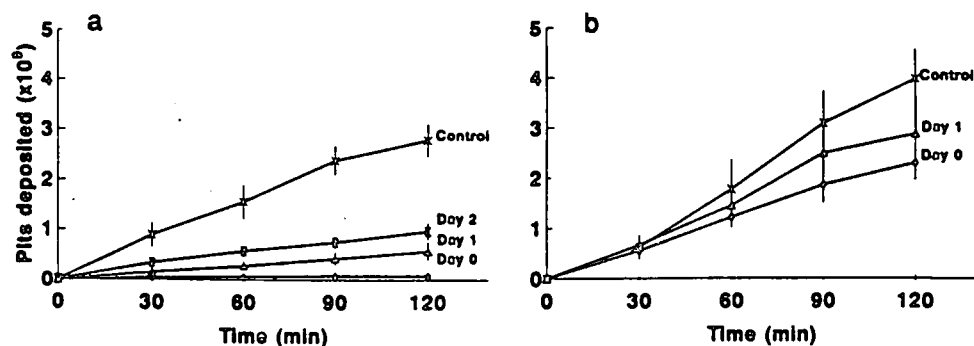


Figure 1. The effects of a bolus injection of 0.3 mg/Kg and 0.1 mg/Kg of 16N7C2 on platelet deposition on a thrombogenic surface are illustrated. The top curve in each graph shows platelet deposition over a 2 hour period before each treatment. A dosage of 0.3 mg of antibody abolished platelet deposition on the first day. Platelet deposition was still inhibited by 79 % after 24 hours and 65 % after 48 hours (a). A dosage of 0.1 mg/Kg resulted in a 42 % reduction in platelet deposition on the first day and 28 % after 24 hours (b).

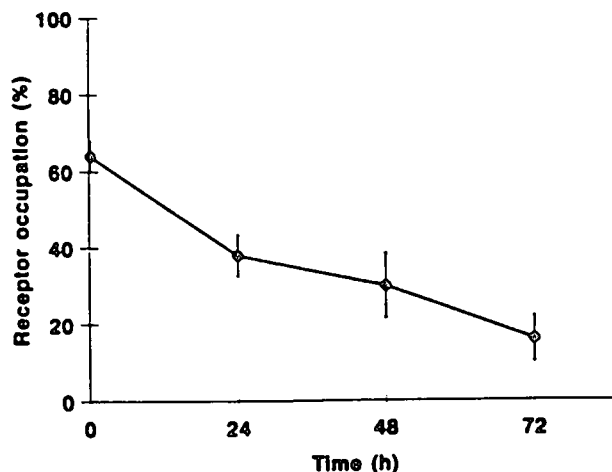


Figure 2. Receptor occupation was measured 10 min after injection of 0.3 mg/Kg of 16N7C2 and then daily for 3 days. Initially 64 % of receptors were blocked. It then decreased in a linear fashion over the next few days.

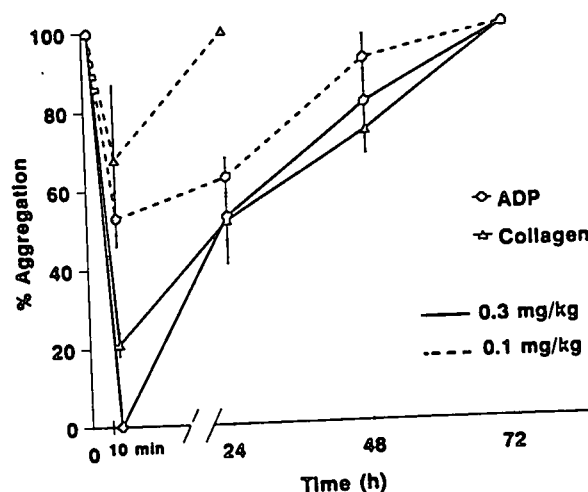


Figure 3. The platelet aggregation responses to 10 μ M ADP and 0.05 g/l collagen are shown for both the 0.3 mg/Kg and 0.1 mg/Kg dosages of 16N7C2. The higher dose inhibited platelet aggregation dramatically on the first day. Thereafter there was a steady return to normal by day 2. The effect of 0.1 mg/Kg of the monoclonal antibody was less pronounced but followed a similar pattern.

DISCUSSION

Glycoprotein IIb/IIIa is a two-chain, calcium dependent platelet surface molecule. Its IIb subunit contains the fibrinogen gamma-chain binding region and the amino globular head of the IIIa subunit contains the RGD-binding region. Sequence analysis of the monoclonal antibody 16N7C2, revealed an RGD-sequence in the hypervariable region of the heavy chain that is probably responsible for antibody binding.

The aim was to reduce the number of functional GPIIb/IIIa receptors to a level that would allow an adequate antithrombotic effect without the danger of a bleeding tendency. We calculated that this could be achieved by a dose of 0.3 mg/Kg of the monoclonal antibody to occupy approximately 70 % of the IIb/IIIa receptors. The measured receptor occupancy after injection of 0.3 mg/Kg of 16N7C2 was 64 %, but it was sufficient to abolish platelet deposition on the thrombogenic surface. This dosage of 16N7C2 produced a long term antithrombotic effect with about 65 % of platelet inhibition after 48 hours (Fig 1a). Receptor occupancy decreased in a linear fashion over days and corresponded with the platelet survival of 5 days in the baboon. This could indicate that the increase in unblocked receptors is caused by an influx of new platelets from the bone marrow.

No adverse effects were encountered: The platelet count did not fall, blood coagulation was not effected, and the bleeding time was only moderately prolonged on the first day.

Although these experiments with 16N7C2 are promising, at least two factors regarding the use of monoclonal antibodies to GPIIb/IIIa as antithrombotic agents have to be considered. First, there is the question of immunogenicity. We are dealing with a mouse antibody that has the potential to elicit an immune response in man. Although only two out of 16 patients showed an immune response in the Phase 1 study with the 7E3 monoclonal antibody², repeat therapy remains a problem. Attempts are being made to modify monoclonal antibodies in such a way that they lose their immunogenicity. Secondly, it must be remembered that GPIIb/IIIa belongs to the super-family of integrin receptors. There is a high homology between the different integrins and one

can anticipate some cross-reactivity to develop which could either have a beneficial or harmful effect. This is certainly an aspect that has to be investigated.

These experiments confirmed the usefulness of ^{111}In -labelled platelets and the baboon model to evaluate antithrombotic agents in vivo. They also showed that 16N7C2 is an effective antithrombotic agent with a long term effect in this model of arterial thrombosis. 16N7C2 seems to block the IIb/IIIa receptors permanently because the increase of unblocked receptors over time corresponded with the influx of new platelets into the circulation. Although monoclonal antibodies to GPIIb/IIIa are excellent research tools, much more development is needed before they can be successfully introduced as antithrombotic agents in clinical medicine.

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A Four-Hour Infusion of Recombinant Hirudin Results in Long-Term Inhibition of Arterial-Type Thrombosis in Baboons

By H.F. Kotzé, S. Lamprecht, and P.N. Badenhorst

Intravenous recombinant (r)-hirudin has a potent antithrombotic effect in aspirin- and heparin-resistant platelet-dependent thrombus formation in baboon models. However, these thrombi reform when therapy is stopped after 60 minutes. To determine if 4 hours of therapy can produce a lasting antithrombotic effect, we investigated the extent of deposition of ^{111}In -labeled platelets onto 0.5-cm² segments of Dacron vascular grafts for 53 hours. These grafts had been incorporated into exteriorized permanent femoral arteriovenous shunts in baboons. Platelet deposition in eight untreated animals was generally sigmoidal. Maximum platelet deposition, $1.7\% \pm 0.9\%$ of injected labeled platelets, was reached after approximately 4 hours. Deposition then gradually decreased to $0.4\% \pm 0.2\%$ of injected labeled platelets after 53 hours. After a thrombus was allowed to form for 15 minutes in six animals, intravenous treatment with r-hirudin at a dose of 20 nmol (0.14 mg)/kg-min⁻¹ (aPTT >300 sec-

onds) was started and maintained for 4 hours. Platelet deposition was interrupted during treatment. After infusion was stopped, platelets accumulated again, but not as much as in the untreated animals. Maximum platelet deposition, $0.7\% \pm 0.2\%$ of injected labeled platelets, was significantly less ($P < .01$), and was reached after approximately 23 hours. Thereafter, deposition decreased to $0.4\% \pm 0.2\%$ at 53 hours. The shunts in all of the untreated animals occluded at some stage during the study, while only one shunt occluded in the treated animals. We conclude that 4 hours of treatment of a freshly formed thrombus with a full antithrombotic dose of r-hirudin markedly reduced the maximum thrombus size and the rate of initial platelet deposition after treatment was stopped. This approach could produce lasting benefits in certain clinical settings.

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THROMBIN PLAYS A pivotal role in thrombosis and hemostasis. It amplifies the thrombotic process by activation of platelets and coagulation factors V and VIII. It also stabilizes and enlarges the thrombotic mass by cleavage of fibrinogen and activation of factor XIII. Fibrin-bound thrombin mediates platelet recruitment into existing thrombi and promotes further activation of coagulation.¹⁻⁶ When complexed to recombinant (r)-hirudin, thrombin loses its effects on platelets and coagulation, and thrombosis cannot proceed.⁷⁻¹⁰ Therefore, it is not surprising that r-hirudin, in high enough dosages, interrupts platelet-dependent arterial-type thrombosis.³⁻¹¹ Although the studies clearly show that r-hirudin inhibits acute arterial thrombosis, it is not clear what the effect on thrombosis is after treatment was stopped, especially in view of its short half-life in plasma.¹² In one study where a native Dacron vascular graft (Bard Vascular Systems Division, Billerica, MA) was treated with D-Phe-Pro-Arg chloromethyl ketone (PPACK), a specific thrombin inhibitor with a similar effect on platelet deposition than r-hirudin,¹¹ platelet deposition proceeded at the same rate as in the untreated studies after treatment was stopped and reached approximately the same level of deposition after 1 hour.³ In another study where a 15-minute old thrombus was treated with either r-hirudin or PPACK for 1 hour, platelets deposited at a slower rate (*v* untreated studies) after treatment was stopped and approximately 20% to 40% less platelets

were deposited onto the thrombus during the following 3 hours when compared with the untreated animals.¹¹

In this study, we investigated if 4 hours of treatment with a full antithrombotic dose of r-hirudin may result in long-term inhibition of platelet deposition after treatment was stopped. This was done in a baboon model of arterial thrombus formation.¹³ The baboon was selected as the experimental model because of its similarity to humans with respect to the molecular and cellular composition and function of the hemostatic apparatus.¹⁴ Thrombogenic devices consisting of Dacron vascular graft material were inserted as extension segments into chronic femoral arteriovenous access shunts.^{13,14} Systemic treatment with r-hirudin was started 15 minutes after the devices were placed and maintained for 4 hours. The devices were kept in place for up to 53 hours. Thrombogenesis was assessed by measuring the deposition of ^{111}In -labeled platelets onto the thrombogenic device for the first 6 hours after placement and then twice daily.

MATERIALS AND METHODS

Animals studied. Thirteen normal male baboons (*Papio ursinus*) were used. The animals weighed 10 to 15 kg and were disease-free for at least 6 weeks before use. All procedures were approved by the Institutional Ethics Committee for Animal Experimentation in accordance with Federal Guidelines (Guide for the Care and Use of Laboratory Animals, Public Health Service National Institutes of Health publication number 85-23, 1985). The baboons supported permanent Teflon-Silastic arteriovenous (AV) shunts (Ven Products, Johannesburg, South Africa) implanted in the femoral vessels.¹³ These shunts do not detectably shorten platelet survival or produce measurable platelet activation.¹³⁻¹⁵ In addition, we used normal baboons without AV shunts to measure the mean platelet life span.

Study protocol. A thrombus was generated using uncrimped, knitted Dacron vascular graft material (0.5 cm²) that was built into the wall of silicone rubber tubing (4 mm inside diameter). The method to construct the thrombogenic device is described in detail.¹⁶ In each experiment, a thrombogenic device, prefilled with saline to avoid a blood-air interface, was incorporated as an extension segment into the permanent AV shunt by means of Teflon connectors.¹³ The devices were kept in place for 53 hours in both the untreated ($n = 8$) and treated ($n = 6$) baboons. Blood flow rate through the

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r-HIRUDIN REDUCES POSTTREATMENT THROMBOSIS

device was 100 to 140 mL/min, producing wall shear rates similar to that found in medium to large sized arteries.¹⁵ In the treatment studies, r-hirudin (Hoechst AG, Frankfurt and Behringwerke AG, Marburg, Germany) was systematically infused at a rate of 20 nmol/kg-min⁻¹ for 4 hours. Treatment was started 15 minutes after placement of the thrombogenic device. The r-hirudin was dissolved in saline and infused into the tubing of the AV shunt distal to the thrombogenic device.

Graft imaging and quantification of platelet deposition. Autologous blood platelets were labeled with ¹¹¹In-chloride as previously described.^{17,18} Imaging and quantification of the deposition of ¹¹¹In-platelets were done as described in detail.^{13,16} Image acquisition of the grafts, including proximal and distal silastic segments, was done with a Searle Pho scintillation camera (Siemens Medical Division, Hoffman Estates, IL) fitted with a high-resolution collimator. The images were stored and analyzed with a Medical Data Systems A³ computer (Medtronic, Ann Arbor, MI) interfaced with the camera. Dynamic image acquisition, 10-minute images (128 × 128 byte mode) for 6 hours, was started simultaneous with the start of blood flow through the devices. Thereafter, a static image of 10 minutes (128 × 128 byte mode) was acquired twice daily until the end of the study. A 10-minute image (128 × 128 byte mode) of a 3 mL autologous blood sample to determine circulating blood radioactivity (blood standard) was also acquired each time the grafts were imaged. A region of interest (1 × 1.5 cm) of the graft segment was selected to determine the deposited and circulating radioactivity in each of the dynamic and static images. Radioactivity in a region of similar size of circulating radioactivity in the proximal segment of the extension tubing was determined and subtracted from the radioactivity in the graft region to calculate deposited radioactivity. ¹¹¹In-platelet deposition was expressed in two ways. First, deposited graft radioactivity was expressed as a percentage of total injected ¹¹¹In-platelet radioactivity. Total injected radioactivity was determined by imaging the injectate for 10 minutes. Deposited graft radioactivity was appropriately corrected for physical decay of ¹¹¹In. Secondly, deposited graft radioactivity was expressed as a percentage of ¹¹¹In-platelet radioactivity in the circulation at the time of imaging. This was determined from the count rate of the blood standard, corrected for plasma ¹¹¹In-radioactivity.¹³ The corrected count rate was multiplied by the blood volume, estimated to be 65 mL/kg.¹⁷

The disappearance of ¹¹¹In-labeled platelets from the circulation was also determined in four normal baboons, and the mean platelet life span calculated. The method is described in detail.^{17,18}

Laboratory measurements. The times at which blood samples were collected for the different determinations can be seen in the Results section. The platelet count and hematocrit of blood samples collected in 2 mg/mL disodium EDTA were determined using a Technicon H1 blood cell analyzer (Miles Inc, Tarrytown, NY). The same blood sample was used to correct whole blood radioactivity by subtracting plasma radioactivity to calculate platelet radioactivity.¹³ Blood was collected in 3.8% sodium citrate (9 vol blood to 1 vol citrate) to determine activated partial thromboplastin time (aPTT), thrombin time (TT) and the plasma r-hirudin concentration. aPTT and TT were measured on a fibrinometer (Clotex II; Hyland Division, Travenol Laboratories, Costa Mesa, CA) using reagents supplied by the manufacturer. The plasma r-hirudin concentration was determined as described.¹⁹

Statistical analysis. Student's *t*-test (two-tailed) for paired or unpaired data was used to test for differences when the data were normally distributed. If not, the Mann-Whitney U-test for nonparametric data was used. Data in the text are given as the mean ± SD.

RESULTS

Platelet count and platelet radioactivity. The mean platelet count before the start of the study was $352 \pm 89 \times$

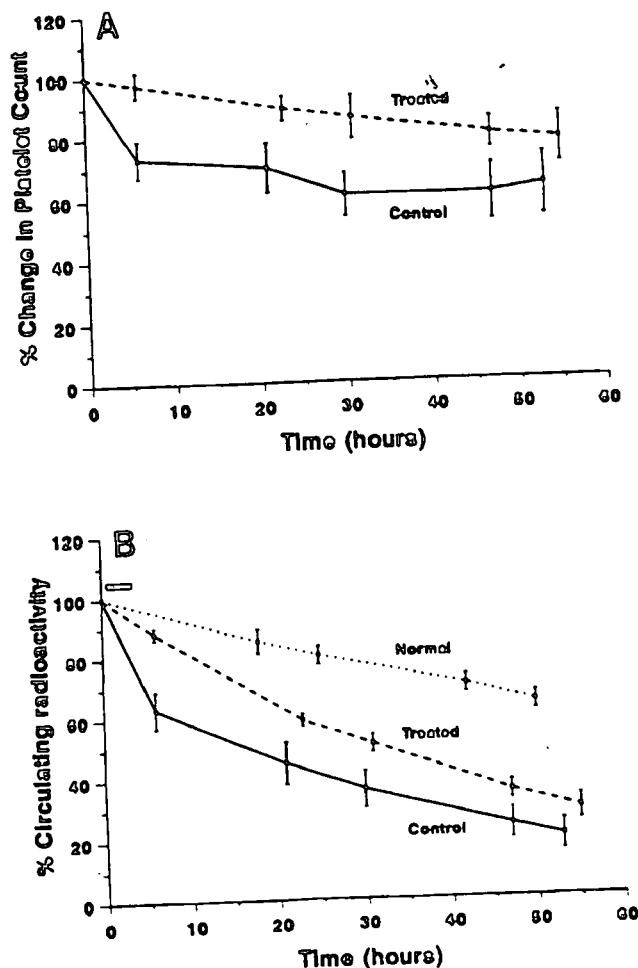


Fig 1. Percent change in platelet count (A) in the untreated (control, $n = 8$) and treated ($n = 6$) animals and the disappearance of ¹¹¹In-labeled platelets from the circulation (B) of normal ($n = 4$), untreated (control, $n = 8$) and treated ($n = 6$) animals. Values are given as a mean ± 1 SEM.

$10^9/L$ in the untreated animals and $326 \pm 65 \times 10^9/L$ in the treated animals. The changes in platelet count following placement of the thrombogenic devices are illustrated in Fig 1A. The largest decrease, 30% (median, range = 9% to 56%) was observed in the untreated animals during the first 6 hours after placement of the devices. The corresponding decrease in the treated animals, 9% (median, range = 0 to 19), was significantly less ($P = .014$, $n = 14$, Mann-Whitney U-test). After 6 hours, the platelet count continued to decrease in both cases until the end of the study. This decrease was similar in the untreated and treated animals, ie, 8% (median range = 2% to 40%) and 10% (median, range = 0% to 25%), respectively ($P = .844$, $n = 14$, Mann-Whitney U-test).

The disappearance curves of ¹¹¹In-platelet radioactivity from the circulation of normal baboons and in the untreated and treated baboons are shown in Fig 1B. The disappearance of labeled platelets from the circulation of both the untreated and treated animals was accelerated when compared with

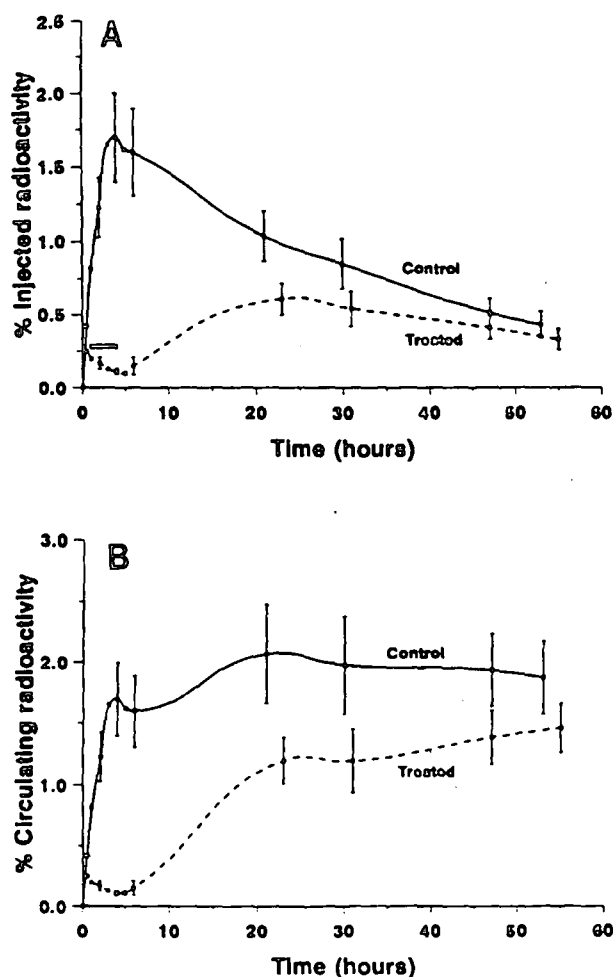


Fig 2. Platelet deposition onto the Dacron vascular graft material in the thrombogenic devices in untreated (control, $n = 8$) and treated ($n = 6$) animals. (A) The results are expressed as a percent of the injected ^{111}In -labeled platelets, and in (B) as the percent of circulating ^{111}In -labeled platelets at the time of imaging. Values are given as a mean ± 1 SEM.

that in the normal animals. We calculated the platelet survival time using the multiple hit model.²⁰ The calculations excluded the data points collected during the first 6 hours of the study. The mean platelet life span (MPLS) of ^{111}In -labeled platelets in the baboons without thrombogenic devices was normal, 138 ± 14 hours.¹⁸ The MPLS in the untreated animals ranged from 17 to 80 hours (median, 60 hours). In the treated animals it ranged from 35 to 79 hours (median, 55 hours). In both cases, it represented a shortening in the MPLS of approximately 40% to 43% when compared with the normal animals.

Thrombus formation. The results are illustrated in Fig 2A and B. Figure 2A represents the time/radioactivity curves where deposited radioactivity was expressed as a percentage of total injected radioactivity. In the untreated animals, platelets rapidly accumulated after the thrombogenic devices were placed. Maximum deposition, $1.7\% \pm 0.9\%$ of injected labeled platelets, was reached approximately 4 hours after

placement of the devices. Thereafter, deposition gradually decreased, reaching $0.4\% \pm 0.2\%$ of the injected labeled platelets after 53 hours. Treatment with r-hirudin, started 15 minutes after the thrombogenic devices were placed (deposition = $0.3\% \pm 0.1\%$ of injected radioactivity), interrupted platelet deposition during the 4 hours of treatment. In fact, it appears that deposited labeled platelets actually detached from the thrombus during treatment. After treatment was stopped, platelets were again deposited, but at a much slower rate, and to a much lesser extent than in the untreated studies. Maximum deposition, $0.7\% \pm 0.2\%$ of injected labeled platelets, was reached after approximately 23 hours. Maximum deposition was approximately 59% less than in the untreated animals. Thereafter, deposition gradually decreased almost in parallel to that observed in the untreated studies. At the end of the study $0.4\% \pm 0.2\%$ of the injected labeled platelets were deposited.

The results in Fig 2B represent the deposition curves when deposited radioactivity was expressed as a percentage of total circulating radioactivity at the time each image was acquired. By expressing the results in this way, one can relate the extent of deposited platelet radioactivity to the circulating radioactivity at the time of imaging. In the untreated animals, the ratio remained relatively constant after a maximum was reached. It does appear that platelets are still being deposited during the first 20 to 25 hours. Thereafter, deposited radioactivity decreased almost in parallel with circulating radioactivity, ie, the relationship remained constant. In the case of the treated animals, maximum deposition was reached approximately 23 hours after the devices were placed. Thereafter, the ratio remained relatively constant indicating that deposited radioactivity decreased in parallel with circulating radioactivity.

Coagulation. Figure 3A summarizes the changes in the plasma concentration of r-hirudin. When infusion was stopped, the plasma levels decreased rapidly to approximately 15% of the maximum plasma concentration 2 hours after treatment was stopped. Figure 3B summarizes the changes in aPTT in the experimental animals over the first 6 hours of the study. Ten minutes after treatment was started, the aPTT was longer than 300 seconds. This was maintained by the infusion. When infusion was stopped after 4 hours, the aPTT shortened, but did not return to pretreatment values (Table 1). There was a slight prolongation in the aPTT (significantly longer at 21 hours) in the untreated animals. The TT remained slightly prolonged for up to 30 hours in the treated animals (Table 1).

DISCUSSION

In the untreated animals platelets were rapidly deposited after the thrombogenic devices were placed and maximum deposition was reached after approximately 3.5 hours. Thereafter, thrombus-associated platelet radioactivity decreased towards the end of the study (Fig 2A). Sigmoidal deposition was also obtained in patients with aorta aneurysms and Dacron vascular grafts implanted for up to 10 years,²¹⁻²³ although maximum deposition was reached at a later stage.²³ This indicates that a fresh thrombus is typically more robust than an older, stabilized one. After maximum deposition was

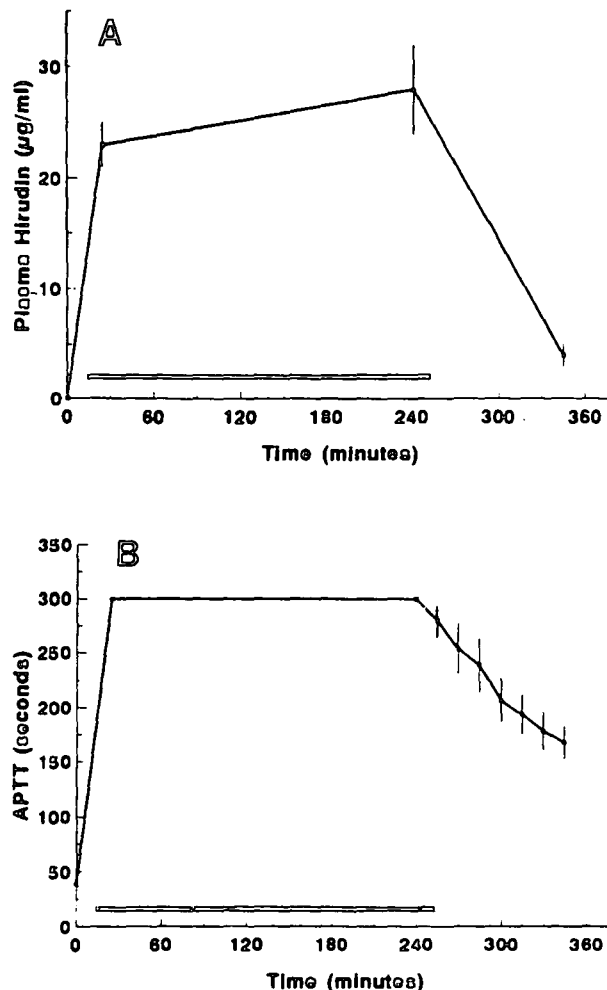


Fig 3. Changes in the plasma concentration of r-hirudin (A) and aPTT (B) in the treated animals. Values are given as a mean $\pm$ 1 SEM.

reached, thrombus radioactivity decreased almost in parallel with circulating radioactivity (Fig 2B). This strongly supports the hypothesis that a dynamic equilibrium exists between deposited and circulating platelets, and that the rate of thrombus formation and dissolution is equivalent. It is unlikely that the loss of radioactivity from the thrombus was due to elution of ^{111}In from deposited platelets, because ^{111}In does not elute from labeled platelets when they are aggre-

gated in vitro, or, when they are incubated at 37°C for 24 hours in plasma.^{24,25}

Thrombus formation was effectively interrupted during the 4 hours of treatment of the 15-minute old thrombus (Fig 2). This is consistent with the view that r-hirudin blocks the activity of thrombin on platelets and coagulation.⁴⁻¹⁰ It also confirms the findings that r-hirudin interrupts platelet-rich, arterial-type thrombus formation induced by Dacron vascular graft material, collagen coated tubing, endarterectomized aorta segments, and following angioplasty.^{6,26}

When treatment was stopped, platelets were again deposited (Fig 1). However, the rate of deposition was much slower and markedly less platelets were deposited than in the untreated animals. It is notable that the short-term treatment with r-hirudin reduced the maximum size of the thrombus by approximately 59% and that maximum deposition was attained at a much later stage. Also important is the finding that at 21 to 30 hours after placement of the devices, the thrombi in both untreated and treated animals were of equivalent size (Fig 2). This, and the fact that the disappearance patterns of circulating and thrombus radioactivity in both the treated and untreated animals were almost parallel after maximum deposition was reached (Fig 2B), suggest that the surface thrombogenicity was essentially the same in the treated and untreated animals after 21 hours. The comparable shortening of the MPLS in both groups (Fig 1B) further supports the finding that surface thrombogenicity is similar after 21 to 30 hours. A shortening in MPLS is generally regarded as a sensitive measure of in vivo platelet activation and increased consumption due to thrombosis.^{27,28}

Our results strongly suggest that the effect of r-hirudin on thrombosis was transient. In spite of this, a long-term benefit of a smaller thrombus and reduced initial thrombogenicity (<21 hours) was achieved. If acute thrombosis is indeed robust, as is suggested by the deposition curves in the untreated animals, the early intervention of thrombosis could produce a lasting benefit in certain clinical settings. This was indeed the case where a 2-hour infusion of r-hirudin reduced restenosis following balloon angioplasty of atherosclerotic arteries in rabbits, possibly as a result of inhibition of platelet-dependent thrombus formation.²⁹ These beneficial effects were obtained when treatment was started before angioplasty. In this study, we started treatment 15 minutes after the thrombogenic devices were placed. Therefore, we simulated a situation where normal hemostasis should have been achieved before treatment was started. This was done to

Table 1. Changes in aPTT and TT in the Untreated and r-Hirudin-Treated Animals

Measurement		Time (h)						
		0	4	6	21	30	47	63
aPTT (s)	Untreated	36 $\pm$ 5	40 $\pm$ 3		41 $\pm$ 4*	39 $\pm$ 3	39 $\pm$ 3	41 $\pm$ 8
	Treated	39 $\pm$ 3	>300	168 $\pm$ 33*	61 $\pm$ 10*	58 $\pm$ 9*	50 $\pm$ 3*	50 $\pm$ 3*
TT (s)	Untreated	29 $\pm$ 2	30 $\pm$ 3		30 $\pm$ 5	29 $\pm$ 2	31 $\pm$ 5	30 $\pm$ 2
	Treated	27 $\pm$ 1	>180	>180	35 $\pm$ 4*	37 $\pm$ 7*	28 $\pm$ 5	26 $\pm$ 3

Values are given as a mean $\pm$ 1 SD.

* $P < .05$ v time 0, Student's *t*-test for unpaired data.

reduce the risk of excessive bleeding as a result of ineffective hemostasis due to treatment. In view of our results, similar beneficial results may be expected in the maintenance of coronary artery patency following successful thrombolysis or after vascular engraftment, without adversely increasing the risk of excessive blood loss.

The mechanism by which the lasting benefit was achieved is unclear. It is unlikely that residual r-hirudin in plasma or, r-hirudin in complex with circulating thrombin, played a part. First, r-hirudin was rapidly removed from the plasma (Fig 1A), and secondly, the half-life of the effect of circulating r-hirudin on aPTT, approximately 47 minutes, was too short to affect the time to reach maximum deposition. It is possible that the characteristics of the thrombus surface played a part. r-Hirudin forms a tight complex with thrombin and can bind and inactivate thrombin bound to fibrin(ogen) in a thrombus.^{12,30,31} In complex with thrombin, r-hirudin also inhibits the thrombin-platelet reaction.³² These characteristics may explain why platelet deposition was interrupted during the treatment period. The long-term benefits with respect to thrombus size may also relate to these inhibitory functions of r-hirudin. It is possible that, because of its high affinity for thrombin,¹² the r-hirudin bound to thrombus-associated thrombin may remain bound for relatively long periods, even when no circulating r-hirudin is present. If this is the case, recruitment of platelets to the thrombus can be mediated by nonthrombin mechanisms like ADP or thromboxane A₂³² as r-hirudin does not affect the activity of these agonists on platelet function.³³ This can also explain the slower rate of platelet uptake because these two platelet agonists are not as potent as thrombin.³³

After the rapid shortening during the first 2 hours after treatment was stopped (Fig 2B), the aPTT remained slightly, but significantly longer than pretreatment times for the remainder of the study (Table 1). It is possible that thrombin, which forms as a result of thrombogenesis, activated protein C.³⁴ It was recently shown that infusion of low doses of thrombin into baboons increases the plasma levels of activated protein C in a dose-dependent manner and that aPTT lengthens accordingly.³⁵ This may explain the slightly prolonged aPTT at later stages of the study. This mechanism could also be responsible for the slower rate of platelet deposition and the decrease in maximum thrombus size in the treated animals. It was shown that activated protein C inhibits platelet-dependent thrombus formation.³⁵

Our results indicated that a 4-hour interruption of platelet deposition onto a freshly formed thrombus produced a lasting effect. After treatment was stopped, the rate of platelet deposition was much slower than in the untreated animals, the size of the thrombus was markedly reduced, and maximum deposition was reached at a much later stage. AV shunt patency was also affected. In the treated animals, only one shunt occluded in comparison with all the shunts in the untreated animals. These benefits were achieved despite the finding that the effect of r-hirudin was transient, and that after 21 to 30 hours, the thrombogenicity of the thrombus surfaces were similar in the treated and untreated animals. Our approach to start treatment after normal hemostasis had been achieved was justified and may be applied in certain

clinical settings to reduce the risk of bleeding. This approach may be used to reduce restenosis after balloon angioplasty, to improve vascular graft patency after implantation, and to maintain coronary artery patency after successful fibrinolysis.

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Determination of the Dosage of Recombinant Hirudin to Inhibit Arterial Thrombosis in Baboons

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ABSTRACT: Recombinant hirudin, a potent and direct inhibitor of thrombin, effectively inhibits platelet-dependent thrombosis. Our aim was to establish the plasma concentration at which r-hirudin expresses its optimal antithrombotic effect. We measured the extent of inhibition of ^{111}In -labeled platelet deposition onto 0.6 cm^2 segments of Dacron vascular grafts. These grafts were incorporated as extension segments into exteriorized permanent femoral arteriovenous shunts in baboons. In six control studies a mean of $1.99 \pm 0.26 \times 10^9$ platelets were deposited at the end of 120 min. In the treatment studies, a thrombus was allowed to form for 10 min in six animals. Treatment for 30 min with r-hirudin at dosages of 140, 70, and $35\text{ }\mu\text{g/kg/min}$, but not $14\text{ }\mu\text{g/kg/min}$, dose dependently interrupted platelet deposition. The relationship between the percent inhibition of platelet deposition caused by r-hirudin and the plasma concentration of hirudin was exponential (i.e., $\% \text{ Inhibition} = 95(1 - e^{0.23 \times [\text{r-hirudin}]})$ ($R^2 = 0.76$). From this, we estimated that 50% inhibition of platelet deposition will occur at a plasma concentration of approximately $3.3\text{ }\mu\text{g r-hirudin/mL}$ and 80% at $8.1\text{ }\mu\text{g/mL}$. The relationship between the inhibition of platelet deposition and the plasma concentration of hirudin makes it possible to estimate the dose of hirudin that will result in a given level of inhibition of platelet deposition. © 2000 Wiley-Liss, Inc. and the American Pharmaceutical Association *J Pharm Sci* 89: 579–585, 2000

INTRODUCTION

Thrombin is pivotal in regulating the processes of thrombus formation at sites of vascular injury and on the surface of vascular graft material. Thrombin is generally regarded as the most important *in vivo* regulator of platelet activation and aggregation and it activates coagulation factors V and VIII to increase activation of prothrombin. Thrombin also stabilizes the thrombotic mass by cleavage of fibrinogen and activation of factor XIII. In addition, it regulates its own formation through activation of protein C.^{1–4}

Thrombin is also an important growth factor that initiates the proliferation of smooth muscle cells and, in addition, modulates the effects of other growth factors.⁵ It is therefore not surprising that thrombin is one of the major targets for the development of agents that can inhibit its function.

When complexed to hirudin, thrombin loses all its prothrombic functions.^{2–7} As a result, hirudin is being aggressively developed for clinical applications in man. Most of this development is based on the anticoagulant effect of hirudin, i.e., its ability to inhibit venous-type thrombosis in which fibrinogen predominates and platelets play a lesser role.^{8,9} There is also evidence that direct inhibition of thrombin or activated factor X inhibits arterial-type thrombosis in which platelets predominate and fibrinogen plays a lesser role.^{10–13} These effects were observed in cases in which

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thrombogenesis is resistant to conventional treatment with aspirin and heparin.⁴ However, the dosages required to inhibit platelet deposition also produced a corresponding impairment of hemostasis,⁴ which may increase the risk of a bleeding tendency developing.¹¹

In this study, we attempted to establish the minimum dose of hirudin that will effectively inhibit platelet-dependent thrombogenesis under arterial blood flow conditions. A baboon model of arterial thrombosis was used because the molecular and cellular composition and function of the hemostatic apparatus closely resembles that of humans.^{14,15} Thrombogenic devices consisting of Dacron vascular graft material were inserted as extension segments into chronic arteriovenous (AV) shunts. The animals were systemically treated for 30 min with different dosages of hirudin, starting 10 min after placement of the devices. Platelet deposition, measured in real time as the deposition of ¹¹¹In-labeled platelets onto the thrombogenic device, was assessed for 120 min.

MATERIALS AND METHODS

Animals Studied

Twelve normal male baboons (*Papio ursinus*) were used. The animals weighed between 10 and 15 kg and were disease-free for at least 6 weeks before use. All procedures were approved by the Ethics Committee for Animal Experimentation of the University of the Orange Free State in accordance with the National Code for Animal Use in Research, Education, Diagnosis and Testing of Drugs and Related Substances in South Africa. The baboons supported permanent Teflon-Silastic AV shunts implanted in the femoral vessels.¹⁴ These shunts neither shorten platelet survival nor cause measurable platelet activation.¹⁴⁻¹⁶

Study Protocol

Platelet-dependent arterial-type thrombus formation was induced with uncrimped, knitted Dacron vascular graft material (0.6 cm²) that was built into the wall of silicone rubber tubing (4 mm inside diameter). The method of preparation of the thrombogenic device is described in detail.¹⁷ In each experiment, a thrombogenic device, pre-filled with saline to avoid a blood-air interface, was incorporated as an extension segment into the permanent AV shunt by means of Teflon con-

nectors.^{14,15} In six baboons, control studies were done in which the devices were placed for 120 min. At the end of the study, the devices were removed and blood flow through the permanent shunt re-established. In six other animals with platelet counts matching those of the control baboons, treatment for 30 min with r-hirudin (Lepirudin, Hoechst AG/Behringwerke AG, Frankfurt, Germany) at dosages of 140 (20), 70 (10), 35 (5), and 14 (2) µg (nmol)/kg/min was started 10 min after placement of the thrombogenic devices. The devices were kept in place for a further 90 min before removal, when after flow through the permanent shunts were reestablished. A washout period of 24 h was allowed between treatments, which were randomized to balance for possible effects of residual hirudin. The hirudin was dissolved in saline and was infused into the tubing of the A-V shunt distal to the thrombogenic device, i.e., the treatments were systemic.

In all the studies, the starting blood flow rate through the devices was 100–140 mL/min, producing wall shear rates similar to that found in medium- to large-sized arteries.¹⁵

Graft Imaging and Quantification of Platelet Deposition

Autologous blood platelets were labeled with ¹¹¹In-tropolone as previously described.¹⁸ Imaging and quantification of the deposition of ¹¹¹In-platelets were done as described in detail.^{14,17} Image acquisition of the grafts, including proximal and distal Silastic segments, was done with a Searle Pho scintillation camera fitted with a high-resolution collimator. The images were stored on and analyzed with a Medical Data Systems A³ computer (Medtronic, Ann Arbor, MI) interfaced with the scintillation camera. Dynamic image acquisition, 10-min images (128 × 128 byte mode) for 2 h, was started simultaneously with the start of blood flow through the devices. A 10-min image (128 × 128 byte mode) of a 3-mL autologous blood sample was also acquired each time that the grafts were imaged to determine circulating blood radioactivity (blood standard). A region of interest of the graft segment was selected to determine the deposited and circulating radioactivity in each of the dynamic images. Radioactivity in a region of similar size of circulating radioactivity in the proximal segment of the extension tubing was determined and subtracted from the radioactivity in the graft region to calculate deposited radioactivity. Platelet deposition was expressed

as the total number of platelets deposited. The method to calculate this is described in detail.¹⁴ The percent inhibition caused by treatment was calculated from the number of platelets deposited at the end of treatment and the number of platelets deposited at 40 min in control baboons. In general, platelet deposition in the control baboons reached a steady state at 40 min. The control baboons were selected to have blood platelet counts that matched that of the treated baboons. This was done to eliminate the effect of differences in platelet count on platelet deposition.¹⁵

Laboratory Measurements

The times at which blood samples were collected for the different determinations can be seen in the Results section. The platelet count and hematocrit of blood samples collected in 2 mg/mL disodium EDTA were determined with a Technicon H2 blood cell analyzer (Bayer Diagnostics, Tarrytown, NY). The same blood sample was used to determine whole blood and plasma radioactivity. Blood radioactivity was corrected by subtracting plasma radioactivity to calculate platelet radioactivity.¹⁴ The radioactivity in the different samples was determined in a well-type scintillation counter. Blood was also collected in 3.8% sodium citrate (9 volumes blood to 1 volume citrate) to determine the activated partial thromboplastin time (aPTT), the plasma levels of hirudin, and thrombin-antithrombin III (TAT) complexes. aPTT was measured on a fibrinometer (Cobas Fibrro, Hyland Division, Travenol Laboratories, Costa Mesa, CA) using reagents supplied by the manufacturer. Hirudin was determined using the ecarin clotting assay¹⁹ and TAT complexes measured by an ELISA method (Enzygnost Combi-pack, Behring, Marburg, Germany).

Analyses

Differences between means were tested using Student's test (two-tailed) for paired data. A difference was regarded as significant if P was <0.05 . Values in the text are given as a mean $\pm$ 1 SD. To calculate the half-life of hirudin in plasma, a one-phase exponential decay function ($H = ae^{kt}$), in which H = plasma concentration of hirudin in $\mu\text{g/mL}$ and t = time in minutes, was fitted to the data. The half-life was calculated as $0.693/k$.

RESULTS

The effect of hirudin on platelet deposition is summarized in Figure 1. Treatment with 140, 70, and 35, but not 14 $\mu\text{g/kg/min}$ hirudin, abolished platelet deposition during infusion. At a dose of 35 $\mu\text{g/kg/min}$ it took somewhat longer than with the higher dosages for the inhibitory effect to become apparent. After the treatment was stopped, platelets started to deposit again after a lag period. This lag phase was longest at the highest dose of hirudin (Fig. 1). The rate at which platelets were deposited during the 80-min period after treatments were stopped was $10 \pm 6 \times 10^6/\text{min}$ at a dose of 140 $\mu\text{g/kg/min}$, $19 \pm 8 \times 10^6/\text{min}$ at a dose of 70 $\mu\text{g/kg/min}$, $22 \pm 7 \times 10^6/\text{min}$ at a dose of 35 $\mu\text{g/kg/min}$, and $22 \pm 6 \times 10^6/\text{min}$ at a dose of 14 $\mu\text{g/kg/min}$. In the control studies, the rate of platelet deposition was $17 \pm 6 \times 10^6/\text{min}$.

The relationship between the percent inhibition of platelet deposition and the plasma concentration of hirudin at the end of the infusion period is illustrated in Figure 2. It is best described by an exponential association function

$$\% \text{ Inhibition} = 95(1 - e^{-0.23 \times [r\text{-hirudin}]}) \quad (1)$$

where $n = 21$ and $R^2 = 0.76$. This relationship implies that platelet deposition will be inhibited by 50% at a plasma concentration of 3.3 $\mu\text{g/mL}$ and by 80% at a concentration of 8.1 $\mu\text{g/mL}$.

In all studies, the half-life of hirudin was 20 ± 3 min. The small standard deviation from the mean indicates that the half-life was not affected by the dosage of hirudin used. The aPTT and plasma levels of the TAT complexes are summarized in Table I. TAT complexes remained low during the infusion of hirudin, irrespective of the dosage. After infusion was stopped, TAT complexes formed during the following 70 min, and the amount formed was inversely related to the dosage used. TAT complexes increased approximately 40-fold in the control baboons.

DISCUSSION

A constant infusion of hirudin inhibited platelet deposition onto the surface of a fresh thrombus in a dose-dependent manner (Fig. 1). The blood flow rates, 120 to 140 mL/min, produce wall shear rates of 400 to 470 s^{-1} , similar to that found in large- and medium-sized arteries.^{11,14} A throm-

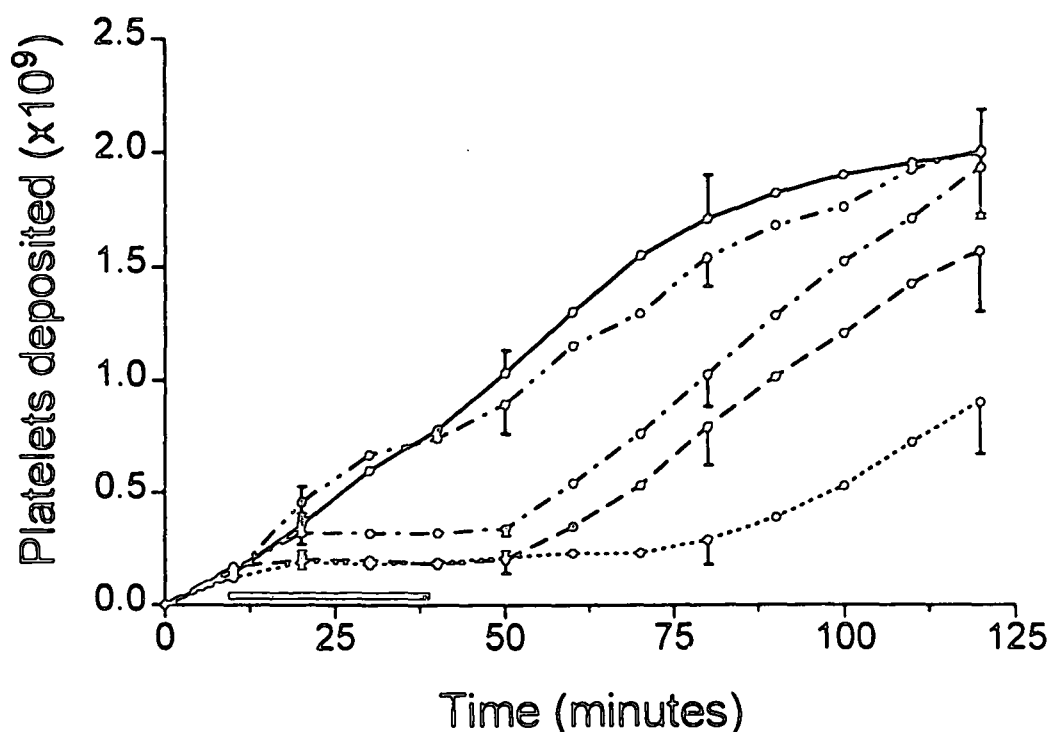


Figure 1. The effect of treatment with r-hirudin on platelet deposition. The solid bar represents the time of infusion. Values are given as a mean $\pm$ 1 SEM. (— Control; ···· 140 μ g/kg/min; --- 70 μ g/kg/min; - · - 35 μ g/kg/min; ···· 14 μ g/kg/min).

bus was allowed to form before treatment was started; we therefore investigated the effect of hirudin on platelet-platelet interactions. Our results are similar to those in which treatment with hirudin was started simultaneous with the placement of Dacron vascular graft material, homologous endarterectomized aorta, and collagen-coated tubing.¹¹ These data clearly show that hirudin is an effective inhibitor of platelet-dependent thrombogenesis, irrespective of the nidus of thrombogenesis. It is not clear whether the inhibition of platelet deposition was due to interruption of platelet deposition or whether a steady state was achieved between platelet deposition and dissolution of platelets from the thrombus, similar to the steady state that is achieved when animals are not treated.^{10,12,14} It is probably a combination of both mechanisms. In the case of the latter, it is reasonable to assume that the residence time of platelets in the thrombus was short, possibly because the complexed thrombin is unable to convert fibrinogen into fibrin to stabilize the forming thrombus. In support of this, one must also bear in mind that platelets can also be activated by nonthrombin mechanisms^{4,17} and that these mechanisms in their own right can cause thrombogenesis, although at a slower rate.

The mechanism of inhibition of platelet deposition by hirudin is unknown. It is known that hirudin binds avidly to thrombin, whether it is present in plasma or in the thrombus, and so inhibits all its effects on platelets and coagulation.^{4,6,20} It is reasonable to assume that, in our study, thrombin was generated during the first 10 min before treatment was started. We did not measure TAT complexes as an indication of thrombin generation, but have shown in a similar study that the plasma levels of TAT complexes increased approximately eightfold during this period.¹² When treatment is started, the hirudin can bind to both thrombus-bound and plasma thrombin, causing the thrombin to lose its effect on coagulation and platelets.²⁰ This will inhibit thrombogenesis. One must bear in mind that thrombin is currently regarded as a major regulator of platelet activation and aggregation *in vivo*.⁴ After this initial inhibition of thrombin, hirudin can inhibit thrombogenesis in at least two ways. First, the thrombus can continue to generate thrombin, which binds to hirudin, similar to that following the initial period after treatment was started. Second, because of complex formation with hirudin, less thrombin is available to activate platelets. This can further inhibit throm-

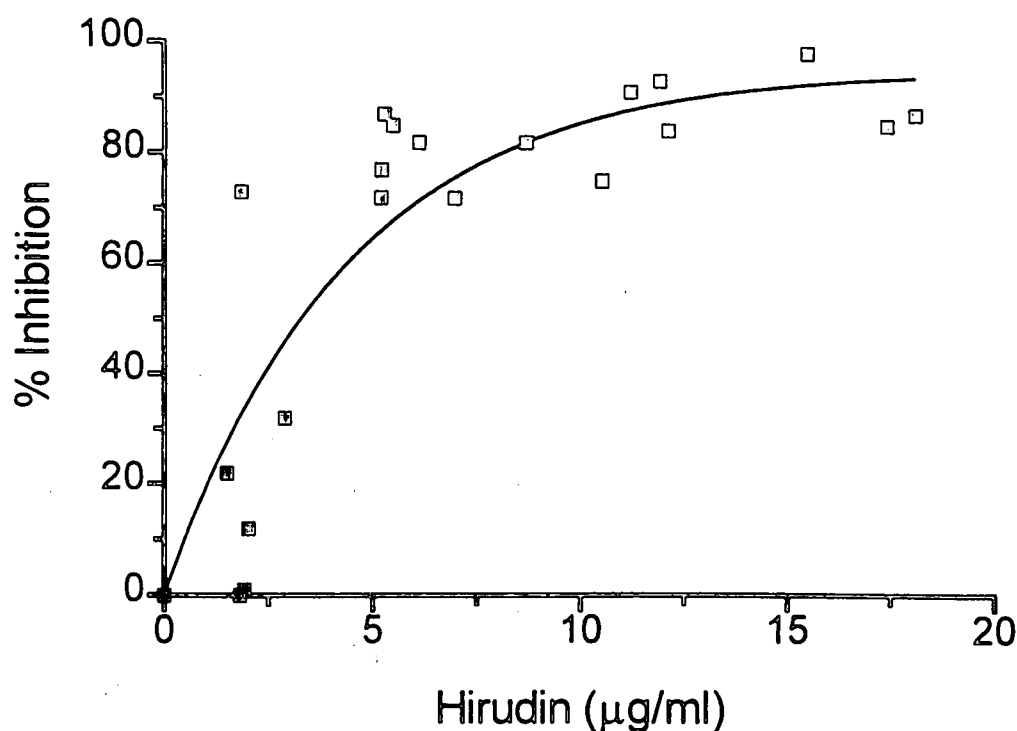


Figure 2. The relationship between the percent inhibition of platelet deposition as a result of treatment with r-hirudin and the plasma concentration of r-hirudin at the end of the infusion period was exponential. The solution was $\% \text{ Inhibition} = 91(1 - e^{-0.23(r\text{-hirudin})})$.

bin generation because activated platelets promote thrombin generation considerably, and hirudin also prolongs the lag phase of the burst of prothrombin activation in recalcified platelet-rich plasma in a dose-dependent manner.⁷ Our results do not identify which of the two mechanisms was

responsible for the inhibition of platelet deposition. It is likely that both are in operation and that the relative contribution of each to inhibit platelet deposition can change during the treatment period. The lack of increase in the plasma levels of TAT-complexes (Table I) can either be

Table 1. The aPTT and the Plasma Concentration of Thrombin-Antithrombin III (TAT) Complexes in the Baboons Where Thrombogenic Devices Were Used (Values are given as a mean $\pm$ 1 SD.)

Measurement	Dosage $\mu\text{g/kg/min}$	Time (min)			
		0	40	85	115
aPTT (s)	140	42 $\pm$ 6	>500	120 $\pm$ 6	101 $\pm$ 5
	70	38 $\pm$ 8	>500	97 $\pm$ 6	83 $\pm$ 7
	35	40 $\pm$ 9	194 $\pm$ 56	81 $\pm$ 8	74 $\pm$ 12
	14	39 $\pm$ 4	119 $\pm$ 17	63 $\pm$ 6	54 $\pm$ 7
TAT complexes ($\mu\text{g/mL}$)	140	7 $\pm$ 1	4 $\pm$ 2	—	11 $\pm$ 3
	70	9 $\pm$ 6	5 $\pm$ 3	—	13 $\pm$ 6
	35	8 $\pm$ 3	6 $\pm$ 2	—	20 $\pm$ 8
	14	12 $\pm$ 4	8 $\pm$ 3	—	32 $\pm$ 10
	Control	4 $\pm$ 1	—	—	123 $\pm$ 16

the result of decreased generation of thrombin because of decreased platelet activation⁷ or the result of sufficient hirudin available to bind to the generated thrombin.⁴ Of interest is the finding that platelet deposition was not inhibited at the lowest dose of hirudin (Fig. 1) despite the fact that the plasma levels of TAT-complexes remained unchanged during treatment (Table I). This may be due to activation of platelets through nonthrombin mechanisms,¹⁷ platelet activation by endogenous thrombin⁷ that was not accessible to hirudin because of low plasma levels of hirudin, or the procoagulant activity of factor Xa in the presence of hirudin.²¹ One must also bear in mind that the minimum concentration of thrombin to initiate platelet activation is much lower than that required to rapidly clot plasma.⁷

The relationship between the magnitude of inhibition of platelet deposition and the plasma concentration of hirudin fitted an exponential association function best (Fig. 2). It is important to note that the asymptote was 91% (i.e., maximum predicted inhibition of platelet deposition that can be attained). Because a thrombus was allowed to form for 10 min, 100% inhibition cannot be achieved. In fact, the number of platelets deposited after 10 min was approximately 7% of maximum deposition in the control studies. The relationship predicts that 50% inhibition of platelet deposition will occur at a plasma concentration of approximately 3.3 $\mu\text{g}/\text{mL}$ and 80% inhibition at approximately 8.1 $\mu\text{g}/\text{mL}$. These estimates can be used to calculate the rate of infusion of hirudin to maintain a plasma concentration that will result in a given inhibition of platelet deposition, i.e.

$$\begin{aligned} \text{Infusion} &= \text{Clearance (ml/min)} \\ &\quad \times \text{plasma concentration } (\mu\text{g/ml}) \\ &= (\ln 2/t_{1/2} \times \text{plasma volume}) \\ &\quad \times \text{plasma concentration} \quad (2) \end{aligned}$$

where $t_{1/2} = 20$ min and plasma volume = $(1 - \text{hematocrit}) \times 65 \text{ mL/kg body mass}$.²² The infusion rate for a 10-kg baboon with a hematocrit of 0.45 will be approximately 40.7 $\mu\text{g}/\text{kg}/\text{min}$ to maintain a plasma concentration of 3.3 $\mu\text{g}/\text{mL}$ to inhibit platelet deposition by approximately 50%. Similarly, an infusion rate of 100 $\mu\text{g}/\text{kg}/\text{min}$ will be required to maintain the plasma concentration at 8.1 $\mu\text{g}/\text{mL}$ to inhibit platelet deposition by approximately 80%. The relationship and calculation of the infusion rates are based on the inhibition of thrombin generated by a thrombus having

a surface area of roughly 0.6 cm^2 . It remains an open question whether the values will be valid if thrombi are of a different size where more, or less, thrombin are generated.

When treatment with hirudin was stopped, platelets again deposited onto the thrombus surface (Fig. 1). Both the time from the end of treatment to the start of deposition and the rate at which platelets deposited were, to a large extent dependent on the dosage. In general, at higher doses the lag phase was longer and the rate of deposition slower. We have no ready explanation for this finding. It is possible that, at the high dose, enough hirudin infused into the thrombus to inactivate the thrombus-bound thrombin and that it also took longer for this hirudin to dislodge from the thrombin. Our results may also suggest that there is a threshold limit of thrombin-hirudin complex below which the inhibitory effect of this complex is not effective to inhibit platelet deposition after treatment was stopped. In another study we have shown that the combined effect of short-term inhibition of platelet deposition and a slower rate of deposition results in long-term benefits of a smaller thrombus with a reduced thrombogenic surface.^{10,12} We now show that the dose has an important effect on platelet deposition after treatment was stopped. It is not clear how this effect is brought about.

It is of interest to note that it took relatively long for TAT complexes to increase after treatment was stopped and that markedly less TAT complexes formed at the end of the study compared with the control studies, even at the low dose of hirudin. This indicates that less thrombin was formed after the treatment was stopped, implying a reduced capacity of the thrombus surface to generate thrombin. It is difficult to explain why TAT complex formation was delayed. It is unlikely that residual hirudin in plasma was responsible because these measurements were made 90 min after treatment was stopped, i.e., approximately after three half-lives of hirudin. It is likely that thrombus-bound hirudin will reduce the capacity of the thrombus to produce thrombin, a hypothesis that is supported by the finding that the amount of complex formed was indirectly related to the dosage, i.e., the plasma concentration of hirudin. It is conceivable to assume that, at higher doses, more hirudin will infuse into the thrombus and bind to thrombin. This can have important benefits with respect to restenosis. It was shown that thrombin modulates gene expression in cultures of endothelial and smooth muscle

cells, including the genes encoding platelet-derived growth factor, whose expression is increased after treatment with thrombin.^{5,23}

In summary, we have shown that hirudin inhibits platelet deposition onto a freshly formed thrombus in a dose-dependent manner at doses greater than 35 µg/kg/min. We were able to calculate, from the relationship between the plasma level of hirudin and the percent inhibition of platelet deposition, that a plasma level of 3.3 µg/mL will reduce platelet deposition by approximately 50%. Similarly, 80% reduction will be achieved at a plasma level of approximately 8.1 µg/mL. These relationship estimates pertain to inhibition of thrombin generated by a thrombogenic surface of 0.6 cm². It remains an open question whether it will also apply if more, or less, thrombin is generated.

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Transient Interruption of Arterial Thrombosis by Inhibition of Factor Xa Results in Long-term Antithrombotic Effects in Baboons S-49

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Summary

Recombinant tick anticoagulant peptide (r-TAP) is a potent and specific inhibitor of activated coagulation factor X which effectively interrupts *in vivo* arterial thrombosis during treatment. It is, however, uncertain if it also affects thrombosis after treatment is stopped. This was tested in a baboon model of arterial thrombosis where platelet deposition onto Dacron vascular graft segments, inserted as extensions into permanent femoral arteriovenous shunts, was measured. The baboons were intravenously treated with 10 µg/kg/min (low dose, aPTT = 39 ± 1 s) and 25 µg/kg/min (high dose, aPTT = 58 ± 2 s) r-TAP for two hours. During treatment the r-TAP inhibited thrombin formation and dose-dependently interrupted platelet deposition onto the graft segment. This effect lasted for up to two hours after treatment with the low dose. Following treatment with the high dose, the graft segments were kept in place for 53 h. After treatment was stopped, platelets again deposited, but at a much lower rate than in control studies. Maximum deposition was approximately 38% lower than in the control studies. Total platelet deposition over 55 h, calculated as the area under the deposition curve, was approximately 40% ($p < 0.05$) less than in the control studies. A significant shortening in the mean platelet life span and an approximately 15-fold increase in thrombin-antithrombin III complexes during the first 31 h indicated that the thrombus surface remained thrombogenic and that the effect of r-TAP was transient. We have shown that 2 h of treatment with a full antithrombotic dose of r-TAP markedly reduced both the rate of platelet deposition after treatment was stopped and the total number of platelets deposited over 55 h. This was in spite of the finding that the antithrombotic effect of r-TAP was transient.

Introduction

The propensity of thrombi, particularly when rich in platelets, to propagate and to induce activation of the coagulation system depends on the activity of thrombin bound to fibrin in a thrombus and the pro-coagulant activity of thrombus-associated factor Xa. Under these circumstances both these factors are protected from inhibition by anti-thrombin III (1, 2). This explains why heparin is relatively ineffective to inhibit arterial-type thrombosis where platelets predominate (3, 4). However, the direct inhibitors of thrombin and factor Xa are able to inhibit these thrombus-bound factors and so effectively inhibit arterial thrombosis (4, 5). As a result, there is considerable interest in the devel-

opment and testing of agents that can achieve inhibition of thrombin and its production.

Tick anticoagulant peptide, a 60-amino-acid protein isolated from the tick, *Ornithodoros moubata*, is a highly specific inhibitor of coagulation factor Xa (6). Its recombinant form, r-TAP, was characterised as a competitive, tight-binding reversible inhibitor of factor Xa (7). It has been shown that its use *in vivo* prevents arterial-type platelet-rich thrombus formation (5), maintains coronary artery patency following thrombolysis (8), and prevents venous thrombosis (9). However, it is not known if the antithrombotic effect exerts itself only during infusion, or, if r-TAP has an effect after treatment is stopped, similar to r-hirudin (10).

In this study, we investigated the efficacy of r-TAP as a potential antithrombotic drug. Special attention was paid to its role in suppressing thrombogenesis after systemic treatment with a full antithrombotic dose was stopped. A baboon model of arterial thrombosis (11) was used because the molecular and cellular composition and function of the haemostatic apparatus closely resembles that of humans (12). Thrombogenic devices consisting of Dacron vascular graft material were inserted as extension segments into chronic arteriovenous (AV) shunts (11, 12). In short-term studies, the effect of two doses of r-TAP on platelet deposition was investigated. In long-term studies, the animals were systematically treated for 2 h with a full antithrombotic dose of r-TAP, starting at the time of placement of the thrombogenic device. Platelet deposition, measured in real time as the deposition of In-111-labelled platelets, onto the device was assessed for 4 h after treatment was stopped and twice daily for 3.5 days.

Materials and Methods

Animals Studied

Twenty normal male baboons (*Papio ursinus*) were used. The animals weighed 10-15 kg and were disease-free for at least 6 weeks prior to use. The peripheral blood platelet count, $315 \pm 111 \times 10^9/l$, was normal. All procedures were approved by the Ethics Committee for Animal Experimentation of the University of the Orange Free State in accordance with Federal Guidelines (Guide for the Care and Use of Laboratory Animals, Public Health Service National Institutes of Health publication number 85-23, 1985). The baboons supported permanent Teflon-Silastic AV shunts implanted in the femoral vessels (11). These shunts do not detectably shorten platelet survival or produce measurable platelet activation (11-13). In addition, we used four normal baboons without AV shunts to measure the mean platelet life span.

Study Protocol

The experimental set-up and schematic representations of the thrombogenic devices that were used in this study is illustrated in Fig. 1.

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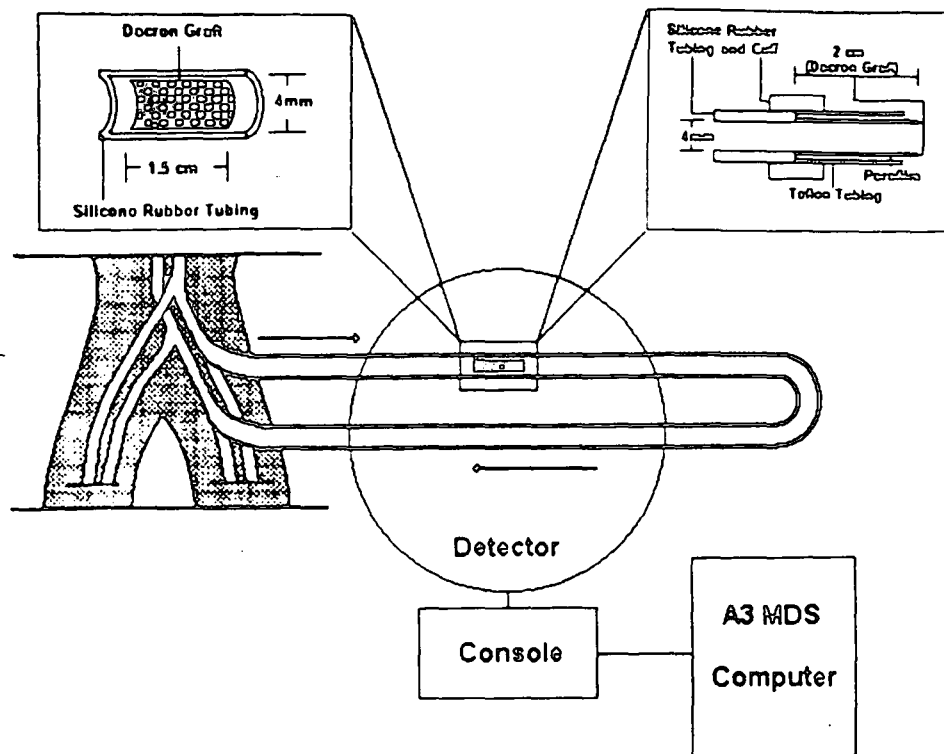


Fig. 1 A schematic representation of the experimental set-up to study platelet deposition. The enlarged device on the left was used for the long-term studies and that on the right for the short-term studies

Short-term studies. Eight baboons were used. Platelet dependent, arterial type thrombus formation was started using uncrimped, knitted Dacron vascular graft material (4 mm inside diameter, 2 cm long) that was built into the wall of silicone rubber tubing (4 mm inside diameter). The method of preparation of the thrombogenic device is described in detail (11). In each experiment, a thrombogenic device, prefilled with saline to avoid a blood-air interface, was incorporated as an extension segment into the permanent AV-shunt by means of teflon connectors (11, 12). A control study was done where the devices were placed until it occluded. When occluded, the devices were removed and blood flow through the permanent shunt re-established. r-TAP (Corvas International, Gent, Belgium) was then systematically infused at a rate of 25 $\mu\text{g/kg/min}$ ($n = 4$, high dose) or 10 $\mu\text{g/kg/min}$ ($n = 4$, low dose) for two hours. Sixty minutes after treatment was started, a thrombogenic device was placed for 180 min, where after it was removed and blood flow through the permanent shunt re-established.

Long-term studies. Twelve baboons were used. Thrombus was generated using uncrimped, knitted Dacron vascular graft material (0.5 cm^2) that was built into the wall of silicone rubber tubing (4 mm inside diameter). The method to construct the thrombogenic device is described in detail (14). We decided to use this device because we have shown that it does not occlude as frequently as the device used in the short-term studies (10), mainly because the surface area to which the blood is exposed is much smaller. The thrombogenic devices were placed and kept in place for 55 h in both untreated ($n = 6$) and treated animals ($n = 6$). In the treatment studies, r-TAP was systematically infused at a rate of 25 $\mu\text{g/kg/min}$ for 2 h. Treatment was started simultaneous with placement of the thrombogenic devices.

In all the studies, the starting blood flow rate through the devices were 100-140 ml/min, producing wall shear rates similar to that found in medium to large sized arteries (12). The r-TAP was dissolved in saline and was infused into the tubing of the A-V shunt distal to the thrombogenic device.

Graft Imaging and Quantification of Platelet Deposition

Autologous blood platelets were labelled with ^{111}In -tropolone as previously described (15). Imaging and quantification of the deposition of ^{111}In -platelets were done as described in detail (11, 14). Briefly, image acquisition of the

grafts, including proximal and distal silastic segments, was done with a Searle Pho scintillation camera fitted with a high resolution collimator. The images were stored on and analysed with a Medical Data Systems A³ computer (Medtronic, Ann Arbor, MI) interfaced with the scintillation camera. Dynamic image acquisition, 10 min images (128 $\times$ 128 byte mode) for 3 h in the short-term and 6 h in the long-term studies, was started simultaneously with the start of blood flow through the devices. For the long-term studies, static images of 10 min (128 $\times$ 128 byte mode) were acquired twice daily until the end of the study. A ten-minute image (128 $\times$ 128 byte mode) of a 3 ml autologous blood sample was also acquired each time the grafts were imaged to determine circulating blood radioactivity (blood standard). A region of interest of the graft segment was selected to determine the deposited and circulating radioactivity in each of the dynamic and static images. Radioactivity in a region of similar size of circulating radioactivity in the proximal segment of the extension tubing was determined. This was subtracted from the radioactivity in the graft region to calculate deposited radioactivity. In the short-term studies, the total number of platelets deposited was calculated. This method is described in detail (11). In the long-term studies, deposited graft radioactivity was expressed in two ways. First, it was expressed as a percentage of total injected ^{111}In -platelet radioactivity and second as a percentage of the labelled platelets circulating at the time of imaging, i.e. at the time platelet deposition was determined. These methods are described in detail (10).

In addition to the 12 baboons used in the long-term studies, the disappearance of ^{111}In -labelled platelets from the circulation was also determined in 4 normal baboons, and the mean platelet life span (MPLS) calculated using the multiple hit model. The methods are described in detail (16, 17).

Laboratory Measurements

The times at which blood samples were collected for the different determinations can be seen in the results section. The platelet count and haematocrit blood samples collected in 2 mg/ml disodium EDTA were determined using Technicon H2 blood cell analyser (Bayer Diagnostics, Tarrytown, NY). The same blood sample was used to determine whole blood and plasma radioactivity. Blood radioactivity was corrected by subtracting plasma radioactivity in order to calculate platelet radioactivity (11, 12). The radioactivity in the dif-

ent samples were determined in a well-type scintillation counter. Blood was also collected in 3.8% sodium citrate (9 volumes blood to 1 volume citrate) to determine activated partial thromboplastin time (aPTT), thrombin time (TT) and the plasma levels of the thrombin-antithrombin III (TAT) complex. aPTT and TT was measured on a fibrinometer (Clotex II, Hyland Division, Travenol Laboratories, Costa Mesa, CA) using reagents supplied by the manufacturer. TAT complexes were determined using an ELISA-method (Enzygnost Combi-pack, Behring, Marburg, Germany) as recommended by the suppliers.

Statistical Analysis

Student's test (two-tailed) for paired or unpaired data was used to test for differences when the data were normally distributed. If not, the Mann-Whitney U-test for nonparametric data was used. Data in the text are given as the mean $\pm$ SD.

Results

Short-term studies. The results of platelet deposition are summarised in Fig. 2. In the untreated animals platelets were rapidly deposited and all the thrombogenic devices occluded between 25 and 50 min (mean = 39 ± 10 min) following their placement. In the case of treatment with the high dose r-TAP, some platelets deposited during the first 10 min following placement of the devices. Thereafter platelet deposition was interrupted and a plateau in deposition was reached. No platelets deposited after treatment was stopped and at the end of the study, i.e. two hours after treatment was stopped, the number of platelets deposited were approximately 93% less than that measured in the untreated animals. Treatment with the low dose did not completely prevent platelet deposition. Platelets were however deposited at a much slower rate than in the untreated animals and deposition reached a plateau approximately 30 min after treatment was stopped. At the end of the study, approximately 60% less (versus untreated animals) platelets were deposited. Treatment with both dosages prevented occlusion of the devices for up to two hours after the treatment was stopped.

The changes in platelet count, aPTT and TAT complexes are summarised in Table 1. TT was not affected by treatment and the results are therefore not given. There was a decrease of approximately 18% in the platelet count of the untreated animals. In the case of the high dose r-TAP, the platelet count was not affected, even after treatment was stopped. The platelet count decreased only slightly during treatment with the low dose. This was followed by a slightly larger decrease when treatment was stopped. The aPTT was only affected by

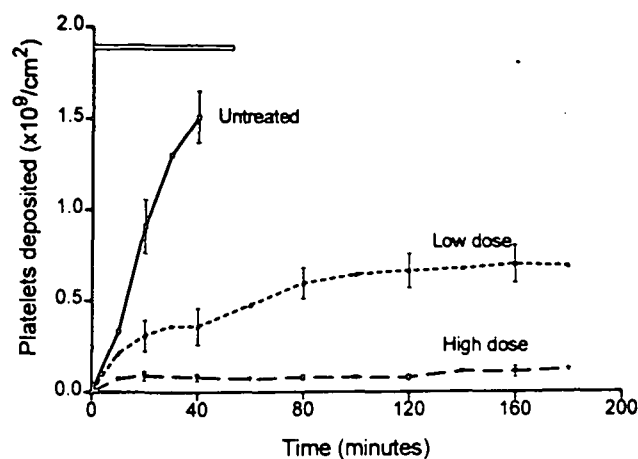


Fig. 2 Platelet deposition onto the thrombogenic devices in untreated animals and the effect of treatment with 10 and 25 μ g r-TAP/kg/min on platelet deposition. The solid bar represents the time of infusion. Values are given as a mean $\pm$ 1 SEM

the high dose r-TAP. It almost doubled during treatment, but returned to approximate baseline 2 h after treatment was stopped. An eightfold increase in the plasma levels of TAT-complexes was measured in the untreated animals. During treatment with either dose, the TAT-complex levels did not change markedly. In the two hours following treatment, the levels almost doubled in the animals receiving the high dose. In the case of the low dose, the levels increased by a factor of four.

Long-term studies. The results on platelet deposition are given in Fig. 3. Figure 3A represents the time/radioactivity curves where deposited radioactivity was expressed as a percentage of total injected radioactivity. In the untreated animals platelet deposition was generally sigmoidal, reaching a maximum of approximately 2% 5-6 h after the thrombogenic devices were placed. Thereafter platelet deposition gradually decreased to approximately 0.5% of injected labelled platelets after 55 h. Platelet deposition was interrupted during treatment with r-TAP. When treatment was stopped, platelets were again deposited. Maximum deposition, approximately 0.6% of injected labelled platelets, was reached between 6 and 23 h following placement. This was significantly less than in the untreated animals. Towards the end of the study, equivalent numbers of platelets were deposited onto the thrombogenic surfaces. Total platelet deposition, measured as the area under the curve, was 55 ± 15 h $\times$ % platelet deposition in the control studies and 33 ± 5 h $\times$ % platelet deposition in the treatment studies. Thus, approximately 40% less platelets were deposited in the treated animals. This difference was statistically significant.

Figure 3B represents the deposition curves where deposited radioactivity was expressed as total circulating radioactivity at the time each image was acquired. By expressing the results this way, the extent of deposited platelet radioactivity relative to circulating platelet radioactivity is given. In both the control and treatment studies the ratio remained relatively constant after maximum deposition was reached, indicating that deposited radioactivity decreased almost in parallel with circulating radioactivity. The ratio in the treatment studies was markedly lower than in the control studies. This also indicates that less platelets deposited after treatment with r-TAP was stopped.

Changes in aPTT and TAT-complexes are summarised in Table 2. TAT-complexes were not measured in the untreated animals. Treatment with 25 μ g/kg/min r-TAP almost doubled the aPTT. Plasma levels of TAT-complexes increased markedly during the first five

Table 1 Changes in platelet count and aPTT and plasma levels of thrombin-antithrombin III complexes in the untreated animals (n = 8) and in the animals that were treated with 25 (n = 4) and 10 (n = 4) μ g r-TAP/kg/min for 2 h

Study	Time (min)	Platelet count (% baseline)	aPTT (sec)	TAT (μ g/l)
Untreated animals	Pre	100	36 $\pm$ 3	10.6 $\pm$ 6.1
	Post	82 $\pm$ 9	38 $\pm$ 3	82.6 $\pm$ 18.0
25 μ g/kg/min	Pre	100	40 $\pm$ 2	5.5 $\pm$ 1.9
	60	103 $\pm$ 4	67 $\pm$ 5	3.9 $\pm$ 2.0
	180	105 $\pm$ 3	48 $\pm$ 3	9.3 $\pm$ 5.3
10 μ g/kg/min	Pre	100	35 $\pm$ 3	4.0 $\pm$ 0.9
	60	97 $\pm$ 4	39 $\pm$ 1	6.3 $\pm$ 1.0
	180	92 $\pm$ 5	35 $\pm$ 1	17.7 $\pm$ 10.6

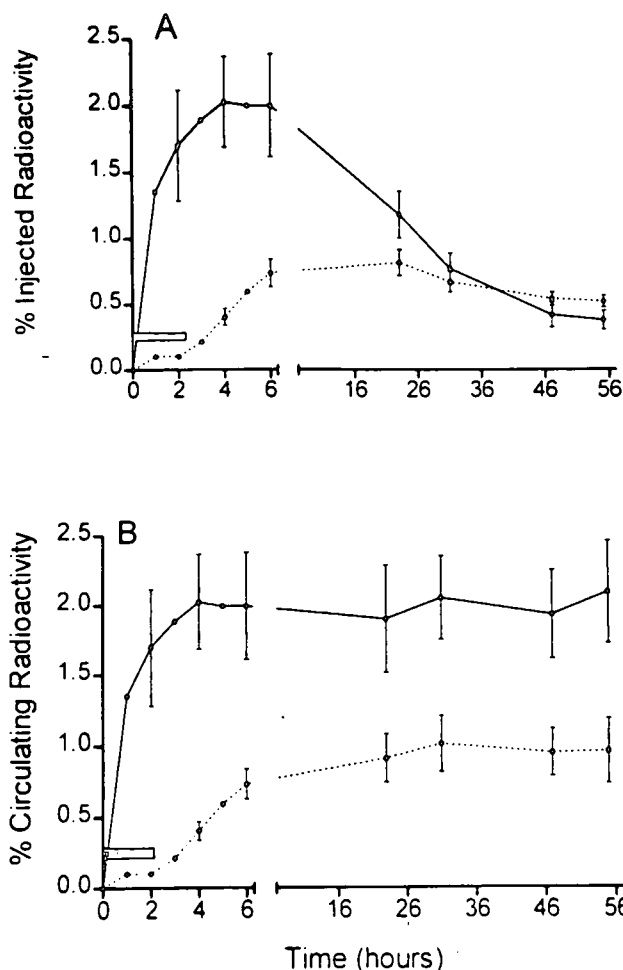


Fig. 3 Platelet deposition onto the thrombogenic devices in untreated ($n = 6$) and animals treated with $25 \mu\text{g r-TAP/kg/min}$ ($n = 6$). In A, the deposited radioactivity was expressed as a percentage of total injected radioactivity and in B, as a percentage of circulating radioactivity at the time of imaging. The solid bar represents the time of infusion. Values are given as a mean $\pm$ 1 SEM

minutes after placements of the thrombogenic devices. Thereafter, treatment decreased the levels to lower than pre-study values, and this was still observed 4 h after treatment was stopped. The TAT-complex levels then increased to reach a maximum approximately 31 h after the devices were placed. At the end of the study the levels approximated pre-study levels.

The MPLS in the normal animals (138 ± 14 h) was similar to that reported for normal baboons (17). In the untreated animals the MPLS (54 ± 34 h) was markedly and significantly ($p < 0.01$) shorter than in the normal animals. The MPLS in the treated animals (85 ± 34 h) was also significantly ($p < 0.05$) shorter than in the normal animals. The mean difference in MPLS between the treatment and control studies, 31 ± 58 h, was not significantly different ($p > 0.05$). The 95% confidence interval from -6 to 68 h however, suggested that the MPLS was slightly longer in the treatment studies.

Discussion

In the short-term studies we confirmed that systemic treatment of baboons with r-TAP dose-dependently inhibits high shear induced platelet deposition onto Dacron vascular graft segments and prevents

Table 2 Changes in APTT in the treated and untreated animals and the plasma concentration of thrombin-antithrombin III complexes in the treated animals used in the long-term studies. Values are given as a mean $\pm$ 1 SD

Time (hours)	APTT (sec)		TAT complexes ($\mu\text{g/l}$)
	Treated	Untreated	
0	33 $\pm$ 2	36 $\pm$ 4	16 $\pm$ 8
0.08			122 $\pm$ 81
2	58 $\pm$ 2*		6 $\pm$ 2
6	44 $\pm$ 2*	39 $\pm$ 1*	9 $\pm$ 4
23	35 $\pm$ 3	41 $\pm$ 3*	47 $\pm$ 21
31	37 $\pm$ 2*	39 $\pm$ 2*	157 $\pm$ 52
47	34 $\pm$ 3	39 $\pm$ 5	68 $\pm$ 56
55	33 $\pm$ 2	38 $\pm$ 8	27 $\pm$ 36

* $p < 0.05$, Student's t-test for paired data

graft occlusion (3). This antithrombotic effect was associated with only a moderate prolongation of the ex vivo aPTT (Table 1). In addition, we showed that these effects lasted for up to two hours after treatment was stopped (Fig. 2). It is noteworthy that platelet deposition continued during treatment with $10 \mu\text{g/kg/min}$ of r-TAP, and that maximum deposition, approximately 40% of that measured in the untreated animals, was reached 30 min after treatment was stopped. In the case of treatment with $25 \mu\text{g/kg/min}$, maximum deposition, approximately 10% of that in the untreated animals, was reached 10 min after the devices were placed. Thereafter only a few additional platelets were deposited, even during the two hours after treatment was stopped.

At the high dose, platelets deposited during the first 10 min following placement of the devices. This happened in spite of the fact that the baboons were treated for 60 min before the devices were placed. Similar results were obtained when native Dacron graft segments, inserted as extension segments into permanent AV-shunts in baboons, were treated with the direct thrombin inhibitor, D-Phe-Pro-Arg CH_2Cl (13). Our results where thrombin production, measured as TAT-complexes (Table 1), was prevented together with those where the actions of thrombin was blocked suggest that thrombin may not play an important part in the initial phase of platelet activation and deposition onto native grafts. There is evidence that fibrinogen, the platelet membrane glycoprotein IIb/IIIa and neutrophils/monocytes mediate the initial deposition of platelets onto biomaterials (18, 19). In addition, platelets in blood anticoagulated with D-Phe-Pro-Arg CH_2Cl deposit onto biomaterials (19). Platelet activation to expose the Gp IIb/IIIa receptor to bind fibrinogen therefore proceed through non-thrombin mechanisms. However, it remains difficult to explain why this "initial" event lasted for approximately 10 min. It is possible that a dynamic equilibrium is reached between platelet deposition and dissolution of platelets from the thrombus, and this takes approximately 10 min to achieve. Thus, in spite of the fact that thrombin formation was inhibited, as measured by TAT-complexes (Table 1), non-thrombin mediated mechanisms (18-20) may have resulted in the activation and deposition of platelets onto the graft segments until a steady state of thrombus formation and dissolution was reached.

Platelets were deposited onto the vascular graft segment during treatment with the low dose of r-TAP (Fig. 2). This was observed in spite of the fact that the plasma levels of TAT-complexes remained unchanged (Table 1) indicating that there was very little thrombin present in the plasma. It is unlikely that non-thrombin mechanisms was responsible for platelet activation and deposition. If this was the case, similar deposition results would have been observed with the high dose.

It is possible that the dose of 10 $\mu\text{g/kg/min}$ was not sufficient to fully prevent activation of prothrombin. The thrombin that is formed may bind to fibrinogen in the thrombus (1, 2) and may recruit platelets into the growing thrombus. The fact that the plasma levels of TAT-complexes remained unchanged may indicate that all the thrombin that formed was incorporated into the thrombus. One must bear in mind that factor Xa may also have prothrombotic properties (2), and that this can also contribute to the recruitment of platelets into the thrombus. It is noteworthy that, in contrast to the untreated animals, platelet deposition reached a plateau approximately 30 min after treatment was stopped and that the rate of deposition to reach the plateau was markedly slower than in the untreated animals (Fig. 2). These findings may imply that the thrombus surface became less thrombogenic as a result of treatment. This assumption is strengthened by the increase of approximately 4.5 fold in TAT-complexes after treatment was stopped. The corresponding increase in the untreated animals, approximately eight-fold, was much higher (Table 1).

On the basis of the results of the short-term studies, especially with the high dose of r-TAP, we postulated that a longer treatment with the high dose of r-TAP may produce lasting benefits with regard to thrombus size and surface thrombogenicity. To test this hypothesis we used a smaller thrombogenic device than in the short-term studies. We showed that platelet deposition onto this device is generally sigmoidal and that it can be kept as an extension segment into the permanent AV-shunt for up to 60 h (10) because it does not occlude as frequently as the larger devices used in the short-term studies. Treatment was started simultaneously with placement of the thrombogenic device in order to simulate a situation where normal haemostasis is attained before the antithrombotic effect of r-TAP becomes apparent, i.e. it takes approximately 10 min following placement of the thrombogenic device before platelet deposition is interrupted. Such an approach can have important implications in patients undergoing cardiac or vascular surgery because it can limit excessive blood loss due to the treatment.

Platelet deposition in the long-term control studies was generally sigmoidal (Fig. 3A). Maximum deposition was reached after approximately 5 h, where after deposition gradually decreased towards the end of the study. Similar kinetics of deposition was obtained in patients with abdominal aorta aneurysms and Dacron vascular graft implants (21). However, it took much longer in these patients to reach maximum deposition. This indicates that a fresh thrombus is typically more robust than an older, stabilised one. In the treatment studies, platelets were deposited for the first 10 min following placement of the grafts. Thereafter platelet deposition was interrupted (Fig. 3A). The initial deposition has to be ascribed to the fact that thrombin formation was not blocked during the first minutes of treatment (Table 2) because the plasma concentration of r-TAP was low shortly after treatment was started. Thrombin was most likely incorporated into the thrombus (1, 22), which would invariably lead to the recruitment of additional platelets into the thrombus. Thrombin is now generally regarded as perhaps the most important *in vivo* factor that recruits additional platelets into a fresh thrombus (22). There are a few possible explanations why platelet deposition was interrupted 10 to 15 min after treatment was started. First, after 15 min of treatment the plasma concentration of r-TAP was probably sufficient to prevent thrombin generation and inhibit the role of factor Xa in thrombogenesis. Second, the recruitment of additional platelets into the thrombus over this initial period probably shielded the thrombus-bound thrombin and so made it ineffective to recruit more platelets. Third, it is possible that inhibition of factor Xa may be sufficient to inhibit thrombus growth in a thrombus rich in thrombin (2).

The finding that platelets were again deposited after treatment was stopped, but at a much slower rate than in the control studies (Fig. 3A), suggests that the thrombus surface was less thrombogenic. Maximum deposition, reached between 6 and 23 h, was significantly less than in the control studies. As a result, total platelet deposition over the 55 h of the study, measured as the area under the deposition curve, was approximately 40% less than in the control studies. Therefore, transient interruption of platelet deposition for 2 h led to the deposition of fewer platelets over the long-term. The mechanism through which this was achieved is unclear, especially in view of the fact that thrombin generation reached a maximum at 31 h and remained elevated until the end of the study (Table 2). Similar results were reported when direct thrombin inhibitors were used (10, 23), which suggests that both thrombin and factor Xa are equally important in thrombus growth.

After maximum deposition was reached in both the control and treatment studies, thrombus radioactivity decreased almost in parallel with circulating radioactivity (Fig. 3B). This strongly supports the hypothesis that a dynamic equilibrium existed between deposited and circulating platelets and that the rates of thrombus formation and dissolution were equivalent. Platelet consumption by the thrombus did therefore not stop, an assumption that is strengthened by the measurement of MPLS. The MPLS was significantly shorter than normal in both the control and treatment studies. A shortening in the MPLS is generally regarded as a sensitive measure of *in vivo* platelet activation and increased consumption due to thrombosis *in vivo* (24, 25). The finding that the MPLS tended to be longer in the treatment studies can suggest that the thrombus surface may have been less thrombogenic as a result of treatment. This assumption is supported by the finding that less platelets deposited after treatment was stopped (Fig. 2).

Our results indicate that the prevention of thrombin formation by inhibition of factor Xa with r-TAP dose-dependently inhibited platelet-dependent thrombus formation without adversely affecting the aPTT. We also showed that a long-term benefit of fewer platelets deposited and reduced initial (<24 h) and possibly later (>24 h) surface thrombogenicity was achieved when the baboons were treated for 2 h with a full antithrombotic dose of r-TAP. These benefits were achieved despite the finding that the effect of r-TAP was apparently transient and that after 31 h the thrombogenicity of the thrombus surfaces was similar in the treated and untreated animals. Our approach of short-term treatment with potent antithrombotic agents may be used to improve graft patency after implantation and to reduce the incidence of occlusion and restenosis after balloon angioplasty and endarterectomy.

Acknowledgements

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New approaches in the treatment of arterial thrombosis

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In the past decade, remarkable strides have been made in establishing the molecular principles that underlie arterial thrombosis, especially with regard to the pivotal role which the blood platelet membrane glycoproteins and thrombin play. Application of this knowledge, together with the rapid development in recombinant DNA technology and the ability to make monoclonal antibodies against specific epitopes on the membrane glycoproteins, have resulted in exciting new approaches to the treatment of arterial thrombosis.

This brief review focuses on selected aspects of arterial thrombosis and is intended to give an indication of the current status of experimental antithrombotic therapy. The first part will explain thrombosis, focusing on the aspects against which therapy is directed. It is followed by a section on the new approaches that are being followed to inhibit thrombus formation. It includes the use of monoclonal antibodies directed against platelet membrane glycoproteins, inhibition of the actions of thrombin by potent antithrombins and prevention of binding of adhesive proteins to the platelet membrane glycoproteins by peptides. Since fibrinolysis forms an integral part of thrombosis, a short summary of innovative approaches to fibrinolytic therapy is also included.

Thrombus formation

In vivo thrombus formation is a pathological consequence of alterations in the blood haemostatic mechanisms and/or vessel wall injury under variable flow conditions. The pathogenesis of arterial and venous thrombosis differs. Typically, an arterial thrombus is rich in platelets and poor in red cells and fibrin and develops in response to vessel wall injury

with activation of platelets and coagulation under high shear flow conditions. On the other hand, a venous thrombus is rich in fibrin and red cells and poor in platelets, and generally forms by activation of coagulation mechanisms in regions of stasis or low blood flow. Fig. 1 depicts the basic components of arterial thrombosis. Platelets and thrombin are important.

Platelets act by their ability to adhere to the subendothelium, i.e. platelet adhesion, to co-adhere to one another, i.e. platelet aggregation, and by providing a negatively charged surface for coagulation factor complexes to assemble to generate thrombin.^{1,4} Thrombin mediates fibrin formation, activates plasma proteins and stimulates platelets and endothelium.⁹

Platelet adhesion is the initial step following endothelial damage. Platelets adhere to the subendothelium through binding of the adhesive protein, von Willebrand factor

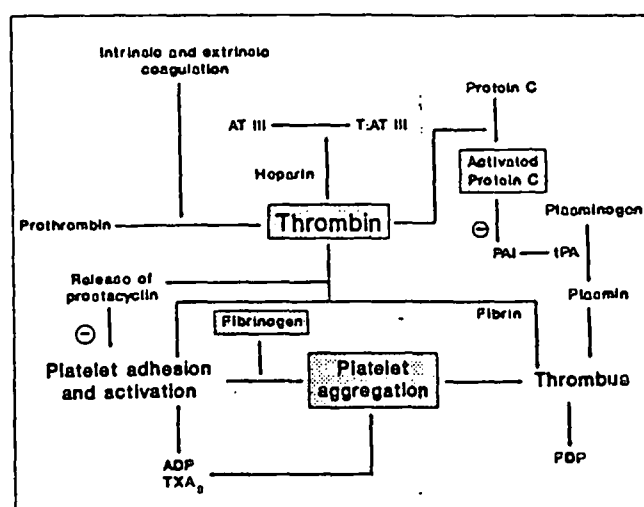


Fig. 1. A schematic representation of the events of arterial thrombosis (TxA₂ = thromboxane A₂; ATIII = antithrombin III; T:ATIII = thrombin : antithrombin III complex; tPA = tissue plasminogen activator; PAI = plasminogen activator inhibitors; FDP = fibrinogen degradation products).

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(vWF) to the glycoprotein (Gp) Ib/IX receptor complex in the platelet membrane. This binding only occurs in resting platelets,¹⁰ and adhesion via the Gp Ib/IX receptor to vWF only happens when vWF is bound to a surface in the sub-endothelium,^{11,12} presumably collagen.^{11,12} Glycolipids and/or heparin-like molecules may also act as subendothelial anchors for vWF.^{13,14}

The initial process of adhesion, in contrast to aggregation, does not require platelet metabolic energy.⁴ It leads, however, to activation of platelets, which synthesise thromboxane A₂ (TxA₂), and secrete their granule contents, both of which amplify platelet activation and recruit additional platelets to the thrombus.

Platelet aggregation requires an agonist such as adenosine diphosphate (ADP) or collagen to react with a specific membrane receptor which leads to the conversion of the membrane Gp IIb/IIIa into a receptor for fibrinogen. Von Willebrand factor, vitronectin and fibronectin are other adhesive proteins that can also bind to Gp IIb/IIIa.^{15,16} Fibrinogen mediates aggregation by binding to Gp IIb/IIIa on adjacent platelets in a reaction that is dependent on extracellular calcium.³

Platelets also have a procoagulant function. The activated platelet membrane serves as a surface for membrane-based coagulation reactions involved in the generation of thrombin, which cleaves fibrinogen to fibrin.¹⁷ Thrombin is generated through the concentrated action of the coagulation factors. The rate of generation is greatly increased in the presence of phospholipid,¹⁸ indicating that the formation of the coagulation factor complexes (tenase and prothrombinase) on platelet, or other cell membranes, is critical for thrombin generation.^{17,18} Thrombin, through binding to Gp Ib on the platelet membrane,¹⁹ activates and aggregates platelets. It stimulates the conversion of fibrinogen to fibrin, activates coagulation factors V, VIII, XIII and proteins C and S. Thrombin further stimulates the synthesis and secretion of prostacyclin, a potent inhibitor of platelet aggregation, from endothelial cells to contain the thrombus at the site of vessel wall injury.^{19,20} Thrombin is inactivated by its binding to antithrombin III. Heparin greatly enhances the activity of antithrombin III.

Antithrombotic therapy

Based on the knowledge of the molecular principles that underlie thrombus formation, it seems reasonable to assume that inhibition of platelet activation and fibrin formation may be an effective way to inhibit thrombosis. This will prevent expression of the aggregation receptors Gp IIb/IIIa on the platelet membrane and inhibit assembly of the coagulation factor complexes on the platelet membrane surface. This is achieved by either the prevention of binding of adhesive proteins to glycoprotein receptors, or inhibition of the function of thrombin by proteins that bind to its active sites. However, such an approach may lead to a serious bleeding disorder. Therefore, the ideal antithrombotic drug should maintain a balance between the prevention of thrombus formation and the precipitation of a bleeding disorder.

The measurements that are used to assess efficacy of treatment with the new-generation antithrombotics include

thrombin time (TT), activated partial thromboplastin time (APTT) and prothrombin time (PT), bleeding time, *in vitro* platelet aggregation tests and the *in vivo* deposition of platelets onto thrombogenic surfaces. In general, TT, APTT and PT are a measure of the functional status of the coagulation mechanisms. A lengthening in these times relates to the degree of anticoagulation attained. Bleeding time measures the ability of platelets to prevent excessive blood loss following vessel injury, and generally relates to the haemostatic mechanism. A lengthening in the bleeding time may therefore be regarded as antihæmostatic. *In vitro* platelet aggregation and platelet deposition onto thrombogenic surfaces measure the ability of platelets to aggregate and form a thrombus. Decreases in these measurements therefore relate to the antithrombotic effect obtained.

Monoclonal antibodies

The development of hybridoma technology has resulted in an extensive library of monoclonal antibodies that are directed against specific epitopes on the platelet membrane glycoproteins. A variety of these antibodies have been tested as potential antithrombotic agents.

Monoclonal antibodies against Gp Ib and vWF have been reported to lengthen the bleeding time and to inhibit *in vitro* the platelet aggregation in response to agonists.^{21,22} API, a monoclonal antibody against Gp Ib, caused immediate and profound thrombocytopenia when infused into baboons.²³ The lengthening in bleeding time, i.e. the antihæmostatic effect, in response to infusion of an anti-vWF antibody is not surprising, since platelet adhesion is blocked by this approach. However, there is evidence that collagen binds directly to Gp Ia/IIa in the platelet membrane,²⁴ and that this in its own right may support platelet adhesion.²⁵ This, and the finding that other adhesive proteins can also support adhesion,⁴ explain why patients with von Willebrand's disease may develop atherosclerosis.

Much more attention has been given to the use of monoclonal antibodies against Gp IIb/IIIa as possible agents to treat arterial thrombosis.^{21,26-28} The major involvement of Gp IIb/IIIa relates to the spreading of platelets onto a thrombogenic surface and the incorporation of platelets into a growing thrombus. When infused, the majority of these antibodies cause a dose-dependent decrease in the blood platelet count.^{21,29,30} The onset, duration and magnitude of thrombocytopenia varies.³¹ Infusion of monoclonal antibodies typically inhibits *in vitro* platelet aggregation response to agonists,^{21,26-28} decreases the deposition of platelets onto thrombogenic surfaces,^{23,26,27,30} and lengthens the bleeding time markedly^{21,26,27} but has little effect on PT and APTT.³² These effects and their duration are dose-dependent.^{21,26,27}

These results demonstrate that infusion of antibodies directed against Gp IIb/IIIa may significantly impair both platelet haemostatic function and the ability of platelets to participate in thrombus formation.²⁶⁻²⁸ Their use may be complicated by either acute or delayed thrombocytopenia. These antibodies may also cause an increased bleeding tendency.

Apart from their potential as antithrombotic agents, these monoclonal antibodies are also used to isolate the membrane glycoproteins, to determine their amino acid sequence

and to identify the peptide sequences that are responsible for the binding of adhesive proteins involved in platelet adhesion and aggregation.

Agents that inhibit thrombin

Because of its crucial role in arterial thrombosis, inhibition of thrombin generation and/or thrombin activity is one of the main strategies being examined in designing antithrombotic agents. Under normal conditions, thrombin is inactivated through complex-formation with antithrombin III, a reaction potentiated by heparin.¹¹ The use of heparin, and low molecular weight heparins, is effective in venous-type thrombosis where fibrin and red cells predominate. In contrast, very high doses are needed to inhibit arterial thrombosis where platelets predominate.¹²

Hirudin, a 65-residue polypeptide extracted from medicinal leeches (*Hirudo medicinalis*), is the most potent inhibitor of thrombin in nature.¹³ In the past 5 - 6 years, exploitation of recombinant DNA technology has provided sufficient quantities of hirudin for experimental studies.¹⁴ Hirudin inhibits thrombin activity through reversible binding to the anion-binding exosite and to regions neighbouring the catalytic site that cleaves fibrinogen.⁹ The functional half-life when infused into humans and animals is approximately 1 hour.^{15,16} Infusion of recombinant hirudin lengthens the APTT and PT significantly^{17,18} without adversely affecting the bleeding time,¹⁹ decreases the deposition of platelets and fibrinogen on platelet-rich thrombi and collagen surfaces²⁰⁻²¹ and decreases the incidence of reocclusion of injured vessels.^{22,23} These effects are dose-dependent.^{15,21} Hirudin also inhibits *in vitro* aggregation of platelets with thrombin as agonist. No influence is exerted on the platelet reactions induced by ADP and collagen, and platelet adhesion is not impaired.²⁴⁻²⁶ Small peptides isolated from the C-terminal of hirudin have similar antithrombotic properties to the parent molecule^{23,27} suggesting that the antithrombotic effect is situated in the Hirudin 54 - 65 region that binds to the anion-binding exosite.²⁸ Hirudin is therefore an effective anticoagulant that reduces the deposition of platelets and fibrin on a thrombus, without adversely affecting the bleeding time. Since hirudin is a relatively large polypeptide, there is some concern regarding its antigenicity. However, it has been shown to be a weak immunogen.²⁹

The observation that treatment with hirudin has resulted not only in effective anticoagulation but also inhibition of platelet-dependent thrombus formation, without affecting the bleeding time adversely,^{19,21} is important. When platelets are activated, they synthesise TxA₂, which is secreted together with ADP. The action of TxA₂ and ADP appears to be important only in the initial stages of thrombus formation before thrombin is generated. This is supported by the finding that ADP- and collagen-induced platelet aggregation is not inhibited by hirudin and that platelet adhesion is not impaired.²⁴⁻²⁶ The contribution of these two agonists of thrombus formation is apparently very small once thrombin is formed, but nevertheless enough to maintain a normal bleeding time through effective platelet plug formation. It is also apparent that the coagulation mechanism *per se* makes no major contribution to maintaining primary haemostasis, thereby indicating that platelets are of major importance in this regard.

D-phenylalanyl-L-prolyl-L-argenyl chloromethyl ketone (FPRCH₂Cl) is a synthetic peptide derivative that binds irreversibly to the active site of thrombin.³⁰ The FPRCH₂-thrombin complex then binds to the high-affinity thrombin receptor on platelets, but does not activate them.^{13,31} FPRCH₂Cl also inhibits fibrin formation.^{30,31} Infusion into baboons abolishes platelet deposition onto native Dacron vascular grafts³² and on freshly formed thrombi.³³ A dose-dependent lengthening of the TT and bleeding time was recorded when FPRCH₂Cl was infused.³² The *in vitro* aggregation response of platelets collected during infusion was abolished when thrombin was used as agonist. Aggregation in response to ADP and collagen was, however, not affected.³² The anticoagulant effect of FPRCH₂Cl, as measured by the TT, returned to normal 15 minutes after infusion was discontinued. Although the bleeding time normalised within 15 minutes after infusion was stopped, inhibition of platelet deposition lasted for approximately 30 minutes.³² FPRCH₂Cl is therefore a potent antihemostatic and antithrombotic agent. Its use may result in a bleeding tendency. However, the anticipated bleeding tendency was not observed in baboons.^{30,32,33}

Agents that inhibit fibrinogen binding to platelets

The other important element of thrombosis is platelet-platelet interactions. Following activation, platelets bind to adhesive proteins (fibrinogen, vWF, fibronectin) through interactions with platelet Gp IIb/IIIa.¹³ Fibrinogen binding mediates platelet aggregation,³⁴ which can also be supported by vWF.³ Fibronectin and vWF may be involved in platelet GP IIb/IIIa adhesion and spreading of platelets at sites of vessel injury.^{13,34} The adhesive proteins interact with platelet Gp IIb/IIIa through a common arginine-glycine-aspartic acid (RGD) peptide recognition sequence.¹ A synthetic RGD-containing peptide, arginine-glycine-aspartic acid-valine (RGDV) selectively inhibits platelet thrombus formation in a dose-dependent manner, without affecting the bleeding time.³⁵ By substituting the aspartic acid with glutamic acid (RGEV), the antithrombotic activity was abolished.³⁶ Thus, the RGD-recognition sequence on adhesive proteins is highly selective for Gp IIb/IIIa, and is currently a focal point of research into antithrombotics.

In this regard, two avenues of investigation are being explored: firstly, the synthesis of peptides representing the carboxy-terminal residues of the gamma chain of fibrinogen that contain the RGD sequence,^{37,38} and secondly, the isolation of peptides from snake venoms that have antithrombotic properties.^{39,40} All the synthetic and isolated peptides inhibit *in vitro* platelet aggregation with different efficiencies. Very few have been tested for their *in vivo* antithrombotic effects. Applaggin, from snake venom, inhibits *in vivo* platelet thrombus formation significantly and markedly lengthens the bleeding time.⁴² A cause for concern is that infusion of this peptide may cause transient thrombocytopenia,⁴² which in its own right may result in a serious bleeding disorder.

Research into the use of inhibitors of platelet Gp IIb/IIIa receptors is still at an early stage, and it will be interesting to see whether agents which inhibit platelet thrombus for-

mation without affecting the circulating platelet count and bleeding time will be found.

Fibrinolysis

Fibrinolysis is a naturally occurring process that degrades fibrin, the principal component of the structural lattice of a thrombus. The pivotal enzyme, plasmin, is formed by the action of plasminogen activators on plasminogen. The natural activator, tissue plasminogen activator (tPA), is under control of the plasminogen activator inhibitors (PAI). When a thrombus is formed, thrombin activates protein C (APC) in the presence of thrombomodulin in adjacent endothelium. APC inhibits PAI with a resultant increase in tPA activity and increased formation of plasmin (Fig. 1). Of interest is the finding that APC inhibits platelet-dependent thrombus formation without affecting the bleeding time.⁶¹ This action may be directly through inactivation of PAI, or indirectly by inhibition of thrombin generation through inactivation of coagulation factors V and VIII.⁶²

Plasminogen activators have been shown to be effective therapeutic agents in coronary artery thrombosis.^{63,67} First-generation activators (streptokinase and urokinase) are compounds found in nature and lack fibrin selectivity. The use of these activators is invariably accompanied by a marked depletion of fibrinogen caused by the generation of excess plasmin.⁶⁷ Second-generation plasminogen activators (tPA and single-chain urokinase-like plasminogen activators) are natural human proteins that possess some fibrin selectivity. However, their half-lives are very short.⁶⁷ Both generations of plasminogen activators have at times caused an extensive loss of fibrinogen and coagulation factors V and VII with resultant serious haemorrhage.^{67,68}

As a result various innovative approaches are currently being followed to produce third-generation plasminogen activators. The main aim is to develop activators that have optimal properties with regard to both recognition of the target components of a thrombus and increased duration of action.^{69,71} These third-generation plasminogen activators include those that have been modified by recombinant DNA technology and those which have increased fibrin selectivity. Plasminogen activators that are modified by DNA technology are formed by domain deletion and site-directed mutagenesis to increase either their fibrin selectivity⁷⁰ or half-life.⁷¹ Fibrin selectivity was increased by the addition of fibrin-binding domains, combining activators with fibrin-specific monoclonal antibodies, or by producing recombinant hybrid molecules containing an antifibrin antibody and the active site of the plasminogen activator.^{69,71} These activators have yet to be tested *in vivo*.

Implications and future directions

We are entering an era where treatment of arterial thrombosis may become effective. The basic knowledge of the molecular mechanisms that underlie thrombosis is being applied to develop agents that are antithrombotic. Inhibition of the two pivotal events of thrombosis, thrombin function and platelet aggregation, inhibits platelet-dependent throm-

bus formation in a dose-dependent manner. The dose-dependency of the effects is important. It will allow one to choose the 'level' of antithrombotic activity needed and design the treatment protocol accordingly. Unfortunately, several of these agents are also antithaemostatic, and lengthen the bleeding time markedly. Their use may therefore result in a bleeding disorder. However, because of their short half-life, the antithaemostatic action may be of short duration, as was found with FPRCH₂Cl.⁵² RGD-containing peptides that inhibit platelet aggregation may be preferable. RGDV peptide inhibits platelet-dependent thrombus formation without a simultaneous lengthening of the bleeding time and inhibition of ADP and collagen-induced platelet aggregation.⁵⁵ However, a suitable RGD-containing peptide with effects similar to the RGDV peptide has not yet been identified.^{52,56-62} Activated protein C seems to be a very promising antithrombotic agent that does not have antithaemostatic effects.⁶³

The short half-life of these antithrombotics obviously precludes their prophylactic use. Since most of the agents show almost immediate and profound interruption of platelet-dependent thrombus formation, they may be candidates for potent, immediate and transient inhibition of arterial thrombosis. However, there are preliminary results that indicate that short-term interruption of thrombus formation with either hirudin or FPRCH₂Cl may limit subsequent thrombosis after treatment is stopped.^{60,72}

Possible uses of these drugs include prevention of reocclusion in patients with acute myocardial infarction who have undergone successful reperfusion of coronary arteries by thrombolysis, and the prevention of occlusion in patients undergoing angioplasty or endarterectomy.

A novel approach to overcome the short half-life of antithrombotic agents might be to transform endothelial cells to synthesise and secrete antithrombotic peptides. These cells could be implanted into de-endothelialised arteries or used to seed vascular grafts to be implanted. This approach would deliver small amounts of the antithrombotics to sites where they are required for a relatively long period. A comparable approach has been used with endothelial cells synthesising recombinant β -galactosidase.^{73,74}

It is evident that molecular pharmacology will be employed to an ever-increasing extent in the treatment of arterial thrombosis. Extensive research is still needed, but the development of several new drugs requiring testing in humans can be anticipated.

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12 - 16 April

Annual scientific sessions of the American College of Cardiology, Dallas, Texas.

30 Aug - 3 Sept

14th Congress of the European Society of Cardiology, Barcelona, Spain. For registration and congress information, write to ECCO, 22, rue Juste Olivier, PO Box 299, CH-1260, Nyon/Switzerland.

16 - 18 September

5th Congress of the SA Society of Nuclear Medicine including sessions on Nuclear Cardiology, Nuclear Neurology, PET and Nuclear Medicine Physics. *Overseas participants* include Dr Terry Jones from Hammersmith Hospital, London, Head of the PET and Cyclotron Unit of the British Medical Research Council, and various other overseas experts have expressed interest. Venue: MRC Headquarters, Parow Valley. Contact person: Dr A. Ellmann, Department of Nuclear Medicine, Tygerberg Hospital 7505, Tygerberg; tel. (021) 931-1519/938-4372.

20 - 24 September

18th Biennial Congress of the Southern African Cardiac Society, Sun City, Bophuthatswana. The programme includes original presentation and state-of-the-art lectures on all major fields of cardiology.

Overseas participants:

Cary W. Akins, M.D. Associate Clinical Professor of Surgery, Cardiac Surgery Unit, Massachusetts General Hospital, Boston, USA.

Blase A. Carabello, M.D., Professor of Medicine, Medical University of South Carolina, USA.

Tirone E. David, M.D., Head, Division of Cardiovascular Surgery, Toronto Hospital, Canada.

Anthony N. De Maria, M.D., Professor of Medicine and Chief Cardiology Division, University of Kentucky, Kentucky, USA.

Bernard J. Gersh, M.D., Professor of Medicine, Cardiovascular Division and Internal Medicine, Mayo Clinic, Minnesota, USA.

Roger B. Mee, M.D., Victorian Paediatric Cardiac Surgical Unit, Royal Children's Hospital, Melbourne, Victoria, Australia.

Frans H. Messerli, M.D., Professor of Medicine, Ochsner Clinic, New Orleans, USA.

Craig D. Millar, M.D., Professor of Medicine, Department of Cardiovascular Surgery, Cardiovascular Research Centre, Stanford University, California, USA.

Peter C. Palrolero, M.D., Thoracic and Vascular Surgery, Mayo Clinic, Minnesota, USA.

Milton Packer, M.D., Professor of Medicine, Division of Cardiology, Mount Sinai Medical Centre, New York, USA.

Julian Rosenthal, M.D., Professor of Medicine, Pharmacotherapy Section, Clinical Pharmacology, ULM University, Donau, Germany.

Allan M. Ross, M.D., Professor of Medicine, Director, Division of Cardiology, The George Washington University Medical Centre, Washington, USA.

Melvin H. Sheinman, M.D., Professor of Medicine, Moffitt Hospital, University of California, San Francisco, USA.

Norman H. Silverman, M.D., Professor of Paediatrics and Radiology (Cardiology), University of California, San Francisco, USA.

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BRIEF TOPICAL APPLICATION OF SYNTHETIC ANTITHROMBIN PREVENTS VASCULAR GRAFT THROMBOSIS

Alan B. Lumsden, MB, ChB, Harry Kotze, PhD,
Stephen R. Hanson, PhD, and Lawrence A. Harker, MD

CURRENTLY, NO SATISFACTORY method exists by which antithrombotic therapy may be applied to sites of vascular injury. Consequently, systemic anticoagulation remains the only option for the prevention of occlusion at high-risk areas, such as vascular grafts, angioplasty sites, intravascular stents, and others. Unfortunately, anticoagulation following such interventions is associated with significant risk of hemorrhagic complications, which may have devastating consequences.

An ideal therapy would, therefore, provide an effective local antithrombotic action, active only at selected sites, without altering systemic hemostasis. We have produced a local antithrombotic effect, by topical application of D-phenylalanine-proline-arginine-chloromethylketone (D-FPRCH₂Cl), a nonreversible inhibitor of thrombin.¹

MATERIALS AND METHODS

Six young male baboons (8–13 kg), with a surgically implanted femoral arteriovenous shunt, were used in this study. Platelets in each animal were labelled with ¹¹¹indium.²

Uncrimped knitted (4 mm inner diameter) Dacron grafts, 2 cm long, were selected as the thrombotic surface for study.³ The grafts were rendered impervious to blood by wrapping with Parafilm and mounting into Silastic tubing. Once the graft device was assembled, it was preclotted using 5 cc of venous blood for 10 minutes. Excess clot was removed with a Fogarty catheter. The lumen of the treated graft was then filled with D-FPRGCH₂Cl (10mg/ml), controls were filled with normal saline solution, and each was incubated at room temperature for 10 minutes. Each graft was then irrigated with 10 cc of normal saline solution. The grafts were then exposed to pulsatile flow by incorporation into the arteriovenous circuit. Two grafts were studied in each experiment and mounted in a series, with the treated graft downstream of the untreated graft to eliminate any effect due to elution of D-FPRGCH₂Cl. Bleeding time, prothrombin time, and partial thromboplastin time were measured in each animal. Platelet deposition onto the grafts was measured using a Picker DC 4/111 Dyna Scintillation camera (Picker, Northford, CT).

From the Departments of Surgery and Hematology, Emory University, Atlanta, GA

RESULTS

Platelet deposition onto the untreated control grafts reached a plateau at 60 minutes with $5.7 \times 10^{-9} \pm 2.7$ platelets. Four of six untreated grafts occluded at 40, 50, and 60 minutes. The treated grafts continued to accumulate platelets up to 120 minutes, with a maximum platelet deposition of $2.87 \times 10^{-9} \pm 2.1$ platelets ($P < .01$). One of the six treated grafts occluded. Significant differences in the number of platelets deposited were evident at 15, 30, and 60 minutes; following this, the number of patent untreated grafts was inadequate to perform valid statistical interpretation. Bleeding time, prothrombin time, and partial thromboplastin times were all within normal limits.

DISCUSSION

D-FPRGCH₂Cl is a synthetic tripeptide antithrombin that produces covalent inhibition of thrombin by nonreversible alkylation of its active site. Thrombin has a central role both in inciting platelet aggregation and in the coagulation cascade. To exploit the profound antithrombotic activity of this agent, we hypothesized that topical application of D-PRGCH₂Cl onto preformed thrombus may produce permanent interruption of the thrombotic process. This process may be mediated by irreversible inhibition of locally generated, clot-bound, thrombin. We have demonstrated that such topical anticoagulation results in a dramatic short-term antithrombotic effect.

Such a therapeutic scheme provides maximal antithrombotic activity at the site where it is most necessary, without producing systemic anticoagulation. We believe, therefore, that local therapy represents a new concept in antithrombotic therapy with a wide range of potential clinical applications. Numerous questions remain to be addressed: how brief an exposure period is necessary, what concentration is required, and will this influence development of neointimal hyperplasia?

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REGULAR ARTICLE

Production of a Recombinant Antithrombotic and Fibrinolytic Protein, PLATSAK, in *Escherichia coli*

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Abstract

The three main components involved in thrombosis and haemostasis are thrombin, platelets, and plasmin. Almost all inhibitors of thrombosis are focused either on the inhibition of thrombin or on the inhibition of platelets. We designed a construct using the fibrinolytic activity of staphylokinase, fused via a cleavable linker to an antithrombotic peptide of 29 amino acids. The peptide was designed to include three inhibitory regions: (1) the Arg-Gly-Asp (RGD) amino acid sequence to prevent fibrinogen binding to platelets; (2) a part of fibrinopeptide A, an inhibitor of thrombin; and (3) the tail of hirudin, a potent direct antithrombin. The amino acid sequence of the 29 amino acid peptide was reverse translated, and the gene was chemically synthesised and cloned into an expression vector as a 3' fusion to the staphylokinase gene. Gene expression was induced in *E. coli* Top 10 cells and the fusion protein, designated PLATSAK, was purified using metal affinity chromatography. The purified fusion protein significantly lengthened the activated partial thromboplastin time and thrombin time and inhibited the

amidolytic activity of thrombin. The fibrinolytic activity was almost equal to that of recombinant staphylokinase as measured with a thrombelastograph. Platelet aggregation was not markedly inhibited by PLATSAK, probably due to the unfavourable three dimensional structure, with the Arg-Gly-Asp sequence buried inside. Our results confirm that it is feasible to design and produce a hybrid multifunctional protein that targets various components of the haemostatic process. © 1998 Elsevier Science Ltd.

Key Words: Staphylokinase; Antiplatelet; Antithrombin; Recombinant

Platelets and thrombin both play pivotal roles in thrombogenesis whereas plasmin dissolves the thrombus. It is therefore not surprising that we have experienced a drastic increase in the design and development of new antithrombotic agents that can either inhibit the action of thrombin or prevent platelet aggregation, and fibrinolytic agents that can enhance fibrinolysis. There are potent new inhibitors of platelet function, whereas powerful inhibitors of thrombin function and production are currently under study. The platelet inhibitors include synthetic Arg-Gly-Asp (RGD)-peptides [1,2], snake venoms [3,4], and the F(ab')₂ fragment of the monoclonal antibody 7E3 [5]. Amongst the thrombin inhibitors are hirudin [6], hirudin fragments [7], the hirulogs [8], hirugen [9], and synthetic hirudin C-terminal peptides [10]. Inhibitors of thrombin production include recombinant tick anticoagulant peptide [11] and recombi-

Abbreviations: SAK, staphylokinase; Pr, primer; FXa, factor Xa; TT, thrombin time; aPTT, activated partial thromboplastin time; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyranoside; NTA, nitrilo-tri-acetic acid; ADP, adenosine diphosphate.

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nant activated protein C [12]. Collen and Lijnen [13] suggested that the effectivity of anticoagulant and antiplatelet agents could be enhanced by conjunctive use with thrombolytic agents like staphylokinase (SAK) or streptokinase. SAK is a 136 amino acid fibrinolytic protein produced by the bacterium *Staphylococcus aureus* which forms a 1:1 complex with plasmin(ogen). This complex then activates other plasminogen molecules [14].

For the purpose of this study, we designed an antithrombotic peptide of 29 amino acids that combines antithrombin and antiplatelet activity and used SAK to provide fibrinolytic activity. SAK was linked to the antithrombotic peptide through a sequence that can be cleaved by activated coagulation factor X. The factor Xa target sequence was introduced to enable release of the antithrombotic peptide *in vitro* and *in vivo*. The peptide was designed to include three inhibitory regions: (1) an amino acid sequence (RGD) derived from fibrinogen, which is essential for binding of fibrinogen to its receptor (Glycoprotein IIb/IIIa) on platelet membranes [15]; (2) a part of fibrinopeptide A (residues 8–16), an inhibitor of thrombin [16]; and (3) the C-terminal tail of hirudin (residues 54–65), a direct antithrombin [6]. We hypothesised that the construct, named PLATSACK (Platelet-Antithrombin-Staphylokinase), would prevent platelet aggregation, inhibit the action of thrombin and enhance fibrinolysis. In this paper we report on the construction, purification, and *in vitro* characterisation of this recombinant fusion protein.

1. Materials and Methods

1.1. Host Strain

Escherichia coli Top 10F' [mcr A, Δ (mrr-hsdRMS-mcrBC), ϕ 80 Δ lacZ Δ M15, Δ lacX74, deoR, recA1, araD139, Δ (ara, leu), 7697, galU, galK, λ^- , rpsL, endA1, nupG, F'] was used for cloning the recombinant genes and for propagation of plasmids. The same strain was also used as host for gene expression.

1.2. Enzymes and Chemicals

Restriction endonucleases, T4 DNA ligase, Taq polymerase and Chromozym TH were purchased from Boehringer Mannheim. Synthetic fibrinopeptide A (residues 8–16) and hirudin C-terminus (res-

idues 54–65) were obtained from Sigma. Chemicals for media were purchased from Difco. All chemicals used were of analytical grade.

1.3. Recombinant DNA Techniques

DNA techniques were performed according to the general criteria currently in use [17]. Fragments for cloning were purified from agarose gels using Qiagen purification kits. *E. coli* transformation were performed as described [18].

1.4. Cloning Strategy

The amino acid sequence encoding the antithrombotic peptide was reverse translated using optimal universal codons [19]. Two 60-mer oligonucleotides, designated primers 1 and 2 (Table 1), were chemically synthesised. These two primers overlap 22 base pairs and the lacking ends were filled in by PCR (Figure 1). The purified gene was subsequently cloned into the Sma I restriction site of pUCBM21 (Figure 2) and the resulting plasmid was designated pWB1. Using primers 2 and 3 (Table 1), the gene was amplified from pWB1 and cloned simultaneously with the SAK gene and the Factor Xa cleavage site into the Eco RI and the Sal I restriction sites of pWB6 (Figure 2). The Factor Xa cleavage site was constructed as two complementary oligonucleotides, primers 4 and 5 (Table 1). The newly constructed plasmid was designated pWBM3. To aid purification of the recombinant fusion protein, PCR was applied to add six consecutive histidine residues to the C-terminus of the protein, using primers 6 and 7 (Table 1). The newly constructed plasmid was called pWBM3H. A plasmid containing only the SAK gene was named pWBM and produced in parallel with pWBM3H. Gene expression was under control of the strongly inducible tac promoter, which can be induced by the addition of 2 mM isopropyl- β -D-thiogalactopyranoside (IPTG). To prevent the promoter from producing a low level of protein during the initial phases of growth, the cells were co-transformed with pREP4, which carries the lacI gene encoding the lac repressor. All constructs were sequenced to verify accurate gene synthesis.

1.5. Media and Cultivation

Following transformation, the cells were plated on SOB agar (2% tryptone, 0.5% yeast extract, 0.05%

Table 1. Summary of primers

Name	Sequence, 5' to 3'	Use
Primer 1 (60-mer)	AGAGGTGACTTCTTGGCTGAA- -GGTGGTGGTGTAGACCAGGT- -GGTGGTGGTAACGGTGAC	Upper strand of anticoagulant gene
Primer 2 (60-mer)	CGCTCGAGCTACAAGTATTCTT- -CTGGAATTTCTTCGAAGTCA- -CCGTTACCACCACCACCT	Lower strand of anticoagulant gene
Primer 3 (30-mer)	GCCCCGGGAGAGGTGACT- -TCTTGGCTGAAG	Primer 1 + Sma I restriction site
Primer 4 (36-mer)	CAAGGTTGTTATAGAAAAG- -AAATCGAGGGAAGGAG	Upper strand of FXa linker
Primer 5 (32-mer)	CTCCTTCCTCGATTTTCTT- -TTCTATAACAAC	Lower strand of FXa linker
Primer 6 (26-mer)	ACAGAATTCAGGAGGC- -CTCATATGTC	Forward primer for adding 6 his
Primer 7 (69-mer)	GCGGTCGACCTAGTGATG- -GTGATGGTGATGCAAGTA- -TTCTTCTGGAATTTCTTC- -GAAGTCACGTTACC	Reverse primer for adding 6 his

NaCl, and 2.5 mM KCl) containing the appropriate antibiotics. For cultivation purposes the cells were grown on TB medium (1.2% tryptone, 2.4% yeast extract, and 0.4% glycerol, pH controlled at 7 with 0.017 M KH_2PO_4 /0.072 M K_2HPO_4), containing 25 $\mu\text{g/ml}$ kanamycin and 100 $\mu\text{g/ml}$ ampicillin for maintenance of the plasmids. A 3-ml culture was

inoculated with a single colony and grown for three hours at 37°C in a shaking incubator. This starter culture was then used to inoculate a 200-ml working culture, which was allowed to grow to an OD_{600} of 0.5. Gene expression was subsequently induced by addition of 2 mM IPTG to the culture. Following growth for an additional hour, the cells were har-

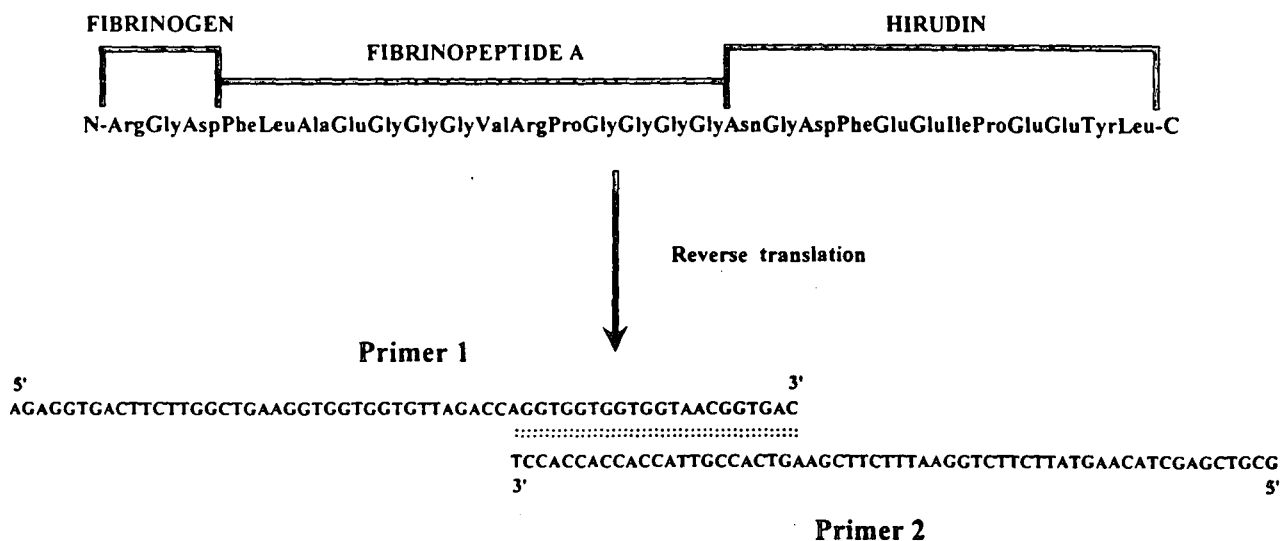


Fig. 1. The amino acid sequence of the antithrombotic peptide was reverse translated and two overlapping oligonucleotides (primers 1 and 2) were chemically synthesised. The gene was constructed by annealing the two primers and filling in the lacking ends by PCR. The resultant gene was subsequently cloned into pUCBM21 resulting in plasmid pWB1.

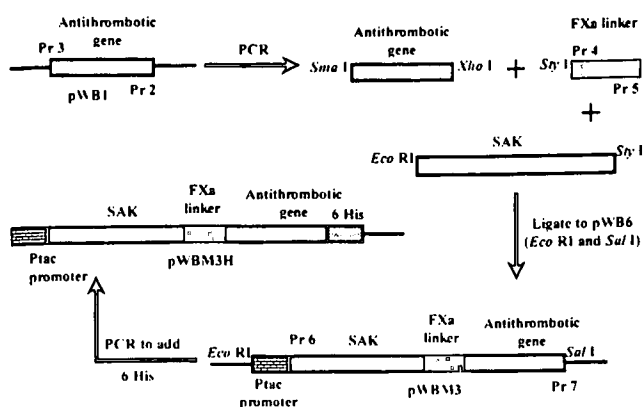


Fig. 2. A schematic representation of the strategy followed for construction of the PLATSAK gene. Cloning vectors are presented as linear figures. Pr, primers.

vested by centrifugation ($3000 \times g$ for 10 minutes). This procedure was also followed in parallel to produce SAK, which was also analysed in all activity assays to ensure that the SAK portion did not contribute to any antithrombotic or antiplatelet activity (results not shown).

1.6. Protein Extraction and Purification

Recombinant proteins were isolated by repeated cycles of freezing and thawing [20]. The cell pellets were frozen for ten minutes in liquid nitrogen and thawed on ice for fifteen minutes. This procedure was repeated twice. Two ml of sonication buffer (50 mM Na-phosphate (pH 8.0), 300 mM NaCl) was added to the cell pellets and left on ice for another hour. Cell debris was removed by centrifugation at 4°C for ten minutes at $10000 \times g$.

The protein extract was allowed to bind to Ni-NTA resin (Qiagen) for one hour at 4°C in a batchwise manner. The resin was left to settle in a column and was washed with sonication buffer until the OD_{280} was below 0.01. To remove other contaminating proteins, the resin was extensively washed with washing buffer [50 mM Na-phosphate (pH 6.0), 300 mM NaCl, 10% Glycerol] until the OD_{280} was below 0.01. The purified PLATSAK was eluted from the column with elution buffer (50 mM Na-phosphate (pH 4.0), 300 mM NaCl) and concentrated using Centricon-10 concentrators (Amicon). Protein concentrations were determined using the BCA Protein Assay system from Pierce. Proteins were separated on 15% SDS-PAGE gels and silver stained with the BDH Insta-view Silver Staining Kit.

1.7. Measurement of Antithrombin Activity

Thrombin time (TT) and activated partial thromboplastin time (aPTT), measured with standard assay systems, were used to test for antithrombin activity [21]. An amidolytic assay of thrombin activity, performed at 30°C using Chromozym TH (Boehringer Mannheim) as thrombin substrate, was also used [22]. The reactions were done in 0.1 M Tris-HCl (pH 8.3), containing 0.2 M NaCl and 0.05% Triton-X100, with 0.25 nM enzyme, 31.25 μg Tos-Gly-Pro-Arg-p-nitroanilide (Chromozym TH), and different concentrations of PLATSAK and SAK in a total volume of 250 μl . The reaction was followed over 60 minutes at 405 nm in an EL 312e Bio-kinetics microplate reader (Bio-tek Instruments). For purposes of comparison, the effect of individual peptides of fibrinopeptide A (residues 8–16) and the tail of hirudin (residues 54–65) on TT, aPTT, and the amidolytic activity of thrombin was also tested.

1.8. Measurement of Antiplatelet Activity

Platelet aggregation in response to 2 mM ADP (final concentration) was turbidimetrically measured in a Monitor IV Plus Platelet Aggregometer (Helena Laboratories) at 37°C , as described in detail [23]. The platelet count in platelet rich plasma was $200 \times 10^9/\text{l}$. The aggregation response, measured at 4 minutes following addition of ADP, was related to the difference in light transmission between platelet poor plasma and platelet rich plasma and expressed as a percentage. Before the ADP was added, the platelet rich plasma was incubated with different concentrations of PLATSAK as well as SAK.

1.9. Measurement of Fibrinolytic Activity

The fibrinolytic activities of PLATSAK and SAK were determined using a Hellige thrombelastograph. Human venous blood (4.5 ml) was collected in polystyrene syringes and anticoagulated with 0.25 ml tri-sodium citrate (9% w/v stock solution). The anticoagulated blood (250 μl) was calcified by the addition of 100 μl CaCl_2 (0.645% w/v working solution). Different concentrations of purified SAK or PLATSAK were immediately added to the reaction mixture and clot formation and subsequent

lysis due to SAK and PLATSAK activity were followed over time. The L-value, which spans the distance measured by the thrombelastograph from the start of clot formation to the end of lysis, was determined for each concentration used.

2. Results and Discussion

The gene encoding the antithrombotic peptide was constructed by reverse translating the synthetic amino acid sequence to the corresponding nucleic acid sequence, keeping optimal codon use in mind [19]. SAK was added not only to improve the stability of the small peptide, but to include digestion of fibrin to the range of activities carried by the construct. The two genes were separated by a cleavable linker, the Factor Xa target site (Figure 2). The fusion construct was cloned into pWB6 and gene expression was controlled by the strongly inducible *tac* promoter, using *E. coli* as expression host. Purification was facilitated by adding six histidine residues to the C-terminal part of the fusion protein. Six consecutive histidine residues have a very high affinity for Ni-ions associated with the Ni-NTA purification resin [24]. Transformants carrying the gene were cultivated and gene expression was induced by addition of IPTG to logarithmically growing cells. It resulted in the appearance of a protein band of approximately 18 kDa on SDS-PAGE gels (results not shown). Maximal protein production was reached after one hour of induction, after which the cells were subjected to freeze-thaw lysis [20]. This resulted in the preferential release of recombinant proteins from the frozen cell pellets. This method has the advantage of having fewer contaminating proteins that could interfere with the subsequent purification steps. Furthermore, it is a very simple, inexpensive and exceptionally efficient method of enriching recombinant proteins produced intracellularly by *E. coli*.

Further purification was performed using Ni-NTA resin. Protein extracts and column fractions were analysed on 15% SDS-PAGE gels and silver stained. Figure 3 shows the efficiency of the purification procedure used. The standard purification experiment yielded about 1 mg of recombinant PLATSAK per 200 ml culture. Of interest was the finding that the yield of PLATSAK, under similar conditions, was markedly lower than in the case of SAK (results not shown). It may be possible

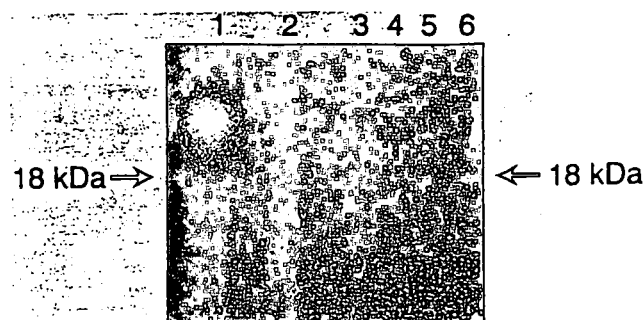


Fig. 3. SDS-PAGE analysis of protein purification procedure. *Lane 1*: total intracellular *E. coli* proteins; *Lane 2*: the enriched fraction obtained from the freeze-thaw lysis; *Lane 3*: the washing fraction from the Ni-NTA resin; *Lanes 4–6*: purified PLATSAK.

that the antithrombotic part of the fusion protein could have an inhibitory effect on protein production by *E. coli*. In spite of all precautions and the application of different elution procedures, a contaminating band of low concentration co-eluted with PLATSAK. The concentration of the contaminant is nevertheless so low that it is not visible in lanes 4 to 6 (Figure 3) and it apparently has not interfered with the subsequent assays.

The antithrombin properties of purified PLATSAK were investigated by measuring TT and aPTT (Figure 4). In both assays equal volumes of elution buffer served as control. Addition of 15 μ M of PLATSAK markedly lengthened TT and aPTT, thereby indicating inhibition of thrombin activity. SAK had no effect on TT and aPTT. The lengthening in TT and aPTT must therefore be due to the two antithrombin sequences present in the peptide fused to SAK. The mechanism of interaction of our peptide with thrombin is not clear and we have no data to show whether the antithrombin activity is due to the hirudin or to the fibrinopeptide A components. It can be assumed that the tail of hirudin binds to the anion binding exosite of thrombin [25,26] and the fibrinopeptide A sequence to the active site [16]. Since these two motifs are adjacent to each other in our construct (Figure 1), one would not expect a single molecule to bind simultaneously to the exosite and the active site. Residues 7 to 16 of fibrinopeptide A are critical to efficient peptide hydrolysis, while residues outside this region are critical for thrombin binding [27]. It appears that the amino acids outside the antithrombotic fragments of PLATSAK contribute to thrombin binding. In the chromogenic assay,

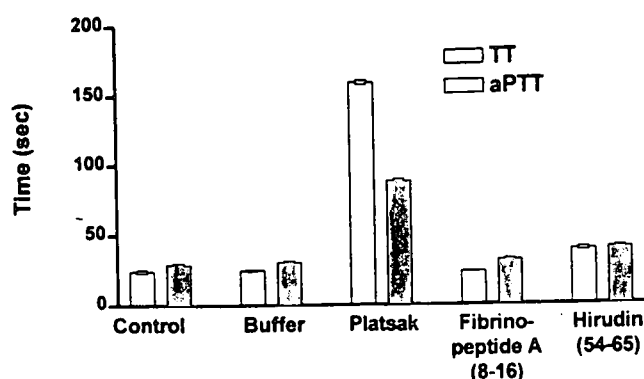


Fig. 4. Determination of antithrombotic activity using TT and aPTT. In both cases 15 μ M of PLATSAC, fibrinopeptide A (residues 8-16) or hirudin (residues 54-65) was used to assay the antithrombotic activity. Results are the average of three individual experiments.

5.5 μ M of the protein was sufficient to inhibit the action of thrombin significantly (Figure 5A). It is known that the C-terminal domain of hirudin is unable to inhibit the amidolytic activity of thrombin towards small substrates [9,28]. Inhibition of thrombin towards substrates like Chromozym TH is, thus, achieved by blocking the active site. In this experiment, thrombin inhibition was, thus, achieved either by the fibrinopeptide A derived part of the antithrombotic peptide binding to the active site or by blocking of the active site by the N-terminal part of PLATSAC, while the C-terminus was bound to the exosite.

It is important to note that PLATSAC markedly lengthened the TT and aPTT (Figure 4) and inhibited the amidolytic activity of thrombin (Figure 5) when compared to equimolar concentrations of the antithrombin part of fibrinopeptide A and the tail of hirudin. This strongly suggests that combination of the fibrinopeptide A part and the hirudin-tail with SAK greatly enhances the antithrombin activity of PLATSAC. We have no ready explanation for this finding.

The RGD sequence is an essential part of all integrins, since it is responsible for binding of the integrin to the glycoprotein IIb/IIIa receptor on the membranes of activated platelets [29], and RGD peptides and monoclonal antibodies containing the RGD motif are effective inhibitors of platelet dependent thrombus formation [30,31]. We introduced the RGD motif at the N-terminus of our peptide (Figure 1) to facilitate antiplatelet activity.

The effect of PLATSAC on platelet aggregation was negligible (Figure 5B). At a concentration of 33 μ M, PLATSAC could not inhibit ADP induced platelet aggregation. On the contrary, 20 μ M of a synthetic RRRRRRRRRRGDV could prevent platelet aggregation induced by thrombin, ADP, collagen, and epinephrine [1]. We could not overcome this problem by prior in vitro digestion of PLATSAC with Factor Xa. There are several possible explanations for the inability of PLATSAC to inhibit platelet aggregation. It could be due to the inaccessibility of the RGD in the tertiary structure of the protein. It is also possible that the RGD motif is situated in non-optimal surroundings [1]. An alternative would be to place the RGD motif on the tip of a loop to increase its accessibility to activated platelets.

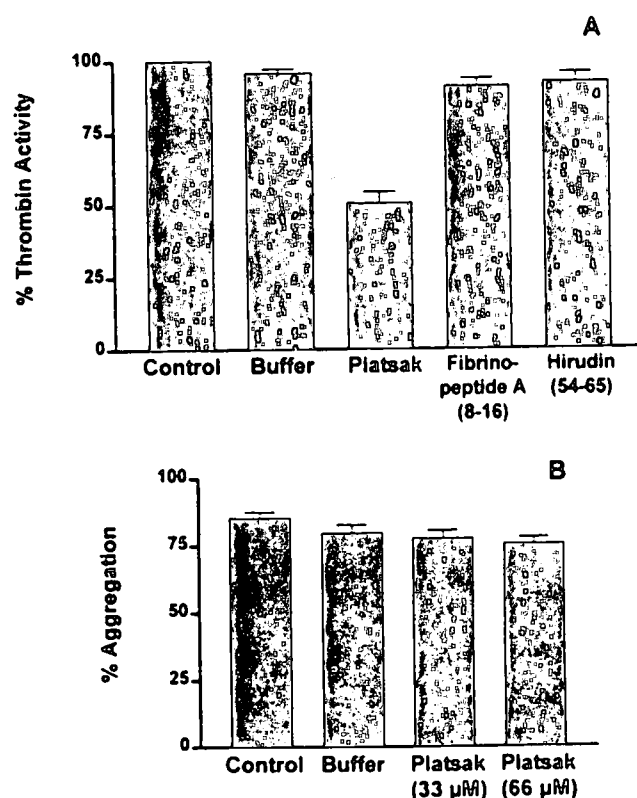


Fig. 5. A. Determination of thrombin inhibition using Chromozym TH as substrate for thrombin. The antithrombotic activity of different concentrations of PLATSAC is expressed as the percentage of thrombin activity retained following incubation of 60 minutes with PLATSAC. B. Determination of the inhibition of ADP induced platelet aggregation. Results are the average of three individual experiments.

Table 2. Comparison of L-values obtained from thrombelastography

Protein concentration	SAK L-value (mm)	PLATSAK L-value (mm)
27 μ M	12.67 $\pm$ 0.47	16.5 $\pm$ 0.41
22 μ M	13.67 $\pm$ 0.47	17.25 $\pm$ 0.82
16 μ M	17.00 $\pm$ 0.82	25.67 $\pm$ 0.47
11 μ M	26.67 $\pm$ 1.25	36.5 $\pm$ 1.48

The fibrinolytic activity of the SAK portion of the protein was determined using thrombelastography, as well as purified recombinant SAK as control. A surprising result was that PLATSAK was slightly less effective than recombinant SAK (Table 2). In theory PLATSAK should inhibit clot formation, due to the antithrombin peptides at its C-terminal end, and activate fibrinolysis, and so have a more pronounced effect on the assay than SAK on its own. A possible explanation for our finding may be that fusion of additional amino acids to the C-terminus of SAK distorts the structure in such a way that it slightly impairs protein activity. However, one would expect that factor Xa would release the antithrombotic peptide from SAK and that the peptide would not have had an effect on SAK. Alternatively, the fact that the fibrinolytic activity was slightly lower and that the inhibition of platelet function was low, strengthens the suggestion that the N-terminal part of the antithrombotic peptide is buried inside the protein, or sterically hindered in some other way. This may also hinder release of the peptide by factor Xa-activity. We have however no data to support this. The C-terminus, with its antithrombin activities, are seemingly more accessible. Unfortunately, no crystal structure for SAK is available at the moment, which could have helped to explain our observations. A possible way of testing the hypothesis would be to rearrange the different motifs of our peptide so that the RGD is at the C-terminal end, or even at the N-terminus of SAK itself.

In conclusion, we were able to produce a hybrid protein in *E. coli* that was able to inhibit thrombin activity and enhance fibrinolysis *in vitro*. Antiplatelet activity was negligible, but can possibly be improved by redesigning or repositioning the RGD motif. The important contribution of this study, we believe, is that we have shown that it is possible to design a multifunctional peptide that can modulate haemostasis and thrombosis. The next step would

be to test the construct in a suitable *in vivo* animal model.

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 THROMBOSIS
RESEARCH

ORIGINAL ARTICLE

PLATSAK, a Potent Antithrombotic and Fibrinolytic protein, Inhibits Arterial and Venous Thrombosis in a Baboon Model

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Abstract

Antiplatelet-antithrombin-staphylokinase (PLATSAK) is a chimeric protein that was recombinantly produced in *Escherichia coli* cells. The protein was designed to target haemostasis at three different levels. It consists of staphylokinase for activation of fibrinolysis, the Arg-Gly-Asp sequence for the prevention of platelet aggregation, and an anti-thrombotic peptide for the inhibition of thrombin. The *in vivo* activity of PLATSAK was evaluated by assessing its effect on platelet deposition in a baboon model of arterial and venous thrombosis. Dacron vascular graft segments and expansion chambers, inserted as extensions into permanent femoral arteriovenous shunts, were used to simulate arterial and venous thrombosis, respectively. PLATSAK (3.68 mg/kg) was administered as a bolus 10 minutes before placement of the thrombogenic devices. Platelet deposition onto the graft surface and in the expansion chamber was imaged in real time with a scintillation camera as the depo-

sition of ¹¹¹In-labeled platelets. After 2 hours, platelet deposition in the graft segments and expansion chambers was inhibited by 50% and 85%, respectively, when compared to control studies. The activated partial thromboplastin time was lengthened to greater than 120 seconds. Interestingly, the level of fibrinogen degradation products in plasma did not increase after administration of PLATSAK. These results demonstrate that PLATSAK effectively inhibited platelet deposition in both arterial- and venous-type thrombosis in an animal model. © 2000 Elsevier Science Ltd. All rights reserved.

Key Words: Antiplatelet; Antithrombin; Fibrinolytic; Baboon model; Hybrid molecule

Several studies in animal models have emphasized the importance and essence of blood platelets and thrombin in thrombogenesis. The pivotal role of the adhesive protein receptor, glycoprotein (Gp) IIb/IIIa, in platelet-platelet interactions, has been demonstrated in canines [1–3] and in non-human primates [4–6] by using selective antagonists. Of particular importance is the fact that Arg-Gly-Asp (RGD) containing peptides and proteins effectively block platelet-platelet interactions [5,7]. The crucial role of thrombin in thrombogenesis has been demonstrated by using direct thrombin inhibitors [8,9]. In other studies, the formation of thrombin was inhibited with activated protein C [10], tissue factor pathway inhibitor [11], or recombinant tick anticoagulant peptide, which inhibits activated factor Xa [12].

Abbreviations: RGD, Arg-Gly-Asp; PLATSAK, Antiplatelet-antithrombin-staphylokinase; Gp, glycoprotein; SAK, Staphylokinase; AV, arteriovenous; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, Sodium dodecyl sulphate polyacrylamide gel electrophoresis; EDTA, ethylenedinitrilo tetraacetic acid; aPTT, activated partial thromboplastin time; FDP, fibrinogen degradation products; TAT, thrombin-antithrombin III.

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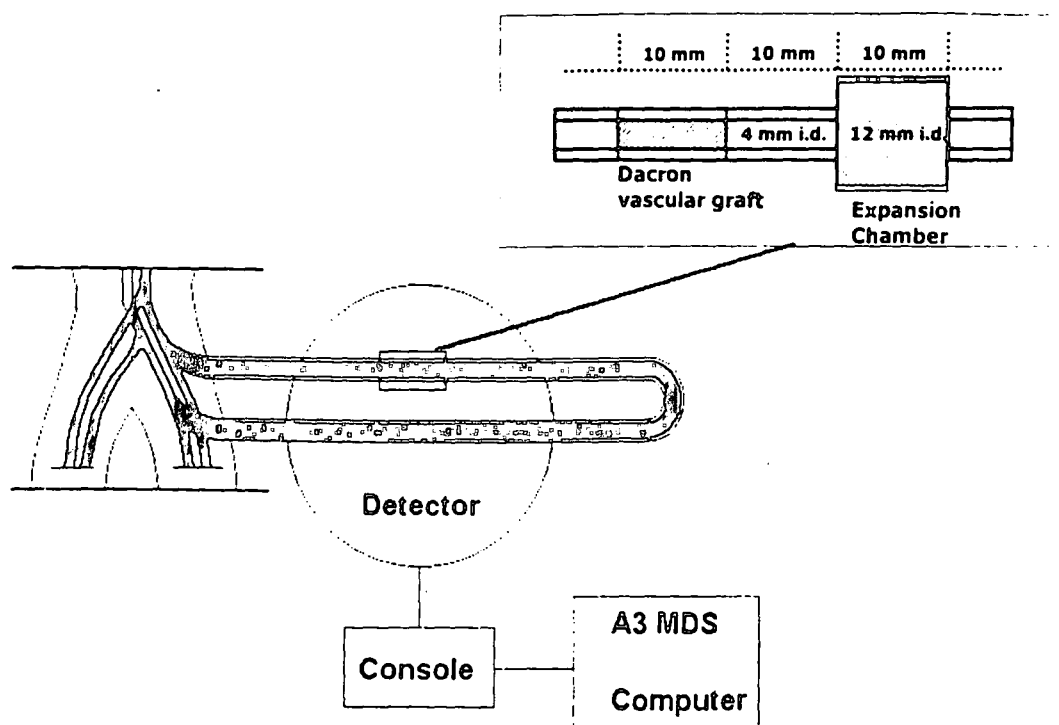


Fig. 2. A schematic representation of the experimental setup to study platelet deposition. The enlarged Dacron graft was used to simulate arterial thrombosis, whereas the enlarged expansion chamber was used to simulate venous thrombosis.

extension regions to calculate deposited radioactivity. The total number of platelets deposited was calculated, as previously described [9,12,24].

1.5. Laboratory Measurements

The times at which blood samples were collected can be seen in the Results section, below. The platelet count of blood samples collected in 2 mg/ml disodium ethylenedinitrilo tetraacetic acid (EDTA) were determined with a Technicon H2 blood cell analyser (Bayer Diagnostics, Tarrytown, NY, USA). The same blood sample was also used to determine whole blood and plasma radioactivity. Blood radioactivity was corrected by subtracting plasma radioactivity in order to calculate platelet radioactivity [24]. The radioactivity in the different samples was determined in a well-type scintillation counter. Blood was also collected in 3.8% sodium citrate (9 volumes blood to 1 volume citrate) to determine activated partial thromboplastin time (aPTT), plasma levels of the thrombin-antithrombin III (TAT) complex, and fibrinogen degradation products (FDP). The aPTT was measured on a

fibrinometer (Clotex II, Hyland Division, Travenol Laboratories, Costa Mesa, CA, USA) using reagents supplied by the manufacturer. TAT complexes were determined using an ELISA method (Enzygnost Combipack, Behring, Marburg, Germany), as recommended by the suppliers. FDP in plasma was determined semiquantitatively using a latex agglutination method following the manufacturer's instructions (Diagnostica Stago, Asnieres-Sur-Seine, France).

2. Results

The results on platelet deposition are summarized in Figure 3. In the three control studies the thrombogenic devices occluded after 42, 39, and 42 minutes, respectively. Whereas platelet deposition onto the graft segment started almost immediately, there was a delay of approximately 6 minutes before they started accumulating in the expansion chamber. In addition, the graft segments accumulated approximately 6× more platelets than the expansion chambers at the end of the control stud-

proteins on thrombogenesis, cells that were transformed with the empty vector were treated in exactly the same manner as the PLATSAK producing cells. These extracts were then used in the control baboons. Protein concentrations were determined using the BCA Protein Assay system from Pierce (Rockford, IL, USA).

1.2. Animals Studied

Three normal male baboons (*Papio ursinus*) were used in this study. The animals weighed approximately 10 kg and were disease-free for at least 6 weeks prior to use. To enable handling, the baboons were sedated with approximately 1 mg/kg ketamine hydrochloride (Premier Pharmaceuticals, Johannesburg, South Africa). The peripheral blood platelet count was normal ($371 \pm 112 \times 10^9/l$). All procedures were approved by the Ethics Committee for Animal Experimentation of the University of the Orange Free State, in accordance with the National Code for Animal Use in Research, Education, Diagnosis and Testing of Drugs and Related Substances in South Africa. The baboons supported permanent Teflon-Silastic AV shunts implanted in the femoral vessels [24]. These shunts do not detectably shorten platelet survival or produce measurable platelet activation [25].

1.3. Study Protocol

The experimental setup is schematically presented in Figure 2. Thrombogenic devices were prepared, as previously described [24,26]. A piece of Dacron vascular graft material (1.26 cm^2) served as a generator of platelet-dependent arterial-type thrombosis. An expansion chamber (3.77 cm^2) was used to generate coagulation-dependent venous thrombosis. Initial wall shear rates were approximately 318 sec^{-1} for the graft section, and less than 10 sec^{-1} for the expansion chamber. Shear rates were calculated using the standard formula and by taking the radius and blood flow rate into account. Initial blood flow rates of 120 ml/min were assumed. Under these conditions, the Dacron vascular graft material and the expansion chamber devices cause arterial-type platelet-dependent and venous-type coagulation-dependent thrombosis, respectively [26]. The thrombogenic device, prefilled with saline to avoid a blood–air interface, was incorporated as

an extension segment in the permanent AV shunt, using Teflon connectors [24].

The control baboons were treated with a bolus of 8 mg/kg of the freeze-thaw extract from *E. coli* cells that did not produce PLATSAK, to show that the proteins did not affect thrombogenesis. The bolus was administered 10 minutes before control devices were placed. The devices were kept in place for 60 minutes, or until it occluded, after which they were removed and blood flow through the permanent AV shunt reestablished. After 60 minutes, the baboons were treated with a bolus of 8 mg/kg PLATSAK extract (i.e., 3.68 mg/kg PLATSAK) to attain a plasma concentration of approximately 0.10 mg/ml plasma. After 10 minutes the thrombogenic devices were placed for 120 minutes, after which they were removed to reestablish blood flow through the permanent AV shunt.

1.4. Graft Imaging and Quantification of Platelet Deposition

Autologous platelets were labeled with ^{111}In -tropolone, as previously described [28]. Imaging and quantification of ^{111}In -platelets were done, as previously described [24]. In short, image acquisition of the grafts was done with a Searle Pho scintillation camera fitted with a high-resolution collimator. The images were stored on and analyzed with a Medical Data Systems A³ computer (Medtronic, Ann Arbor, MI, USA) interfaced with the scintillation camera. Dynamic image acquisition, 3-minute images (128×128 byte mode) for 1 hour in the control studies, and for 2 hours in the PLATSAK studies, was started simultaneously with the placement and start of blood flow through the devices. A 3-minute image of a 3-ml autologous blood sample was also acquired each time the grafts were imaged to determine circulating blood radioactivity (blood standard). This blood was transferred to a thrombogenic device and imaged in the same geometry as the thrombogenic device incorporated into the AV shunt. Regions of interest of the graft and expansion segments were selected to determine the deposited and circulating radioactivity in the dynamic image. Radioactivity in regions of similar sizes was determined for the graft and extension segments of the blood-filled device to determine circulating radioactivity. This was subtracted from the radioactivity in the graft and

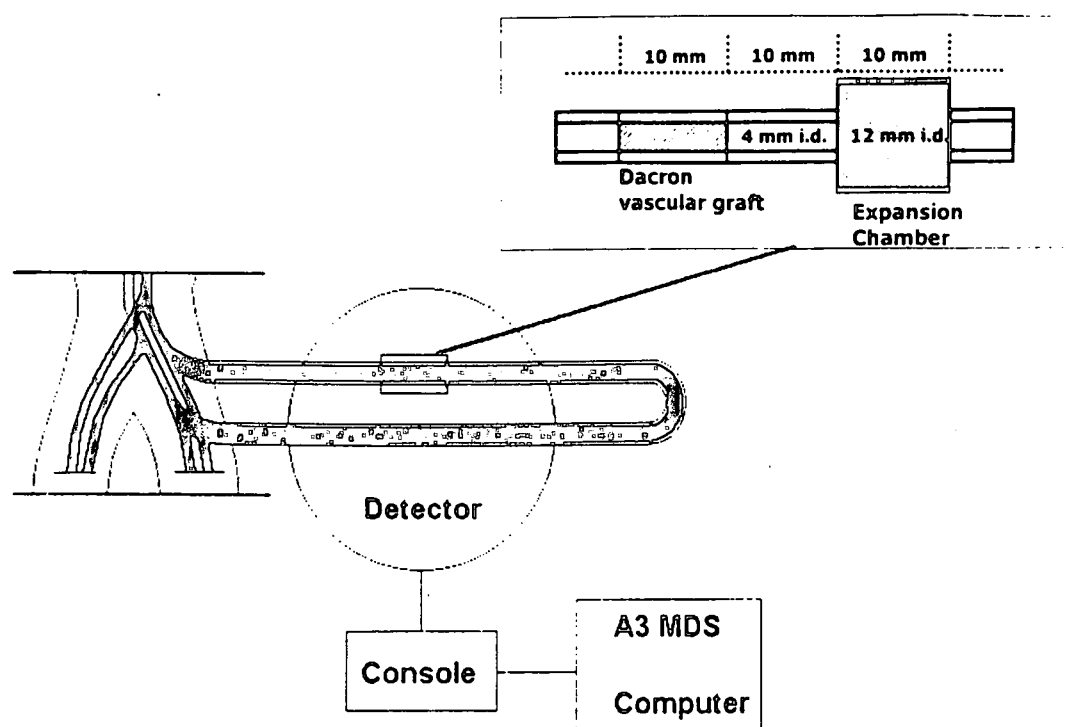


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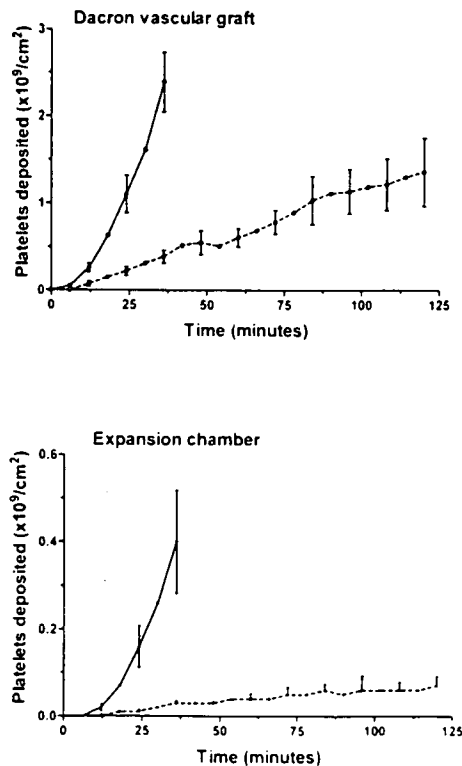


Fig. 3. The effect of PLATSAK on platelet deposition in control animals and after treatment. Values are given as a mean $\pm$ SEM.

ies. Treatment with PLATSAK inhibited platelet deposition onto the Dacron vascular graft by approximately 84% after 39 minutes (i.e., at the time of occlusion of the control devices). Platelet deposition, however, was not completely interrupted but deposited at a much slower rate than in the control studies (i.e., $1.43 \pm 0.71 \times 10^7$ platelets/cm²/min vs. $8.85 \pm 1.83 \times 10^7$ platelets/cm²/min). The inhibitory effect of PLATSAK on platelet deposition in the expansion chambers was more pronounced (i.e., 94% less platelets deposited at 39 minutes than in the control studies). In addition, the start of deposition was delayed by approximately 12–15 minutes. The rate of platelet deposition following treatment was also much slower than in the control studies ($0.22 \pm 0.12 \times 10^7$ platelets/cm²/min vs. $4.39 \pm 1.97 \times 10^7$ platelets/cm²/min). None of the thrombogenic devices occluded during the 120 minutes of the study after treatment with PLATSAK.

The changes in platelet count, aPTT, TAT complexes, and FDP levels are summarized in Table 1. Infusion of the freeze-thaw extract from PLATSAK nonproducing *E. coli* decreased the platelet

count slightly to approximately 80%. Placement of the thrombogenic devices caused a further decrease of approximately 21% during the 39–42 minutes for which the devices remained potent. After treatment, placement of the thrombogenic devices for 120 minutes decreased the platelet count by 8%. The aPTT was not affected by thrombogenesis in the control studies. It was markedly prolonged by treatment with PLATSAK, especially toward the end of the study. The TAT complexes increased approximately 20-fold in the control study. After the thrombogenic devices were removed, and before placement of the second thrombogenic device 30 minutes later, the complexes decreased to normal values. After treatment with PLATSAK, placement of the thrombogenic devices increased the TAT complexes two- to threefold. The changes that we observed in the plasma levels of FDP are of interest. The test only became positive toward the end of the control study. Thereafter, the FDP levels remained constant in spite of treatment with PLATSAK.

3. Discussion

The thrombogenic devices that were used in this study typically represent conditions under which an arterial-type (graft segments) and a venous-type (expansion chambers) thrombus forms. The wall shear rate in the graft segment ($\sim 320 \text{ sec}^{-1}$) approximates that found in medium- to large-sized arteries, while the wall shear rate in the expansion chamber ($< 10 \text{ sec}^{-1}$) represents that found in the venous blood vessels [26,29]. The finding that platelet deposition onto the graft segment was approximately sixfold more than in the expansion chamber (Figure 3) further indicates arterial-type platelet-dependent thrombogenesis vs. venous-type coagulation-dependent thrombogenesis [29]. One must also bear in mind that anticoagulants such as heparin and low-molecular-weight heparin markedly inhibit platelet deposition in the expansion chamber but have little effect on platelet deposition onto graft or collagen surfaces [4,5,8,26]. On the other hand, blockade of the Gp IIb/IIIa receptor markedly inhibits platelet deposition onto the graft surface [4,6] but not in flow expansion chambers [4].

We have no indication that the extract from

Table 1. Summary of the changes that were observed in platelet count, aPTT, TAT complexes, and FDP levels (Mean $\pm$ 1SD)

Study	Time (minute)	Platelet count ($\times 10^9/l$)	aPTT (seconds)	TAT ($\mu g/l$)	FDP ($\mu g/ml$)
Control	Prebolus	371 $\pm$ 112	33 $\pm$ 3	13.1 $\pm$ 4.4	Negative
	Pregraft	340 $\pm$ 94	34 $\pm$ 3	21.5 $\pm$ 15.5	Negative
	10	ND ^a	34 $\pm$ 4	36.6 $\pm$ 16.7	Negative
	39-42	268 $\pm$ 55	40 $\pm$ 5	251.5 $\pm$ 26.9*	20
PLATSAK	Pre-graft	267 $\pm$ 55	65 $\pm$ 8	27.6 $\pm$ 16.8	20
	10	ND	72 $\pm$ 12	25.5 $\pm$ 13.4	20
	120	244 $\pm$ 62	>120	61.0 $\pm$ 21.0*	20

* $p < 0.05$, Student's *t*-test. End vs. pregraft.^a ND=not done.

PLATSAK nonproducing *E. coli* cells inhibited platelet deposition in the control studies. First, platelet deposition onto the graft segments and in the expansion chambers was rapid (Figure 3), and all three devices occluded within 42 minutes following their placement. Second, the number of platelets deposited onto the graft surface and in the expansion chamber compares favorably with the results obtained in studies in which similar devices were used [26,29]. Third, there was no significant lengthening in the aPTT (Table 1). The injected proteins also did not cause thrombocytopenia, since the peripheral blood platelet count was not markedly affected by infusion (Table 1).

PLATSAK markedly inhibited platelet deposition onto the graft segment and in the expansion chamber when the thrombogenic devices were placed 10 minutes after a bolus infusion (Figure 3). In both cases the onset of platelet deposition was delayed when compared to the control studies. Furthermore, none of the devices occluded during the 120 minutes of the study. The differences that were observed in the inhibition of platelet deposition are of interest. At 39 minutes, when the control devices occluded, platelet deposition onto the graft segment was inhibited by approximately 84%, and in the expansion chamber by approximately 94%. At 120 minutes, inhibition was approximately 50% and 85%, respectively, when compared to the number of platelets deposited at 39 minutes in the control studies. These results suggest that the extent of inhibition of platelet deposition onto the graft segment was markedly less than in the expansion chamber. However, when inhibition of the rates of platelet deposition is compared, it seems that

inhibition onto the graft surface and in the expansion chamber was equivalent. In the case of inhibition of platelet deposition onto the graft surface, the rate of deposition was inhibited by 83 $\pm$ 8%. The rate of platelet deposition in the chamber was inhibited by 94 $\pm$ 4% (i.e., slightly, but not significantly, more than in the case of the graft surface). This is difficult to explain, especially in view of our in vitro results with PLATSAK [19], in which we have shown that the antithrombin activity was much more pronounced than the antiplatelet activity. One must also bear in mind that direct inhibition of thrombin with, for example, hirudin requires a much higher dose to inhibit platelet-dependent thrombosis than coagulation-dependent thrombosis [30]. Therefore, one would have expected that the effect of PLATSAK should have been more pronounced in the expansion chamber, since the in vitro studies suggest it [19], and in vivo results with direct thrombin inhibition support it [30]. A plausible explanation for our finding that platelet-dependent and coagulation-dependent thrombosis were equivalently inhibited by PLATSAK may be that its antiplatelet effect is more pronounced in vivo than in vitro. Alternatively, one may conclude that aggregation studies in vitro are not entirely suited to test antiplatelet effects of the blockade of the Gp IIb/IIIa receptor, especially when the antiplatelet effect is not very pronounced. It was clearly shown that not all platelets have to be functional to cause a normal platelet aggregation in response to agonists added in vitro [31].

After 10-20 minutes of the infusion of PLATSAK the aPTT lengthened from approximately 33 seconds to 65 seconds to 72 seconds. It only length

ened greater than 120 seconds toward the end of the study (Table 1). In general, when an antithrombin is infused, the aPTT lengthens to near steady-state levels within 10–15 minutes [9,32]. We do not know whether the design of PLATSACK can explain the results of treatment on the aPTT. The antithrombin/antiplatelet peptide is coupled to SAK with a factor Xa cleavage site (Figure 1). It is plausible that when coupled to SAK, the antithrombin activity of PLATSACK is not optimal. Initially, before placement of the thrombogenic device, and during the first 10 minutes after the device was placed, most of the antithrombin part could still be attached to SAK, because not enough factor Xa is available to free the peptide. At later stages, on the other hand, factor Xa cleavage could provide enough of the peptide to inhibit thrombin and to <<?1>> lengthen the aPTT.

Treatment with PLATSACK decreased the production of thrombin, or PLATSACK formed complexes with thrombin to make it inaccessible for binding to antithrombin III, or both. After treatment with PLATSACK, appreciably less TAT complexes formed (Table 1). FDP in plasma could only be measured at the end of the control studies, indicating some activation of fibrinolysis through normal mechanisms. It does not appear that SAK resulted in additional activation of fibrinolysis, since the FDP levels in plasma does not seem to increase in the studies where PLATSACK was used (Table 1). One must, however, not overinterpret these results, since the method to measure FDP is semi-quantitative.

We have not measured indices that could assess whether PLATSACK increased the risk of developing a bleeding tendency. There were indications that this was the case. When we removed the permanent AV shunts after each study we had some difficulty stopping bleeding at the sutures where the shunt access sites were closed. We do not know whether this was the result of inhibition of thrombogenesis by the peptide or activation of fibrinolysis by SAK. Indications are that it was activation of fibrinolysis. First, the half-life of small peptides is very short [5,8], whereas that of SAK is relatively long [20]. Secondly, one baboon developed a hematoma at the site of surgery 24 hours after the AV shunt was removed, and bleeding effectively stopped. If this was indeed the case, one must redesign the thrombus targeting characteristic of PLATSACK.

In conclusion, we have shown that PLATSACK inhibited platelet deposition in both arterial- and venous-type thrombosis. Our results show that it is feasible to combine into the same molecule different antithrombotic strategies with success. We could not distinguish which strategy was responsible for inhibition. The results do, however, indirectly suggest that both the inhibition of thrombin and the blockade of Gp IIb/IIIa played a part. It does not appear that fibrinolysis contributed to the lower number of platelets that were deposited. One must bear in mind that these conclusions are based on the results obtained in three baboons. In view of the difficulties to produce sufficient amounts of PLATSACK, and because the effect of PLATSACK on platelet deposition was so dramatic, we decided not to study more baboons. The results on the three baboons were regarded as sufficient proof that combination of different inhibitory moieties into one molecule is a viable strategy for the development of antithrombotic drugs.

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