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EVALUATION OF CONSTRUCTED RECOMBINANT MENGOVIRUSES AND OTHER HIV VACCINE CANDIDATES IN MURINE AND PRIMATE MODELS

by

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Due to the nature of most of the work described, a large number of people were involved in most studies. However, the candidate played a coordinating role for the studies and was responsible for day to day planning and execution of the studies. The candidate was also responsible for collation and analysis of the results, and did numerous experiments personally. At the end of each chapter, the specific contribution of the candidate to the studies discussed is given.

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LIST OF ABBREVIATIONS

aa – amino acids

AIDS – acquired immunedeficiency syndrome

AI – avidity index

BCG – Baccille Calmette Guérin

bp – base pairs

BSA – bovine serum albumin

CAEV – caprine encephalitis-arthritis virus

CHO – Chinese hamster ovary

cDNA – complementary DNA

CID_{50} – 50% chimpanzee infectious dose

CTL – cytotoxic T-lymphocyte

DMEM – Dulbecco's modified Eagle medium

DNA – deoxyribonucleic acid

ECHO – enterocytopathic human orphan

EIA – enzyme immunoassay

EIAV – equine infectious anaemia virus

EU – EIA units

FBS – foetal bovine serum

FIV – feline immunodeficiency virus

FMDV – foot-and-mouth disease virus

3Gly – tri-glycine

gp - glycoprotein

HIV-1 and -2 – human immunodeficiency virus types 1 and 2

HLA – human leucocyte antigen

HMA – heteroduplex mobility assay

hsp – human seminal plasma

IC – infectious cells

IFA – incomplete Freund's adjuvant

ip – intra-peritoneal

IRES – internal ribosomal entry segment

LCMV – lymphocytic choriomeningitis virus

LD_{50} – 50% lethal dose

LEMSIP – Laboratory for Experimental Surgery in Primates

LTNP – long-term non-progressors

LTR – long terminal repeat

Il-2 – interleukin-2

im – imtramuscular

ip – intraperitoneal

IRES – internal ribosomal entry segment

iv - intravenous

MHC – major histocompatibility complex

min – minute

moi – multiplicity of infection

MPL-A – monophosphoryl lipid A

NIH – National Institutes of Health

NK – natural killer

OD – optical density

on – oro-nasal

PBMC – peripheral blood mononuclear cells

PBS – phosphate buffered saline

PCR – polymerase chain reaction

pfu – plaque forming units

rgp – recombinant glycoprotein

RNA – ribonucleic acid

RSV – respiratory syncytial virus

RT-PCR – reverse transcription polymerase chain reaction

sc – subcutaneous

SDS-PAGE – sodium dodecyl sulphate polyacrilamide gel electrophoresis

SHIV – HIV/SIV chimaeric virus

SIV – simian immunodeficiency virus

STD – sexually transmitted disease

STLV-I – simian T-cell lymphotropic virus type I

$TCID_{50}$ – 50% tissue culture infectious dose

TCLA – T-cell line-adapted

USA – United States of America

VEEV – Venezuelan equine encephalitis virus

VLP – virus-like particles

WB – Western blot

ABSTRACT

The development of an effective vaccine against HIV is a formidable challenge. The overall objective of this work was to evaluate different HIV-1 vaccine approaches in primate and murine models. In a first approach recombinant Mengoviruses expressing several HIV-1 and SIV gene products were evaluated for their immunogenicity in mice and macaques. Results indicated that Mengovirus recombinants expressing HIV-1 Nef or SIV CTL epitopes are weak immunogens. This was disappointing in light of the promising results previously obtained using other Mengovirus recombinants and indicated that the nature of the insert might play an important role in the immunogenicity of Mengovirus recombinants. As a second approach, protection of chimpanzees from intravenous and vaginal challenge by immunisation with a recombinant canarypox virus expressing the HIV-$1_{IIIB/LAI}$ gp120/TM, *gag* and protease genes was evaluated. In animals challenged by the iv route protection from homologous challenge was seen in one of two animals and this correlated with the neutralising antibody levels. One of five females resisted a total of 3 vaginal challenges, while two further animals resisted 2 challenges. However, only low levels of HIV-1-specific neutralising antibodies were present at time of challenge. This suggests that neutralising antibodies may have little importance for protection from mucosal infection in chimpanzees, in contrast with what was seen for iv challenge. Finally, macaques were immunised with a primary isolate of HIV-1 in order to evaluate the breadth of the immune response induced by HIV-1 in its "native" state. The animals developed moderate to high titers of total anti-HIV-1 antibodies as measured by EIA, which was mainly Gag directed. However, no antibodies capable of neutralising HIV-1_{BX08} were demonstrated, and sera from the animals induced strong facilitation of HIV-1 replication in PBMC, raising the concern that whole virus based HIV vaccines might induce facilitating antibodies that can result in facilitation of transmission and/or evolution of disease.

CHAPTER 1

INTRODUCTION

In 1981 a report in the Centers for Disease Control and Prevention's *Mortality and Morbidity Weekly Report* described 5 cases of *Pneumocystis carinii* pneumonia occurring in previously healthy men in Los Angeles. These cases were followed by several more, as well as an increase in other immunedeficiency-associated conditions such as Kaposi's sarcoma, mucosal candidiasis, disseminated cytomegalovirus infection and disseminated perianal herpes simplex virus infection. The patients all had T-cell dysfunction and all were homosexual men or intravenous drug abusers.[1] However, it soon became clear that this syndrome, now called acquired immunedeficiency syndrome (AIDS), was not limited to these two defined populations, but that the affected populations included Haitian immigrants, haemophiliacs, transfusion recipients, sexual partners of at-risk persons and babies born to at-risk mothers. All of these observations indicated that the cause was an infectious agent spread through genital secretions and blood.

In 1983 a group from the Pasteur Institute in Paris isolated a retrovirus from lymph node tissues of an AIDS patient.[2] The virus was initially called lymphadenopathy-associated virus. A year later scientists at the National Cancer Institute in Bethesda claimed to have isolated another retrovirus (named human T-lymphotropic virus type III) from an AIDS patient,[3] but this virus was soon proven to be identical to the virus isolated by the French group. By this time the virus was firmly entrenched as the etiologic agent of AIDS and was renamed human immunodeficiency virus (HIV). In 1986 a second retrovirus associated with AIDS was isolated from patients in West Africa and named HIV-2.[4]

The time interval between infection with HIV and the development of clinical symptoms (and eventually AIDS) is long compared to conventional infectious agents. This period also varies between individuals. Up to 70% of infected individuals have an acute flu-like syndrome shortly after infection.[5] This seroconversion illness occurs at the period of maximum viral replication, and is followed by an asymptomatic period that can last from a few months to more than 10 years. However, the virus continues to replicate at high levels during the asymptomatic phase, and there is a gradual decrease in $CD4^+$ T-cell numbers over time.[6] The asymptomatic period is followed by the development of clinical symptoms including weight loss, chronic diarrhoea, fevers and opportunistic infections; and eventually the development of overt AIDS. The great majority of HIV-infected people, especially in the absence of any therapeutic interventions, will eventually die from AIDS, but a small percentage (<5%) (designated long-term non-progressors [LTNP]) will survive for more than 15 years without any evidence of immunological deterioration.[7] HIV-2 causes a similar spectrum of disease, but the average time from infection to the development of AIDS appears to be longer and in general it appears to be less pathogenic than HIV-1.[8]

Both HIV-1 and –2 are transmitted via the following routes: i) sexual contact, ii) parenteral inoculation or transfusion of blood and blood products, and iii) perinatal transmission. However, HIV-2 appears to be transmitted less efficiently than HIV-1.[8]

Since the recognition of AIDS in 1981, and the subsequent isolation of HIV-1 in 1983, the number of HIV-1 infections has increased rapidly, resulting in a global pandemic with enormous humanitarian and financial implications. In contrast, HIV-2 has remained relatively confined to West Africa. The rapid expansion of the HIV-1 pandemic has made it clear that mechanisms to control the spread of the infection are desperately needed. Traditionally

vaccines have been the most effective way to control virus diseases, but the development of an effective vaccine against HIV is a formidable challenge. Only a single case of natural clearance of overt infection (as defined by virus isolation from peripheral blood mononuclear cells [PBMC] and detection of proviral deoxyribonucleic acid [DNA] in PBMC) has been reported;[9] and this, together with the absence of any documented cases of recovery from the disease caused by HIV, even raises the question of whether any vaccine against HIV could possibly be effective in preventing infection with the virus. It is clear that the development of such a vaccine presents the greatest challenge vaccine developers have ever had to face.

1.1. The need for a vaccine

The relentless global expansion of the HIV pandemic is claiming thousands of lives each year, and the financial cost is adding to the economic burden of the already poor developing countries. It is estimated that there are currently 33.6 million people living with HIV globally (Fig. 1).[10] Of these more than 90% live in the developing world, and 23.3 million in sub-Saharan Africa. Approximately 5.6 million new infections occurred in 1999, while the total number of AIDS deaths since the beginning of the epidemic is estimated at 16.3 million. It is estimated that 2.6 million people died from AIDS in 1999 alone.

It is clear that sub-Saharan Africa is the area of the world worst affected by HIV. This region has more than 70% of the world's infected people, in spite of the fact that it is home to just 10% of the global population. In fact, AIDS is now the leading cause of death in Africa. HIV transmission in Africa occurs mainly through heterosexual sexual contact, and more women than men (ratio 1.3:1) are infected. In addition, more than 90% of the 500 000 babies infected with HIV through vertical transmission in 1999 were born in sub-Saharan Africa. A major

reason for this is the fact that preventive anti-retroviral therapies are not available to pregnant women in many of these countries.

In spite of the advances in antiretroviral drug therapy, HIV/AIDS remains a challenge in developed countries. For the past few years the number of AIDS deaths in the United States of America (USA) has fallen sharply (for example a decrease of 42% between 1996 and 1997), as treatment with highly active anti-retroviral therapy has resulted in increased survival of HIV-infected patients. However, the emergence of drug resistance and an increase in high-risk behaviour has resulted in the fall in AIDS deaths tapering off. In the USA, the decrease in AIDS deaths from 1997 to 1998 was only half of that seen from 1996 to 1997. There is also no evidence that the number of new infections in industrialised countries is decreasing to a significant extent.[10]

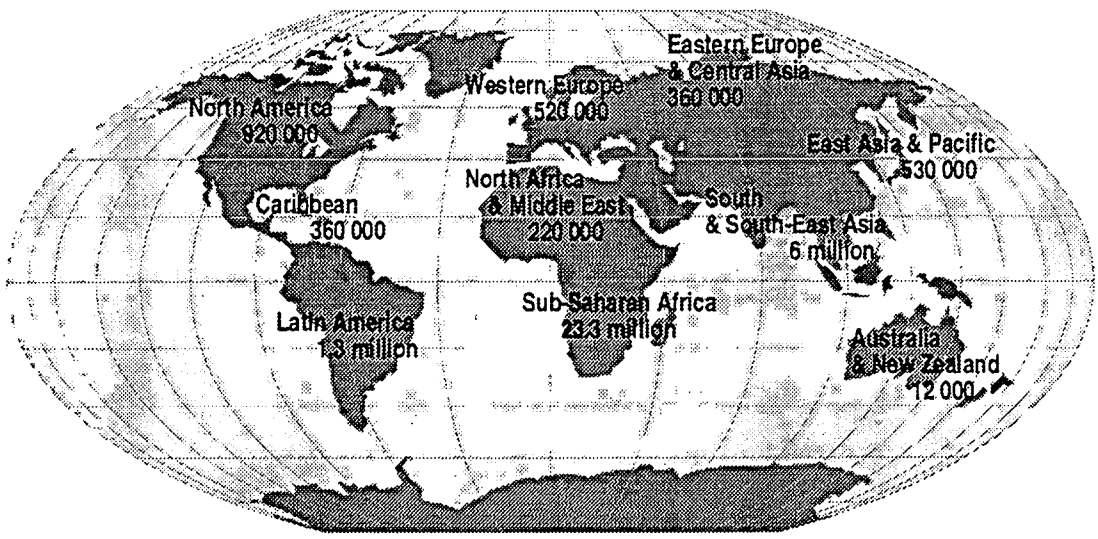


Figure 1. Estimated number of HIV-infected people globally, and geographical distribution of cases, at the end of 1999.[10]

1.1.1. The HIV epidemic in South Africa

South Africa has one of the fastest (if not the fastest) growing HIV epidemics in the world. At the end of 1998 it was estimated that 22.8% of women attending public sector antenatal clinics were infected with HIV, a significant rise from the 17.04% reported at the end of 1997. This number has risen rapidly since the results of the first survey in 1990 was reported (Fig. 2).[11]

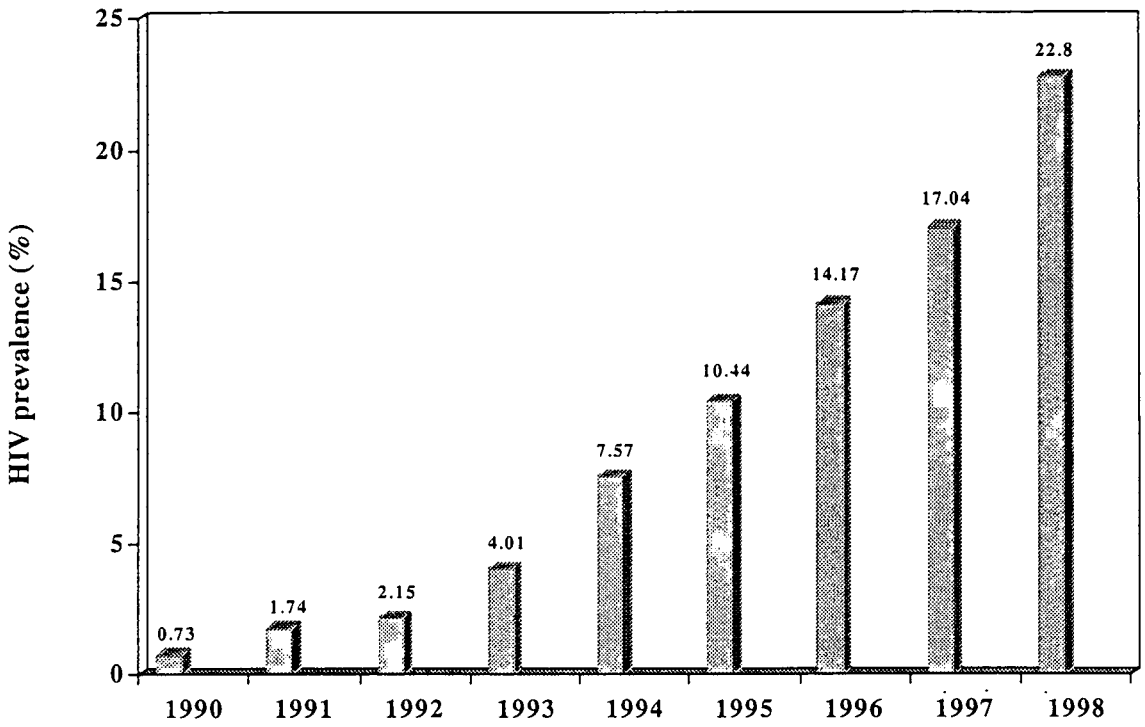


Figure 2. The increase in HIV seroprevalence from 1990 to 1998 in women attending antenatal clinics in South Africa.[11]

All the regions of South Africa are affected by the epidemic. Although the seroprevalence is the highest in KwaZulu/Natal and Mpumalanga, all provinces have experienced an increase during the last year. Four provinces have reached seroprevalences of >20% in antenatal women, with a staggering 32.5% recorded in KwaZulu/Natal (Table 1). Perhaps the most worrying trend is the increased seroprevalence in young women 15-29 years old, in whom the

rates increased from 12.7% in 1997 to 21% in 1998 (Table 2).[11] This increase is most likely an indication that educational programmes are not having an impact in this population. In a study in military recruits from South Africa, it was demonstrated that high-risk behaviour was practiced in spite of knowledge regarding the dangers of contracting HIV.[12]

Table 1. HIV seroprevalence in antenatal clinic attendees by province in 1997 and 1998[11]

	HIV prevalence (%) and 95% confidence interval	
	1997	**1998**
KwaZulu/Natal	26.9 (29.3 – 35.7)	32.5 (29.3 – 35.7)
Mpumalanga	22.6 (20.5 – 24.8)	30 (24.3 – 35.8)
Free State	20 (17.1 – 22.2)	22.8 (20.2 – 25.3)
Gauteng	17.1 (15.1 – 19.2)	22.5 (19.2 – 25.7)
North West	18.1 (16.2 – 20.1)	21.3 (19.1 – 23.4)
Northern Province	8.2 (6.9 – 9.7)	11.5 (9.2 – 13.7)
Eastern Cape	12.6 (11 – 14.4)	15.9 (11.8 – 20)
Northern Cape	8.6 (6.4 – 11.3)	9.9 (6.4 –13.4)
Western Cape	6.3 (5.2 – 7.5)	5.2 (3.2 – 7.2)
National	17.04	22.8

Table 2. HIV seroprevalence in antenatal clinic attendees by age group in 1997 and 1998[11]

	HIV prevalence (%) and 95% confidence interval	
	1997	**1998**
<20	12.7 (11.3 – 14.2)	21 (18.4 – 23.8)
20 – 24	19.7 (18.4 – 21)	26.1 (24.1 – 28.1)
25 – 29	18.2 (16.8 – 19.6)	26.9 (24.7 – 29)
30 – 34	14.5 (12.9 – 16.2)	19.1 (17.1 – 21.1)
35 – 39	9.5 (7.7 – 11.5)	13.4 (11.2 – 15.6)
40 – 44*	7.5 (4.4 – 11.8)	10.5 (6.8 – 14.1)
45 – 49*	8.8 (1.9 – 23.7)	10.2 (0.4 – 20)

** The wide confidence intervals are a reflection of the small number of samples from older women included in the survey.*[11]

1.1.2. The humanitarian and economic impact of the HIV epidemic in Africa

In the 1999 Human Development Index the rankings reflecting health, wealth and education of many African countries declined. Almost all of these downward changes could be ascribed to decreased life expectancy as a result of HIV and AIDS. In the early 1950s life expectancy in Southern Africa was 44 years, and rose to 59 years in the early 1990s. However, it is expected to decline to just 45 years in the early 2000s (Figure 3). For example, it is estimated that only 50% of South Africans currently alive will live until their 60th birthday. The projected average is 70% for developing countries, and 90% for industrialised countries. By contrast, in other poor areas of the world, such as southern Asia, life expectancy is on the increase.[10]

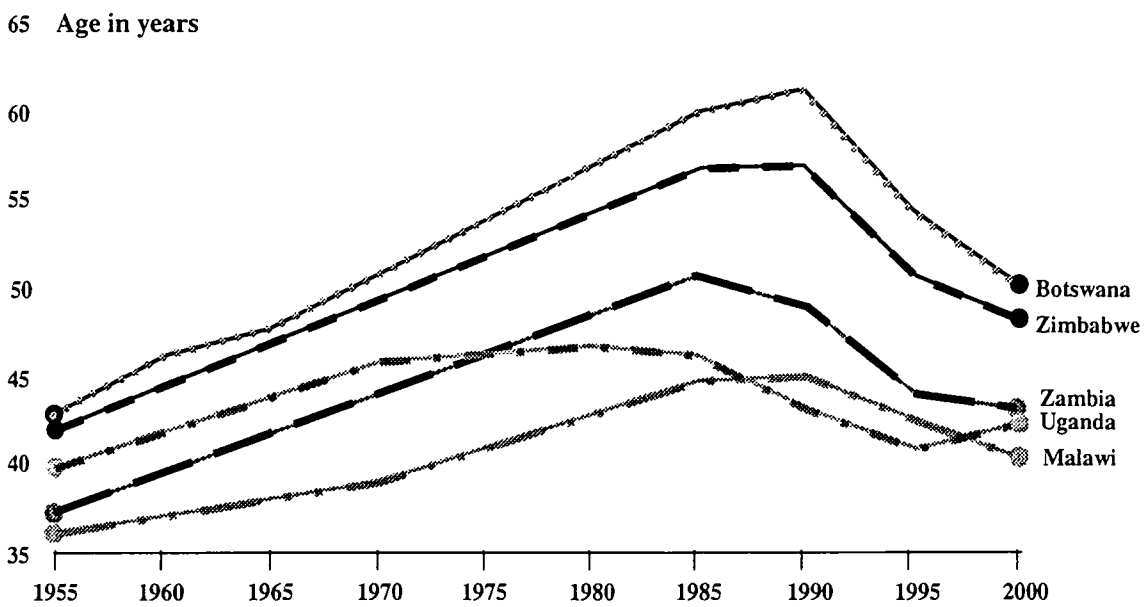


Figure 3. Projected changes in life expectancy in selected African countries with high HIV prevalences, 1995–2000[10]

AIDS has recently become the major cause of death in Africa, surpassing both malaria and armed conflict. It is currently responsible for 19% of deaths in Africa, compared to 4.2% globally (Fig. 4).

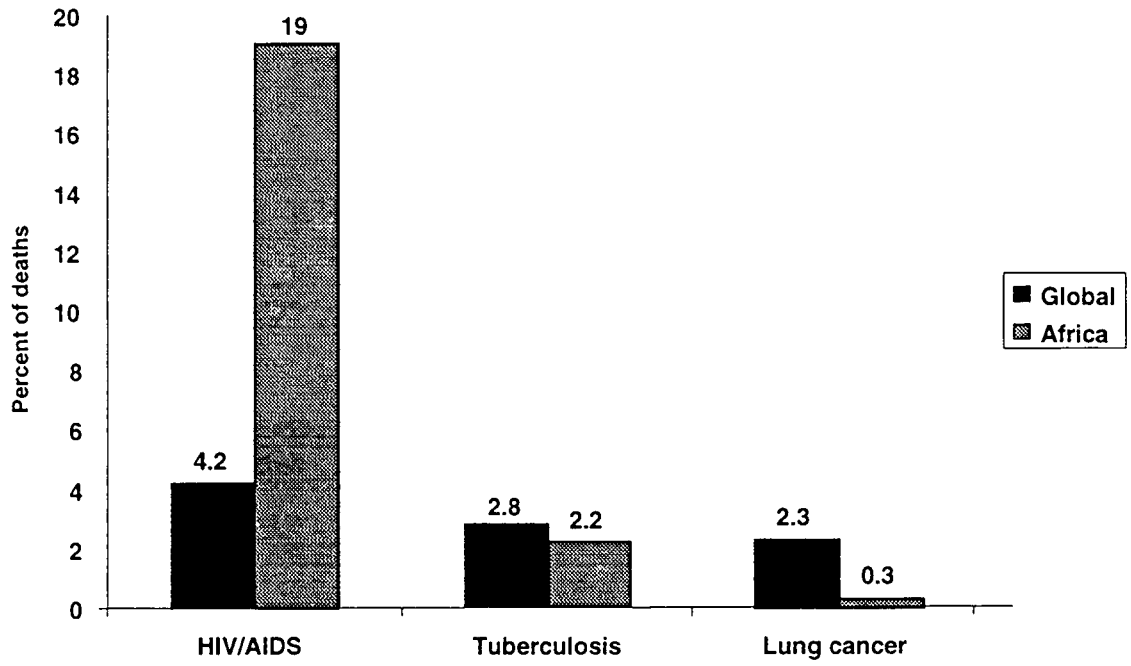


Figure 4. Causes of death, globally and in Africa[10]

It is inevitable that massive rises in death rates among young, economically active adults will affect national economies. In many of the countries worst affected by HIV poor economic management, high inflation rates, rampant corruption, population displacement and deteriorating infrastructure are commonplace. Political conflict and war further add to the precarious economic state of several African countries. It is clear that AIDS will place a further burden on these already severely stretched economies. Another result of the HIV epidemic in Africa is the skill drain caused by the premature death of trained workers.[10]

1.1.3. Limited success of prevention programs

Education and prevention programmes worldwide have had limited success. In Africa the only country that has seen some stabilisation in the growth of the epidemic is Uganda. In Asia some successes have been recorded. Thailand has established an aggressive prevention program, and a fall in the proportion of HIV-infected pregnant women was recorded from

1994 to 1997. This fall was especially steep in younger women, with a 40% fall in HIV prevalence recorded in women under 25 years experiencing their first pregnancy. This is consistent with a slightly earlier decline in HIV prevalence among young male military conscripts in Thailand (Fig. 5).[10,13]

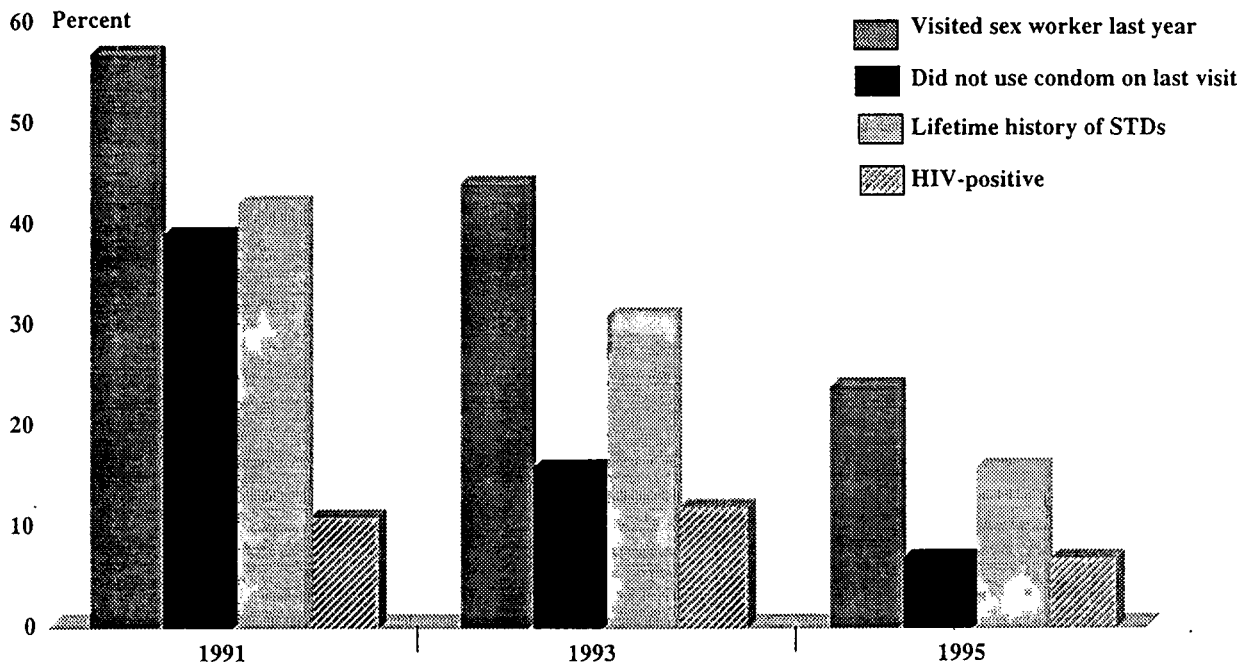


Figure 5. Sexual behaviour, STDs and HIV in 21-year-old men, northern Thailand, 1991–1995[13]

In the Philippines HIV infection appears to remain contained at low levels, with no significant growth even in groups traditionally at high risk for infection. Registered sex workers are screened every 2 weeks for other sexually transmitted diseases (STD), and treated accordingly. The resultant low levels of STDs and the high reported rates of condom use might play a role in the in the slow growth of the epidemic. However, in other parts of Asia such as Vietnam, India and Bangladesh epidemics are growing rapidly.[10]

It is clear that efforts to control the pandemic through education and behaviour modification have had limited success, and the need for a vaccine is desperate.

1.2. HIV-1 genomic structure and biology

HIV-1 belongs to the Lentivirus genus. This group of retroviruses can infect a broad range of animals including monkeys (simian immunodeficiency virus [SIV]), cats (feline immunodeficiency virus [FIV]), sheep (Visna/Maedi virus), horses (equine infectious anaemia virus [EIAV]) and goats (caprine encephalitis-arthritis virus [CAEV]).[14] The HIV-1 genome is about 9.5kb in length. It contains three major structural genes: i) *gag*, encoding the matrix (p17), capsid (p24) and nucleocapsid (p9) proteins; ii) *pol*, encoding the viral enzymes reverse transcriptase (p66), RNAse H (p51), protease (p11) and integrase (p32); and iii) *env*, encoding the external surface envelope (gp120) and the transmembrane (gp41) proteins. There are six additional genes named *tat, rev, nef, vif, vpr* and *vpu*. The long terminal repeat (LTR) regions contain the transcription initiation (5') and termination (3') signals. Precursor polyproteins Gag-Pol, Gag and Env are enzymatically processed to yield mature virion proteins. Gag-Pol and Gag undergo several cleavage steps mediated by the viral protease to produce eight smaller proteins. Env is cleaved once by a cellular protease producing the soluble gp120 and transmembrane gp41. The HIV-1 virion is a spherical particle about 100nm in diameter and consists of a lipid bilayer membrane surrounding a conical nucleocapsid (Fig. 6). The inner core of the viral particle contains the diploid ribonucleic acid (RNA) genome in association with reverse transcriptase (p66/p51) and the nucleocapsid protein (p9).[15]

HIV-1 enters host cells via membrane fusion following attachment to the CD4 receptor on the cell surface.[16] Target cells include cells of the monocyte/macrophage lineage, dendritic cells and $CD4^+$ T-lymphocytes. The virus can also infect $CD4^-$ cells such as glial, mammary, NK, brain endothelial cells, and some gut endothelial cells in culture. In several of these cases the receptor molecule has been demonstrated to be galactosylceramide.[17]

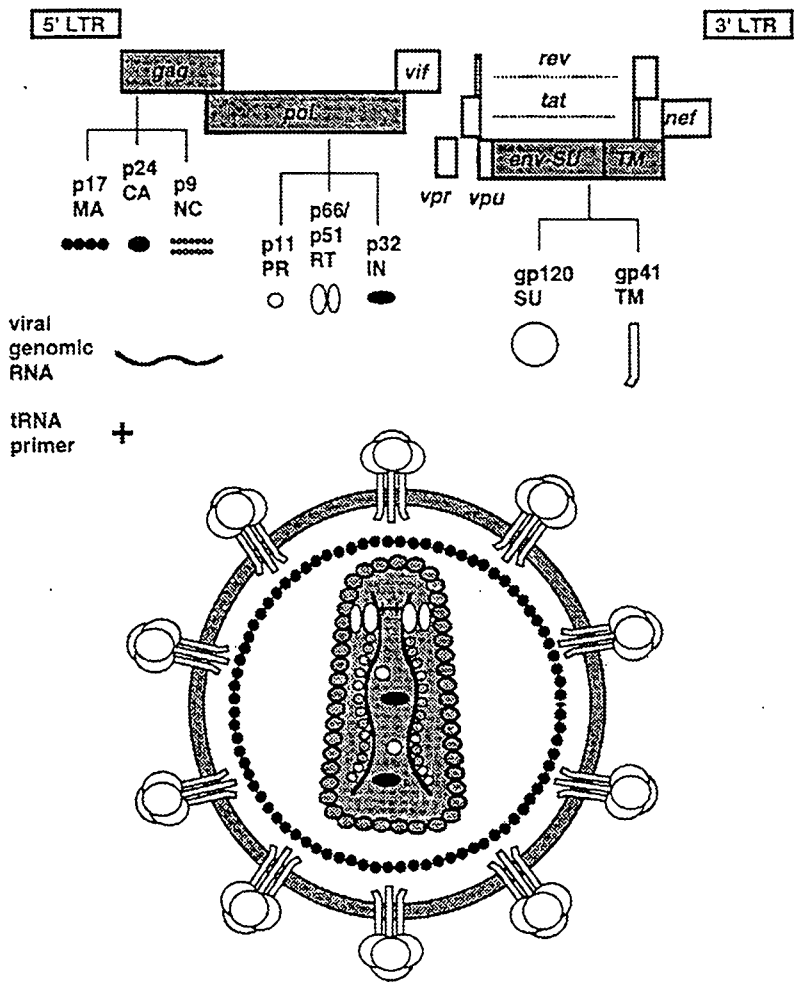


Figure 6. HIV-1 genomic organisation and virion structure.[15]

MA – matrix protein
CA – capsid protein
NC – nucleocapsid protein
PR – protease
IN - integrase
SU - soluble
TM – transmembrane
RT – reverse transcriptase

The CD4 binding domain of gp120 is a complex conformational motif consisting of discontinuous parts of the gp120 molecule. Following gp120/CD4 binding a series of conformational changes occur in the molecule, resulting in interaction between a fusogenic domain of gp41 and the host cell membrane.[18,19] Most primary HIV-1 isolates obtained from patients in the asymptomatic stages of infection are macrophage tropic. These M-tropic strains replicate in PBMCs, but do not form syncytia and cannot replicate in T-cell lines. In

some patients T-cell tropic viruses emerge later in the course of infection. These T-tropic viruses replicate in both PBMCs and T-cell lines and induce syncytia.[20] Some T-tropic viruses have been adapted to replicate to high titers in $CD4^+$-transformed T-cell lines. These T-cell line-adapted (TCLA) virus strains have lost their ability to replicate efficiently in PBMCs. The M- or T-tropic phenotype of HIV-1 isolates depend on specific determinants located in the third hypervariable loop (V3 loop) of gp120.[21] This area is involved in syncytia formation by TCLA viruses, and is the principal neutralisation determinant for these isolates.[22] However, it appears to be less important for neutralisation of primary isolates.[23] It was recently demonstrated that two distinct chemokine receptors, the β-chemokine receptor CCR5 and the α-chemokine receptor CXCR4, act as co-receptors for M- and T-tropic HIV-1 isolates respectively.[24,25] M-tropic isolates use CCR5 as their primary co-receptor, while T-tropic primary isolates can use both CCR5 and CXCR4. TCLA viruses use CXCR4 as co-receptor. The importance of these co-receptors for HIV-1 entry into cells has been confirmed by several studies showing that individuals carrying genetic variants of genes coding for these receptors have different susceptibility to HIV-1 infection and/or different rates of disese progression.[26,27,28]

Following entry into the cell, the viral RNA is transcribed into complementary DNA (cDNA) by the reverse transcriptase enzyme. This enzyme has no proofreading capacity, resulting in misincorporations during the transcription of RNA into cDNA. Based on observed error frequency rates, 0.3 to 10 errors per genome of HIV could be introduced during a single cycle of replication.[29] This results in considerable variation in the viral genome, especially in regions that are targeted by the host immune response, such as *env*. The cDNA is then integrated into the cellular DNA by the viral integrase. Progeny viral RNA is transcribed from the integrated cDNA by cellular DNA polymerases in conjunction with viral elements.

Following translation of the viral proteins, progeny virions are assembled in the cellular cytoplasm and released from the cell by budding through the cell membrane.[15]

1.3. Immune responses to HIV-1 infection

Of particular concern for the development of a vaccine, is the fact that HIV can persist in the host despite a vigorous and apparently normal immune response.[30] Infection with HIV elicits, in most individuals, a cell mediated immune response that includes natural killer (NK) cells and cytotoxic T-lymphocytes (CTL) targeted to cells expressing HIV antigens, as well as non-lytic suppression of HIV by $CD8^+$ cells through the secretion of chemokines or other as yet unidentified mechanisms.[31,32,33,34] Most HIV-infected individuals also eventually develop a neutralising antibody response,[35] as well as antibodies that mediate antibody-dependent cell-mediated cytotoxicity and complement-dependent lysis of infected cells.[30] In a small percentage of HIV-1 infected individuals long-term suppression of virus replication is seen, and these individuals do not develop AIDS (the so-called LTNP).

Why these antiviral immune responses fail to clear the virus remains unknown. It has been suggested that the extremely high rates of $CD4^+$ T-cell turnover eventually exhaust the immune system,[36] and that this, together with depletion of virus-specific CTL by overwhelming virus replication,[37,38] might lead to the development of AIDS. It is unsure whether these immune responses will be more efficacious when induced by a vaccine, rather than by natural infection.[30]

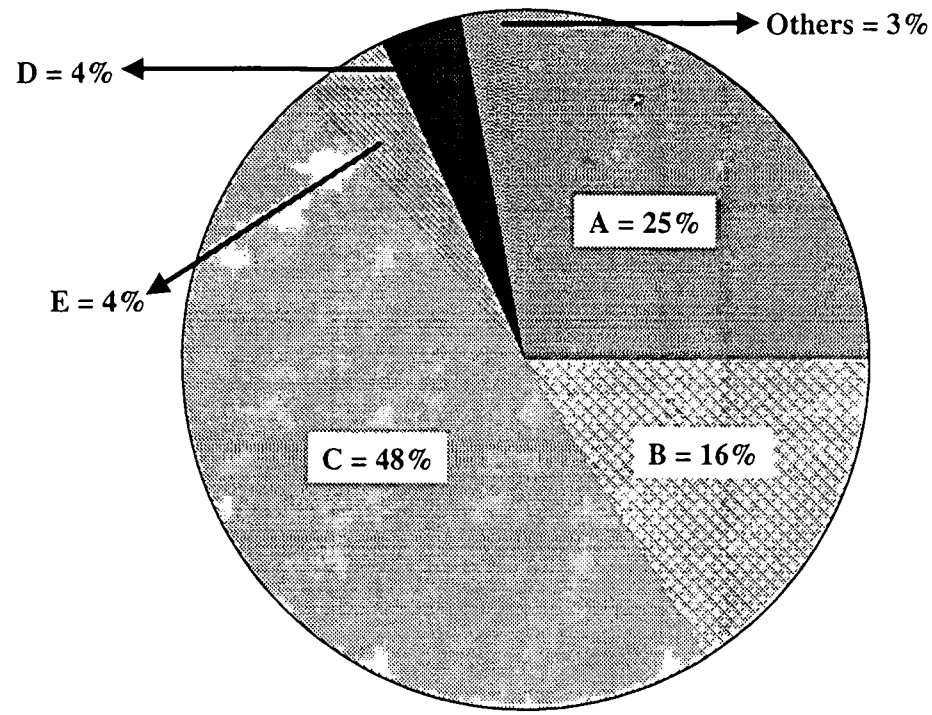
There is reason to believe that both a humoral, as well as a cell-mediated immune response, will have to be induced if a vaccine for HIV-1 is to be truly efficacious. On the one hand, it is important that the virus should be rapidly neutralised, and if possible, prevented from

multiplying at the portal of entry. Therefore, an HIV-1 vaccine should induce high levels of neutralising antibodies, including high levels of antibodies in mucosal secretions. High neutralising antibody titers were reproducibly induced in chimpanzees and in human volunteers by priming with gp160 followed by boosting with V3 peptides.[39] On the other hand, the fact that HIV-1-specific T-helper cells could be detected in HIV-1 seronegative sex partners of seropositive individuals has led to the hypothesis that the immune system might be able to successfully clear a low-dose HIV-1 infection via a cell-mediated immune response.[40,41] This implies that HIV-1 vaccines should also elicit a cell-mediated immune response, particularly CTL, which are able to recognise and destroy virus-infected cells. Unfortunately, a single vaccine is unlikely to be able to elicit both a strong neutralising antibody response and a strong CTL response, and it will probably be necessary to use two vaccines in a prime/boost combination. In fact, a clear synergistic effect between two successive vaccines was observed in human volunteers who were primed with live poxvirus/gp160 recombinants and then boosted with a gp160 subunit vaccine.[42]

1.4. Virus variability

A serious potential problem for the development of an HIV-1 vaccine remains that of virus variability, particularly the hypervariability of the envelope. HIV-1 isolates have been found to form three groups, the M group and the recently identified O[43] and N[44] groups. The M group is sub-divided into at least 9 subtypes (designated A through H and J), on the basis of sequence homologies in the *env* and *gag* genes.[45] Although certain subtypes are found preferentially in certain countries, there does not appear to be a strict localisation of subtypes to precise geographical areas.[46,47] Subtype C accounts for 48% of HIV-1 isolates (Fig. 7a), and is mostly found in southern Africa and India (Fig. 7b).

7a



7b

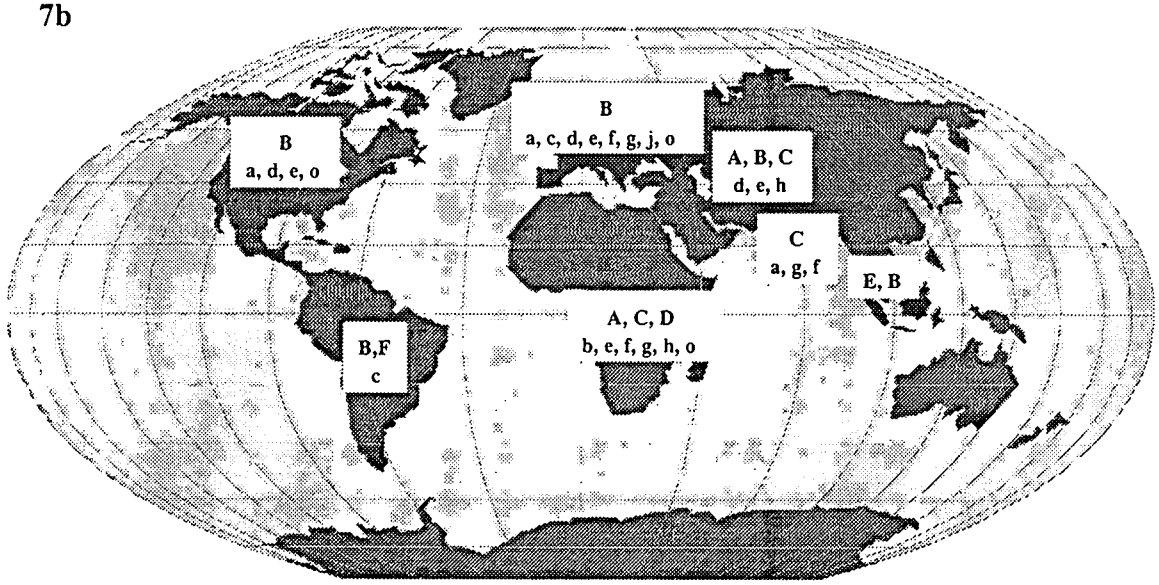


Figure 7. Distribution of HIV-1 group M subtypes (personal communication, S Osmanov).

a: Proportion of total HIV-1 infections represented by each subtype.

b: Geographical distribution. Major subtypes in a region are shown in capitals, and minor subtypes in lower case.

This uneven global distribution of the subtypes, together with unequal rates of spread, has led to considerable debate as to whether the genetic differences observed between the subtypes relate to biological differences. However, it is more likely that the uneven geographical distribution is the result of a founder effect, and that unequal rates of spread merely reflect the risk profile of the population into which a subtype was first introduced.[48] Numerous intersubtype recombinant viruses have also been identified, and it is estimated that at least 10% of all HIV-1 strains have a mosaic genome.[47] It is clear that an HIV-1 vaccine will have to elicit immune responses that recognise viruses from multiple subtypes.

Several studies in the chimpanzee model have shown that inter-subtype cross-protection might be difficult to achieve. Chimpanzees that were protected from an intra-subtype heterologous challenge (using HIV-1_{SF2}) by a vaccine regimen consisting of rgp160 MN/LAI and V3 MN peptides were not protected from inter-subtype cross-challenge using a subtype E virus strain (E90/CR402), showing that no cross-protection exists between HIV-1 subtypes B and E, at least using this model.[39] Furthermore, it was demonstrated that chimpanzees infected with a subtype B (IIIB/LAI) HIV-1 strain, could be superinfected with a subtype E (E90/CR402) isolate by both the intravenous and cervicovaginal routes.[49,50] Perhaps more worrying was the fact that superinfection of subtype B (SF2 or IIIB/LAI) infected chimpanzees could be achieved using heterologous isolates (IIIB/LAI or DH12) from the same subtype, demonstrating that under certain conditions even intra-subtype cross-protection might be difficult to achieve.[50] Evidence for dual HIV-1 infections (and possibly superinfections) with HIV-1 strains from the same and different subtypes in humans also exists.[51,52,53] These results would indicate that virus variability might be an important obstacle to HIV vaccine development. On the other hand, several studies have demonstrated that neutralisation of primary isolates of HIV-1 is not related to their genetic subtype. In

neutralisation studies using panels of isolates containing subtypes A-F and sera from patients infected with diverse isolates in a checkerboard fashion, it was demonstrated that some sera were able to broadly cross-neutralise all HIV-1 isolates tested, irrespective of subtype. Similarly, some isolates appeared to be more sensitive than others to neutralisation.[54,55] Furthermore, sera from HIV-1 group M-infected individuals could neutralise isolates from HIV-1 group O, and extensive cross-neutralisation is seen between HIV-1 and SIV_{cpz} isolates, reflecting their common genetic origin.[56] Broad spectrum neutralisation of HIV-1 is also seen using monoclonal antibodies directed to discontinuous epitopes in gp120 or gp41. These antibodies include IgG1b12, 2G12 and 2F5.[57] This suggests that a vaccine could provide broad spectrum protection if it induces these types of antibodies.

1.5. Candidate vaccine approaches

Traditionally vaccines to prevent viral diseases consist of either live attenuated or whole killed virus preparations. Current exceptions are the hepatitis B vaccine, where the HBs antigen is used, and influenza, where both complete and subvirion preparations of whole inactivated particles are used. Most classical vaccines do not lead to sterilising immunity, but limit replication of the pathogen and prevent clinical disease.[30] It might be too ambitious to expect an HIV vaccine to provide sterilising immunity, and it might be more feasible to turn infected individuals into long-term non-progressors through immunisation. It might also be necessary to use less conventional vaccine approaches.

1.5.1. Whole inactivated vaccines

This is one of the oldest and most widely used vaccine technologies, and initial trials demonstrating protection of macaques from SIV using a whole inactivated virus preparation have raised hopes that the development of a vaccine against HIV might be easy. However, it

was found that the antibodies involved in protection were directed against the human cells in which the vaccine virus was prepared. It was later demonstrated that the animals were protected from challenge with SIV grown in human cells, but not from SIV grown in monkey PBMC, and that immunisation with human leukocyte antigen-DR had similar effects.[58,59] This approach has also failed in protecting chimpanzees from HIV-1 challenge. This, together with safety concerns has resulted in this approach not being seriously considered in HIV vaccine development at the moment.[30,60]

1.5.2. Live attenuated vaccines

Several studies have demonstrated protection from SIV infection using live *nef*-deleted SIV vaccines. These attenuated viruses cause persistent infection of the host and afford a high-level of protection from subsequent challenge via both the intravenous and mucosal routes.[61,62] These promising results have led to calls for moving forward with trials of attenuated HIV-1 in humans. Unfortunately, the safety concerns when using a live attenuated retrovirus vaccine are daunting. Firstly, the live SIV deletion mutants, although attenuated for adults, can still cause AIDS in neonatal macaques (admittedly when given at relatively high doses).[63] Secondly, the attenuated viruses might be transmitted to other individuals, and could even be pathogenic for them. A case of a female long-term survivor, who appeared to harbour an attenuated virus, but nonetheless transmitted the virus to her baby who subsequently died of AIDS, has been described.[64] Most recently it has also been demonstrated that live attenuated SIV vaccines can form virulent recombinants following challenge with virulent virus.[65] However, the most worrying fact remains the integration of HIV into host DNA, leading not only to the likelihood of lifelong persistence of the vaccine virus, but also carries the risk of insertional mutagenesis.[66]

1.5.3. Live recombinant vector vaccines

Live recombinant vaccines consist of a live attenuated viral or bacterial strain that is used as a vector to express genes that encode the relevant antigens of the infectious agent of interest. A live recombinant vaccine is an interesting approach for HIV, as it has the ability to stimulate both humoral and cell-mediated immune responses.[30] Poxviruses are attractive viral vectors due to their large size and the fact that they can tolerate insertion of a relatively large number of foreign genes. The safety concerns associated with vaccinia virus have been overcome by using more attenuated strains such as NYVAC, or modified vaccinia virus Ankara.[67,68] Another alternative is the use of avian poxviruses that are unable to replicate in mammalian cells.[69] Venezuelan equine encephalitis virus (VEEV) has recently come to the fore as an excellent vector for expression of HIV-1 antigens and human clinical trials of a VEEV recombinant expressing regions of a subtype C HIV-1 isolate may soon begin in South Africa.[70,71] VEEV has several properties that makes it particularly attractive as a viral vaccine vector; i) VEEV based vectors can induce protective immune responses via both the parenteral and mucosal routes, ii) most of the human population does not have pre-existing immunity to VEEV, and iii) it is lymphotropic allowing specific targeting of the heterologous protein to lymphoid tissues where it can initiate a vigorous immune response.[72] Other viruses that are under investigation as vectors are poliovirus and adenovirus.[73,74] Bacille Calmette Guerin (BCG) is an attractive bacterial vector as it is easy and inexpensive to manufacture and has been proven to be extremely safe.[30] Recombinant BCG strains induced good cell-mediated immune responses as well as neutralising antibodies in mice and/or monkeys.[75,76] A potential problem, however, with live recombinant vectors is their relative lack of efficacy in individuals previously exposed to the vector.[30] This would not be a factor for VEEV or avian poxvirus vectors, but can be a problem for Vaccinia, BCG, poliovirus and adenovirus vectors.

1.5.4. Protein subunit vaccines

Many HIV-1 and SIV proteins have been produced as recombinant soluble proteins and tested in animal models, but only a transient immune response was introduced in primates. This has prompted research into appropriate formulations or adjuvants to increase their immunogenicity.[77] Liposomes, immunostimulating complexes, and several adjuvants, including the classical Freund's adjuvant and more novel adjuvants such as saponin and muramyl dipeptide derivations, have been studied. These studies have demonstrated the importance of the three-dimensional structure of the envelope glycoprotein for the induction of antibodies able to neutralise primary isolates of HIV-1, as oligomeric gp160 or gp140 can induce antibodies that neutralise primary isolates, probably due to conservation of critical conformational epitopes in these molecules.[30,78] Recently, Nunberg *et al.* reported on a "fusion-competent" immunogen capturing the transient envelope-CD4-coreceptor structures that arise during HIV attachment and fusion. This immunogen elicited neutralising antibodies capable of neutralising primary isolates from several subtypes in a transgenic mouse model.[79] The advantage of protein subunits is the possibility of inducing a strong humoral immune response and they are likely to form an important component of a prime-boost strategy.

1.5.5. Synthetic peptides

Most synthetic peptides used in HIV-1 vaccine programs have been derived from the V3 loop and have induced antibodies that could neutralise T-cell line adapted strains of HIV-1.[30] Some peptides, such as *env*T1-V3 were also able to induce $CD8^+$ major histocompatibility complex (MHC) class I-restricted CTL responses.[80] The major concerns with this approach are that peptides provide a limited base of CTL and B-cell epitopes, which might be problematic in the light of the wide HLA diversity in the target populations. Furthermore, the

responses induced by small peptides are mono-specific, and escape mutants might emerge rapidly. Very small peptides will also not provide the helper T-cell epitopes needed for an optimal CTL response.[30,81]

1.5.6. DNA vaccines

Plasmid DNA encoding a gene of interest under the control of a mammalian transcription promoter is injected intramuscularly or subcutaneously. The antigen is taken up by host cells and an immune response (mostly cellular) is triggered.[82] Vaccination with naked DNA has resulted in protection of macaques from an SIV/HIV chimaeric virus (SHIV)[81] and chimpanzees from HIV-1 (albeit only when using virus strains of low pathogenicity for challenge).[84] Pure DNA is stable, simple to prepare and design is relatively easy, making DNA vaccines an attractive option. However, the question of long-term safety needs to be addressed.[30]

1.5.7. Virus-like particles

Viral antigens in assembled or particulate form are likely to be more immunogenic than non-assembled purified antigens,[30] and to induce an immune response more similar to that seen following natural infection or immunisation with live attenuated or whole inactivated virus. Subvirion particles can form virus-like particles (VLP) that carry the virus antigens on the surface, but do not replicate. There are several ways to obtain HIV-1 VLPs: i) expressing the HIV-1 *gag/gag-pol* and *env* genes in cells infected with a recombinant baculovirus or vaccinia virus;[85] and ii) stable transfection of mammalian cells (eg. Vero or chinese hamster ovary [CHO]) with an appropriate expression vector.[86] These pseudovirions are attractive because they contain most of the HIV-1 protein components in a native conformation without the potential risks caused by the presence of the complete virus genome.[30]

1.5.8. Prime-boost strategies

It is unlikely that any single vaccine approach will lead to both a strong humoral and a strong cellular immune response. It, therefore, makes sense to use an approach combining two different vaccine modalities, one aimed at inducing a strong humoral immune response and the other to induce a cellular immune response. A prime-boost strategy using a live recombinant vector for priming, followed by a recombinant subunit boost is the approach most likely to be successful, based on current knowledge.[30]

1.6. Results of vaccine studies in animal models

The chimpanzee is the only animal that can be reliably infected with HIV-1, albeit only with certain strains. HIV-1-infected chimpanzees are persistently infected, but usually do not develop AIDS, making this a bad model to study the effect of a vaccine on disease progression. Chimpanzees are also very expensive and the fact that they are endangered animals raise ethical concerns regarding their use in HIV-1 vaccine experiments where other models exist. The SIV/macaque model probably better reflects human infection with HIV-1, but the immunogenicity of HIV antigens cannot be tested in this model. The development of SHIVs has gone some way towards making the evaluation of the immunogenicity of HIV antigens possible in macaques.

In spite of these limitations, valuable knowledge has been obtained from studies in animal models of HIV infection. Several groups have succeeded in demonstrating that gp160- or gp120-based HIV-1 vaccines can protect chimpanzees from experimental challenge with cell-free virus. It was also shown that immunisation could protect chimpanzees from experimental infection with HIV-$1_{IIIB/LAI}$-infected lymphocytes. Remarkably, the common denominator of the immune response in all the protected animals was a high V3-targeted neutralising

antibody response at the time of challenge.[87,88,89] Direct evidence that neutralising antibodies might play a major role in protection of chimpanzees from experimental HIV-1 infection stems from passive protection experiments. A V3-specific monoclonal antibody could prevent HIV-1 infection in chimpanzees when given either before, or directly after challenge, with the virus.[90] Similar results have been obtained in the case of HIV-2.[91] Of particular importance is that protection from mucosal challenge was demonstrated in several studies, using different vaccine formulations and different routes of immunisation.[92]

Furthermore, it was demonstrated that in spite of the total absence of anti-HIV-2 neutralising antibodies, cross-protection from HIV-2 infection could be achieved in rhesus macaques by vaccination with a recombinant vaccinia virus (NYVAC) expressing HIV-1 *gag, pol* and *env*, followed by HIV-1 p24 plus gp160. This result reopens the question of immune correlates of protection and also suggests that broad vaccine protection might be achievable, at least in certain animal models.[93] This is supported by the study of Travers *et al.* which demonstrated that a group of high-risk HIV-2 seropositive women had a lower risk of becoming HIV-1 seropositive than an HIV-2 seronegative control group.[94]

Macaque monkeys infected with SIV are widely used as an animal model for AIDS. Protection from SIV infection in macaques has proved to be difficult thus far, and success has only been obtained by vaccination with live non-pathogenic strains of SIV, such as the SIV *nef*-deletion mutants described by Desrosiers and colleagues.[61,95] Unfortunately, the immune correlates of the protection conferred by these live attenuated viruses remain undefined. In the SIV-macaque model, the V3 region of *env* does not seem to play a particularly important role since antibodies to V3 do not have neutralising activity. In fact the importance of neutralising antibodies in protection from SIV infection is unsure. Recent studies on clinical

isolates of HIV-1 suggest that the apparent dominance of V3 might be an artefact caused by adaptation of virus strains to growth on T-cell lines. It is possible that neutralisation of wild-type HIV-1 strains may be more similar to that observed for SIV, than to that of the laboratory-adapted IIIB/LAI or MN strains used in chimpanzee studies, and that antibodies targeted to the V2 loop, the CD4 binding site, or neutralisation epitopes in gp41, play a greater role than those targeted to V3. This implies that the relevance of the SIV-macaque model for the development of HIV vaccines may be much greater than initially anticipated.[96] To complicate matters even further, it was shown that non-neutralising antibodies to *env* antigens can have an enhancing effect in ponies challenged with EIAV.[97]

Perhaps the most important contribution of these primate studies is the fact that they have disproved two early pessimistic predictions: i) a vaccine is a theoretical impossibility as HIV attacks and degrades the immune system itself; and ii) even if an effective vaccine against intravenous transmission could be obtained, it would be much more difficult to develop a vaccine against sexually transmitted HIV infection.[98] The positive outcomes in animal studies have not only demonstrated that a vaccine that affords at least some degree of protection is possible, but also that protection from mucosal challenge is feasible.

1.7. The need for human clinical trials

Although studies of HIV vaccines in animal models are essential, selection of the most appropriate vaccine, or combination of vaccines, will only be possible through phase I clinical trials in human volunteers. This should hopefully pave the way to efficacy trials in persons at risk for HIV-infection. Only through such trials, conducted in a fully coherent and ethical manner, will we eventually be able to assess the true value of candidate HIV vaccines. However, the issues surrounding clinical efficacy trials of HIV vaccines are many and

complex. Ethical issues include, among other, the issue of true informed consent, lack of coercion, protection of confidentiality, and the fact that the patient will test positive for antibodies to HIV.

Results of more than 26 phase I/II trials between 1987 and 1997, including more than 3000 humans volunteers, have shown that the candidate vaccines tested up to now are safe and immunogenic, but that the immune responses induced are, in general, of narrow spectrum and short duration. A summary of the vaccine candidates that have been tested in humans is given in table 3. The most promising results have been obtained in a phase I trial using a prime/boost regimen consisting of a canarypox virus-gp160 recombinant and a gp160 subunit vaccine. Neutralising antibodies developed in >90% of the volunteers and CTL in 40%, but perhaps the most encouraging result of this trial is the fact that several volunteers developed broadly-reactive CTL.[99] This has prompted the announcement of a phase II trial using a canarypox virus/protein subunit combination, which started in the USA in 1999.[42] Immunogenicity results for this trial should be available soon. The first phase III clinical efficacy trial of a candidate HIV-1 vaccine is underway in Thailand (bivalent gp120 of subtypes E and B) and the USA (bivalent gp120 subtype B). The trial is designed to be able to show at least 30% efficacy and results should be available by 2004/2005. If the trial is successful (defined by an interpretable result) this will pave the way for future trials (J Esparza, personal communication).[100]

In conclusion, although human trials are essential, basic research into mechanisms to overcome the problems in developing a vaccine against HIV should not be neglected. New ways to overcome the problem of antigenic variability of the virus must be sought. One solution would be to develop vaccines capable of inducing antibodies targeted to conserved

epitopes such as gp41 or the CD4 binding site. Unfortunately, the CD4 binding site in gp120 is a complex 3D conformational site, which is partly masked on the surface of wild-type virions and is poorly immunogenic. Similarly, it has thus far not been possible to achieve significant titers of gp41-targeted neutralizing antibodies with the immunogens currently available. Whether it will be possible to achieve significantly better results using new antigenic formulations, such as liposomes or pseudo-virus particles, remains unknown at this time.

Table 3: HIV-1 vaccine candidate approaches tested in humans[30,101]

Concept	Product	Subtype	Development status
Protein subunits	gp120 (Chiron)	B	Phase I
	gp120 (Chiron)	B and *E*	Phase I
	gp120 (Genentech)	B	Phase II
	gp120 (VaxGen)	B and *E*	Phase II/III
	gp120 (VaxGen)	B and *B*	Phase II/III
	p24 (Chiron)	B	Phase I
Synthetic peptides	Lipopeptides (ANRS)	B	Phase I
	HGP-30 (CelSci)	B	Phase I
	V3 (Cuban program)	B	Phase I
Live vectors	VV *env/gag/pol* (Therion)	B	Phase I
	ALVAC *env* (PMC)	B	Phase I
	ALVAC *env/gag/pol* (PMC)	B	Phase I
	ALVAC *env/gag/pol/nef/prot*	B	Phase I
	Salmonella *env* (Univ of MD)	B	Phase I
DNA	*Env/rev* (Apollon)	B	Phase I
	Gag/pol (Apollon)	B	Phase I
Prime-boost*	VV *env* +gp160	B	Phase I
	ALVAC *env* + gp160	B	Phase I
	ALVAC *env* + gp120	B	Phase II
	ALVAC *env/gag/pol* + gp120	B	Phase I
	ALVAC *env/gag/pol/nef/prot* + gp120	B	Phase I

**Most significant prime-boost trials performed to date*

1.8. Objectives of study

The overall objective of the study was to evaluate different HIV-1 vaccine approaches in primate and murine models. In a first approach recombinant Mengoviruses expressing several HIV-1 and SIV gene products were evaluated in mice and macaques. The main objective of this was to evaluate this approach for induction of a cellular immune response. As a second approach, protection of chimpanzees from intravenous and vaginal challenge by immunisation with a recombinant canarypox vector was evaluated. The rationale for this was to determine whether a recombinant canarypox virus on its own, without subunit boosts, can result in protective immunity. Finally, an attempt was made to characterise the nature of the immune response induced by a primary isolate of HIV-1 (live or whole inactivated) in macaques. As HIV-1 cannot replicate in macaques, a live virus preparation will in effect be an inactivated virus, but the antigen will be "native" and the immune response induced should be similar to that seen in infected patients.

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1.10. Review papers related to topic (see appendix)

1. Girard M, Barré-Sinoussi F, **van der Ryst E**, Fultz P. Vaccination of chimpanzees against HIV-1. *Antibiotics and Chemotherapy* 1996; **48**: 121 –124 (review).

2. Girard M, Barré-Sinoussi F, **van der Ryst E**, Fultz P. An Approach to HIV vaccines. *AIDS Res Hum Retroviruses* 1996; **12**: 461-463 (review).

3. **van der Ryst E,** Girard M, Barré-Sinoussi F, Fultz P. The development of a vaccine against HIV-1. In: Rousselet F, Chappuis P, Poupon J (eds.). *Le biologiste face au SIDA, XXXX*^{émes} *Journées Internationales de Biologie*. CNB, Paris, 1995: 199-226 (review).

4. **van der Ryst E**. HIV-1 vaccines; where are we now? *Pharmaceutical Physician* 1998; **9**: 20 - 22 (review).

CHAPTER 2

IMMUNOGENICITY OF RECOMBINANT MENGOVIRUSES EXPRESSING HIV-1 NEF OR SIV POL, GAG AND NEF CTL EPITOPES

2.1. Introduction

The possibility of using live replicating viruses as vaccine vectors is attractive as they permit the presentation of foreign antigens via the MHC class I pathway, and the subsequent induction of CTL responses.[1] Interest in picornaviruses as viral vectors stems from the demonstration that a cDNA copy of the viral RNA, or an RNA transcript synthesised in vitro, can give rise to a complete virus cycle once transfected into susceptible mammalian cell cultures.[2] Recombinant picornaviruses can therefore be readily engineered by the manipulation of cDNA clones.

In 1948 Dick and colleagues reported the isolation of a previously unknown virus from several sources (including a wild mongoose and two mosquitoe pools) from the Mengo region in Uganda. This agent caused encephalomyelitis in mice, and was named Mengo encephalomyelitis virus, later shortened to Mengovirus.[3] It is a member of the cardiovirus genus in the family Picornaviridae.[4] The small non-enveloped virion is an icosahedron with a diameter of 30nm.[5]

The single stranded RNA genome of positive polarity is 7750 nucleotides in length and encodes a large polyprotein that is proteolitically cleaved into three regions: P1 is the precursor to the capsid proteins (including VP4/1A, VP2/1B, VP3/1C and VP1/1D); P2 and P3 are the precursors to the non-structural proteins, including the protease (3C) and the polymerase (3D).[4,6] P1 is preceded by the 67 amino acid (aa) leader (L) protein. The function

of the L protein is unknown.[6] Like all picornaviruses, Mengovirus has been shown to possess an internal ribosomal entry segment (IRES) that allows initiation of translation.[7]

In contrast to other members of the Picornaviridae, Mengovirus is able to infect a wide range of animal species, including rodents, pigs and primates.[4] Mengovirus is able to replicate in human cells, and has been shown to infect humans[8], but rarely causes serious disease in humans.[9] The pathogenic potential of Mengovirus is determined by a homopolymeric, polyribocytidylic acid [poly(C)] tract in the 5' noncoding region of the genome, and truncation or deletion of the poly(C) tract leads to a loss of pathogenic potential (Table 1).[10,11,12,13] The poly(C) tract of wild-type Mengovirus contains $C_{50}UC_{10}$ and the virus has a 50% lethal dose (LD_{50}) of only 9 plaque forming units (pfu) for 4 week old BALB/c mice when inoculated intracranially. In contrast, a virus containing a truncated poly(C) tract, vM16 ($C_{13}UC_{10}$) has an LD_{50} of $8x10^6$ by the same route, while a virus with a deleted poly(C) tract, vMC0 has an LD_{50} of $>10^9$.[10,11,12,13]

It was, furthermore, demonstrated that the short poly(C) strains of Mengovirus can induce lifelong protective immunity against challenge with virulent wild-type Mengovirus or encephalomyocarditis virus in mice and pigs.[13,14] The RNA genomes of Mengovirus and other picornaviruses are, moreover expressed exclusively in the cytoplasm of infected cells, and do not undergo reverse transcription.[15] Altogether, these properties make Mengovirus an attractive candidate for development as a potential vaccine vector.

Table 1. Attenuation of Mengovirus by deletion of the poly(C) tract of the 5' non-coding region as demonstrated by Palmenberg and colleagues.

		LD_{50} (pfu)		
		Route		
		Intracerebral	Intracerebral	Subcutaneous
Virus	**Poly(C)**	**4-week old mice**	**Newborn mice**	**4-week old mice**
ECMV	$C_{115}UCUC_3UC_{10}$	<1	ND	$<10^2$
Mengo	$C_{50}UC_{10}$	9	ND	10^5
VMWT*	$C_{50}UC_{10}$	9	6	10^5
VM37*	$C_{26}UC_{10}$	$7x10^2$	ND	ND
VM30*	$C_{19}UC_{10}$	$6x10^4$	ND	ND
VM16*	$C_{13}UC_{10}$	$8x10^6$	$3x10^2$	$>10^{11}$
VM18*	C_8	10^7	ND	$>10^{10}$
VMC0*	None	$>10^9$	$4x10^4$	ND

LD_{50} of various Mengovirus strains for SWISS mice (≥50/group). The exact dose of virus in the inoculum was determined by titration on HeLa cells.[5,10,11,12,13]

ND = not done

** genetically engineered viruses*

It was previously demonstrated that a recombinant Mengovirus expressing a CTL epitope from lymphocytic choriomeningitis virus (LCMV) induced strong CTL responses in mice and afforded protection from lethal LCMV challenge.[6] Furthermore, a recombinant Mengovirus expressing a 450 bp region from the HIV-1_{LAI} gp120 gene induced humoral and cellular immune responses in mice, and humoral immunity in macaques.[16] The aim of the current

study was to test the ability of recombinant Mengovirus vectors to induce CTL responses to various HIV and SIV proteins.

The HIV-1 Nef protein plays an important role *in vivo*, both for high levels of viral replication and for progression to disease.[17,18,19] *In vitro*, several functions have been described for Nef: the downregulation of CD4[20,21]; the enhancement of viral infectivity[22]; and the alteration of cellular pathways.[23] Nef is also highly immunogenic in HIV-1-infected individuals.[24,25]

Several SIV CTL epitopes have previously been described in macaques. These inlude epitopes from SIV Pol (aa 590 - 598) (N Letvin, personal communication), Gag (aa 182 - 190)[26,27,28] and Nef (aa 155 - 178).[29] The epitopes from Pol and Gag each contained a single CTL epitope, while the longer 24 aa sequence from Nef contained 3 overlapping CTL epitopes. Several different recombinant Mengoviruses that expressed either a large region of HIV-1 Nef, or previously described SIV CTL epitopes were therefore constructed. The heterologous proteins were either fused in-frame into the N-terminus of the L polypeptide of the vectors, or expressed as a cleaved product. Cleavage of the heterologous proteins from L were obtained by introduction of a sequence coding for the foot-and-mouth disease virus (FMDV) 2AF protein, which undergoes autocatalytic cleavage[30], immediately following the heterologous gene. The immunogenicity of each different recombinant was then tested in mice and/or macaques.

2.2. Materials and Methods

2.2.1. Construction of recombinant viruses

All DNA manipulations for construction of recombinant plasmids were performed according to standard procedures.[31] The following parental vector plasmids were used: i) pMCS[6] which contains a mutagenesis casette containing unique *XhoI, SnaBI* and *NheI* sites inserted in the *NcoI* site of the infectious Mengovirus cDNA plasmid (pM16)[10]; ii) pMC0S which was constructed by cloning the *PflMI/BglII* fragment of pMCS (containing the mutagenesis cassette) into the *PflMI* and *BglII* sites of the plasmid pMC0 that contains no poly(C) tract[11]; and iii) pM2AF which contains the FMDV 2AF protein coding region inserted into the *SnaBI* and *NheI* sites of pMCS (A Habel, unpublished results). This FMDV 2AF peptide has previously been shown to undergo autocatalytic cleavage[30], and was thus included in certain constructs to allow cleavage of the heterologous protein from the Mengovirus L-protein (Fig. 1).

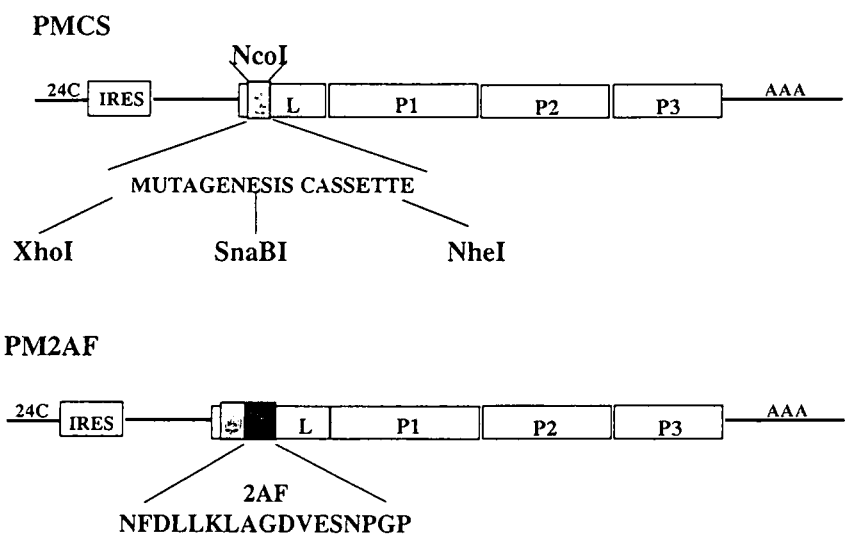

Figure 1. Organisation of the parental Mengovirus plasmids

The position of the mutagenesis cassette, and the (amino acid) aa sequence of the FMDV autoproteolytic peptide (2AF) is shown. The third parental plasmid, pMC0s was organised identically to pMCS, apart from the fact that it contained no poly(C) tract.

A 444 bp cDNA fragment from the HIV-1_{LAI} Nef gene (coding for aa 65 - 206) was amplified by PCR from plasmid pTG1147[20], using oliconucleotides Nef-s (5'-ATT AAC TCG AGG GTG GGT TTT CCA GTC ACA CCT-3') and Nef-as (5'-AT TAA AGC TAG CCA GAG CTC GCA GTT CTT GAA GTA CTC CGG-3') to create unique *XhoI* and *NheI* sites. The PCR product digested at these sites was introduced into the *XhoI* and *NheI* sites of pMCS and pMCS-2AF to construct plasmids pMCS/nef and pM2AF/nef, respectively (Fig. 2).

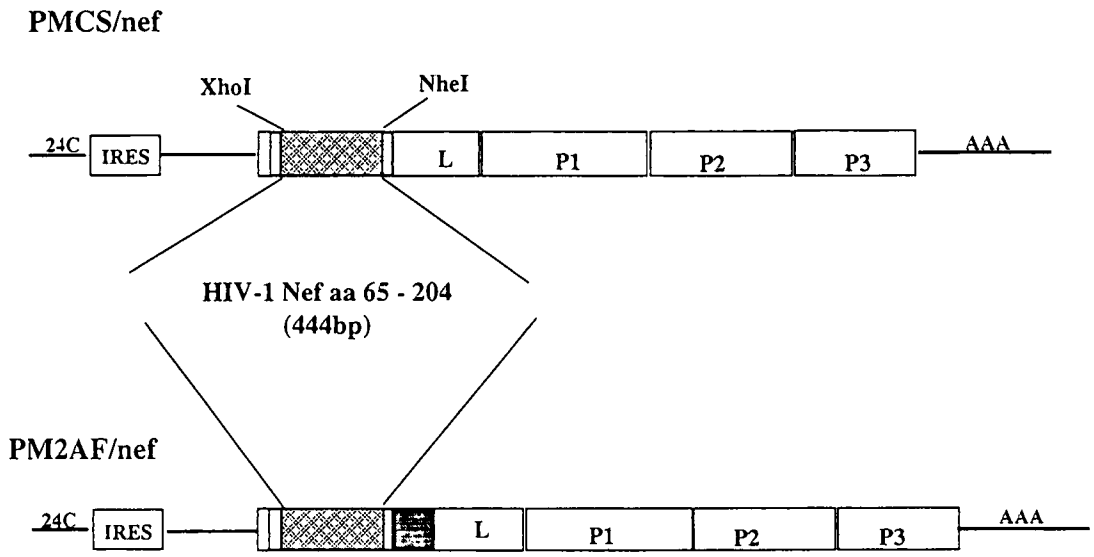

Figure 2. Organisation of the recombinant plasmids expressing HIV-1 Nef.
A 444-bp region from the HIV-1_{LAI} nef gene was generated by PCR and cloned into the NheI and XhoI sites of the parental vector plasmids.

In order to examine the possibility that the insertion of the HIV-1 *nef* gene fragment might interfere with Megovirus translation, a bicistronic Mengovirus/HIV-1 Nef recombinant plasmid was constructed. The bicistronic recombinant was created by the insertion of the IRES of enterocytopathogenic human orphan (ECHO) virus type 25 (strain JV4) into the *NheI* restriction site of pMCS/nef. The plasmid was constructed in such a way that translation of HIV-1 Nef was driven by the Mengovirus IRES, and tranlation of Mengovirus

proteins from the ECHO virus 25 IRES. A 646 bp fragment was amplified from the ECHO virus 25 JV4 IRES template DNA[32] using oligonucleotides IRES-s (5'-TAT GGG CTA GCG TTA ACT ACG TAA TAG TGA CCT TTG TGT GCC TGT-3') and IRES-as (5'-ATA CCA GCT AGC ATT GTT GTA GCC ATA TTA TAG CTA TAT GGC AAC-3'). To allow for termination of Mengovirus IRES driven translation, 3 stop codons were engineered into the plasmid immediately preceding the ECHO virus 25 IRES sequence (Fig 3).

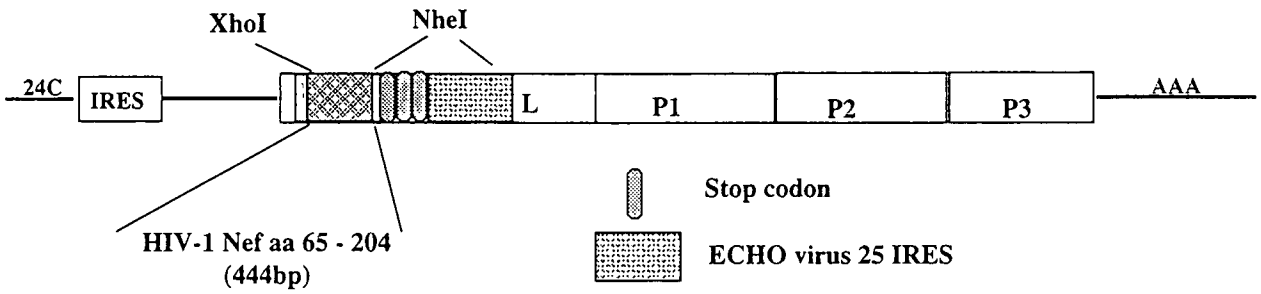

Figure 3. Organisation of the bicistronic recombinant plasmid expressing HIV-1 Nef.
A 646 bp region encoding the ECHO virus 25 IRES was generated by PCR and cloned into the NheI site of the pMCS/nef plasmid as shown.

Three different SIV Mengovirus recombinants carrying SIV Gag, Nef and Pol CTL epitopes, expressed in tandem and separated by a 3Gly linker, were constructed. In a first step, plasmid pMC0S-Δnef was constructed by the introduction of the synthetic oligonucleotides +*SnaBI*/SIVnef/*NheI* (5'-GTA TAT TCC TGA TTG GCA GGA TTA TAC TTC TGG TCC TGG TCC TGG TAT TAG ATA TCC TAA GAC TTT TGG TTG GTT GTG GAA GT TG GT GC CT GT GAC G-3') and -*SnaBI*/SIVnef/*NheI* (5'-CAT ATA AGG ACT AAC CGT CCT AAT ATG AAG ACC AGG ACC ATA ATC TAT AGG ATT CTG AAA ACC AAC CAA CAC CTT CAA CCA CGG ACA CTG CGA TCG-3') encoding aa 153 - 180 from SIVmac251 *nef*, and with unique *SnaBI* and *NheI* sites, into the *SnaBI* and *NheI* sites of pMC0S. The sequence coding for aa 182 - 190 of the SIVmac251 *gag* gene product was then

cloned into the *XhoI* and *SnaBI* sites of pMC0S-Δnef to construct pMC0S-Δgag/nef, using synthetic oligonucleotides +*XhoI*/*SacI*-SIVgag/*SnaBI* (5'-TCG AGT GAG CTC ACT CCT TAT GAT ATT AAT CAA ATG TTG GGT TGG TGG TAC-3') and -*XhoI*/*SacI*-SIVgag/*SnaBI* (5'-GTA CCA CCA CCC AAC ATT TGA TTA ATA TCA TAA GGA GTG AGC TCA C-3') to create *XhoI* and *SacI* sites at the 5' terminus and a 3Gly spacer and a *SnaBI* site at the 3' terminus. Similarly, pMC0S-Δpol/gag/nef was constructed by cloning synthetic oligonucleotides +*XhoI*/SIVpol/*SacI* (5'-TCG AGT TTT ATT TCT ACT CCT CCT TTG GTG AGA TTG GTG TTT AAT TTG GTG GGT GGT GGT GAG CT-3') and -*XhoI*/SIVpol/*SacI* (5'-CAC CAC CAC CCA CCA AAT TAA CAC CAA TCT CAC CAA AGG AGG AGT AGA AAT AAA AC-3'), encoding aa 587 - 601 from the SIVmac251 *pol* gene product with unique *SacI* and *XhoI* sites, and with a 3Gly spacer at the 3' terminus, into the *XhoI* and *SacI* sites of pMC0S-Δgag/nef. Plasmid pMCS-Δpol/gag/nef was constructed by replacing the *PflMI/BglII* fragment of pMCS with the corresponding fragment of pMC0S-Δpol,gag,nef. Finally, pMCS-Δpol/gag/nef-2AF was constructed by replacing the *NheI/BglII* fragment of pMCS-Δpol/gag/nef with the corresponding fragment from pMCS-2AF.

2.2.2. Preparation and characterisation of recombinant virus stocks

All plasmids were sequenced using the T7 sequencing kit (Pharmacia) to verify integrity of the inserted fragments. The recombinant plasmids were linearized with *BamHI* and infectious RNA transcripts obtained using the T7-RNA-polymerase kit (Promega). The RNA-transcripts were transfected into HeLa cells as described by Duke and Palmenberg[10] to obtain recombinant viruses vMCS/nef, vM2AF/nef, vMC0S-Δgag/nef, vMCS-Δpol/gag/nef and vMCS-Δpol/gag/nef-2AF. Sequential passage stock preparations of the recombinant viruses were obtained by infection of confluent HeLa cell monolayers, titrated, and plaque assays performed as described.[33] Briefly, sequential dilutions of virus stocks were cultured on HeLa

cells under an agarose overlay containing a 1:1 mixture of gilose and Dulbecco's minimum essential medium supplemented with 5% foetal bovine serum (FBS). Following 72 hours of culture at 37°C in a 5% CO_2 atmosphere, the agarose overlays were removed and the cell monolayers stained with a crystal violet solution. Virus stocks were analyzed for the presence of the inserted sequences and genetic stability by RT-PCR using Mengovirus-specific oligonucleotides M-609 (5'-A TCT GAT CTG GGG CCT CGG T-3') and M-1212 (5'-GT CTT GAG ACA CTC GGT C-3').

BamHI-linearized DNA's were trancribed *in vitro* using T7 RNA polymerase (Pharmacia)[34] and purified RNAs were used at different dilutions in reticulocyte lysate (Flexi-Retic, Promega) *in vitro* translation reactions essentially as described.[35] The reactions, with a final volume of 10 µl, contained 80% (by volume) of reticulocyte lysate, final concentrations of added KCl and $MgCl_2$ of 100 mM and 0.5 mM, respectively, and 2 mM of aa (except methionine). *De novo* synthesised proteins were labelled with [^{35}S]-methionine. To study Mengovirus-directed expression of HIV-1 Nef in infected cells, HeLa cells were infected at a multiplicity of infection (moi) of 50 - 60, and [^{35}S]-methionine-labelled cytoplasmic extracts prepared as described.[36] The labelled proteins were analysed on 20% polyacrylamide gels followed by exposure of dried gels to Biomax film (Kodak) for 16 - 20 hours. Densitometric analyses of radiolabelled proteins was performed using a Sharp JX-330 analysis system linked to NIH Image software. For the calculation of efficiency of polyprotein cleavage of the 2AF sequence, densitometric values were adjusted to take into account the relative numbers of methionine residues in the fusion proteins and their cleavage products.

Protein G sepharose beads (Gammabind G, Pharmacia Biotech) labelled with a polyclonal sheep anti-HIV-1 Nef serum (MRC AIDS Reagent Research Program) were used for immunoprecipitation[37] of the translation products obtained for the HIV-Nef recombinants.

2.2.3. Immunisation of mice and macaques

Seven-week-old BALB/c (H-2^d) mice (4 per group) were immunised intra-peritoneally (ip) with 10^6 pfu (in 200 µl) of either the recombinant viruses, or the parental vector viruses vM16 and vM0S, obtained from the plasmids pM16 and pM0S.[11] A group of mice were also immunised via the iv route with 10^7 pfu each of recombinant vaccinia viruses expressing HIV-1 Nef aa 73 - 147 and 148 - 206 (Transgene, Strasbourg), respectively. Virus dilutions for the immunisation of the animals were made in DMEM supplemented with 50 mM $MgCl_2$.

Adult rhesus macaques (*Macacca mulatta*) of Indian origin were housed under P3 conditions at the primate center of the Pasteur Institute. Animals were housed and cared for according to institutional guidelines for the humane care and use of primates in biomedical research and consent for all experiments was obtained from the institutional ethics committee. Animals were anaesthetised using ketamine hydrochloride 10mg via the intramuscular (im) route prior to all procedures. Animals were immunised with the appropriate dose of recombinant virus, or the parental virus vM16, in a total volume of 1ml via the im, intravenous (iv) or oronasal (on) route. For on immunisation, the virus preparation was slowly dripped into the mouth and nose, and smeared over the buccal areas. Leakage of the inoculum from the mouth and nose were not quantified, but did not appear significant. Following immunisation of the macaques and mice, aliquots of the diluted virus stocks that were used for inoculation of the animals were back-titrated to confirm that the animals received the correct dose.

2.2.4. Neutralisation and enzyme immunoassays

Anti-Mengovirus neutralising antibody activities were determined by incubating successive two-fold or four-fold dilutions of serum from vaccinated and control animals together with a constant amount of virus (100 50% tissue culture infectious doses [$TCID_{50}$]) at 37°C for 1h. The virus serum-mixture was then added to 10^4 HeLa cells (previously maintained in complete DMEM supplemented with 5% FBS) in 96-well culture plates and incubated for 6 days at 37°C. Eight wells were used per serum dilution and the neutralising antibody titer was determined using the Reed and Muench formula.[38]

Antibody responses to the expressed proteins were evaluated by enzyme immunoassay (EIA). Briefly, 96-well microtiter plates (Nunc Maxisorp) were coated with 100 ng per well of recombinant HIV-1 Nef (British MRC AIDS Reagent Research Program) or the apprpriate peptides (SIV: Nef 155-178, Gag 182-190 and Pol 590-598; or HIV-1: Nef 66-100, 93-120, 115-146, 137-168, 155-185 and 182-206). Empty binding sites were blocked by addition of phosphate buffered saline (PBS) containing 5% bovine serum albumin (BSA). Serial two-fold dilutions of sera from the immunised and control animals were then added to the wells. Wells containing only PBS were included as negative controls. After a 2h incubation at 37°C, the plates were extensively washed and alkaline phosphatase-labelled goat anti-human or anti-mouse IgG (a 1:1000 dilution in PBS) added to the wells and incubated for 1h. Following extensive washing, p-nitrophenyl phosphate substrate was added to each well and the plates were incubated for 30 min in the dark. The optical density (OD) was measured at 405nm, and the positive cut-off was arbitrarilary defined as two times the average OD of the negative control wells.

2.2.5. Cytotoxicity and T-cell proliferation assays

CTL activity in BALB/c mice was evaluated essentially as described by Sauzet *et al.*,[39] except that only $2x10^6$ responding cells were mixed with $4x10^6$ stimulating cells in 2 ml of culture medium supplemented with 10% FBS, and peptides were used at 10 μg/ml. Target cells were P815 (H-2^d, DBA/2) cells that were either peptide-pulsed (final peptide concentration 10 μg/ml) or infected with recombinant vaccinia viruses at an moi of 10 $TCID_{50}$/cell and incubated overnight. Determination of anti-SIV CTL activity in macaques was measured using an *in vitro* peptide restimulation assay as described.[29] Target cells were peptide-pulsed, autologous hepesvirus papio transformed B-lymphocyte cell lines.

T-cell proliferative responses to HIV-1 Nef were evaluated using an interleukin-2 (Il-2) release assay on CTL.L2 cells as described[39] (Sauzet 1996) and also by measuring direct incorporation of [^{3}H]-thymidine into peptide-stimulated splenocytes[40] from mice immunised either with the Mengovirus or the vaccinia virus HIV-1 Nef recombinants. Six overlapping HIV-1 Nef peptides, covering the entire protein from aa 66 - 206 (66-100, 93-120, 115-146, 137-168, 155-185 and 182-206) (Neosystems, Strasbourg), were used at final concentrations of 3, 10 and 30 μg/ml. Concanavalin-A at 2.5 and 5μg/ml final concentrations was used as a positive control in the proliferation assays, while medium alone, or equivalent concentrations of an irrelevant peptide corresponding to the V3 loop of HIV-1_{SF2} gp120 were used as negative controls.

2.3. Results

2.3.1. Characterisation of HIV-1 Nef recombinant viruses and immunogenicity in mice

Recombinant Mengovirus cDNA plasmids encoding a large region of HIV-1 *nef* were constructed. In order to avoid the complications of the reported oncogenic potential of the

Nef protein[20], the recombinants were designed to express a truncated form of the protein, lacking the first 64 amino acids. Infectious RNA transcripts were prepared and transfected into HeLa cells as described, to obtain the recombinant viruses vMCS/nef and vM2AF/nef. The recombinant viruses (vMCS/nef and vM2AF/nef) were viable and transfected cells showed a clear cytopathic effect at about 72 hours after transfection, as compared to 48 hours for the parental Mengovirus RNA. However, all of the recombinants had a very small plaque phenotype (≤25% that of vM16) as determined by infection of HeLa cells under an agarose overlay, followed by crystal violet staining of the cell layers (Fig. 4).

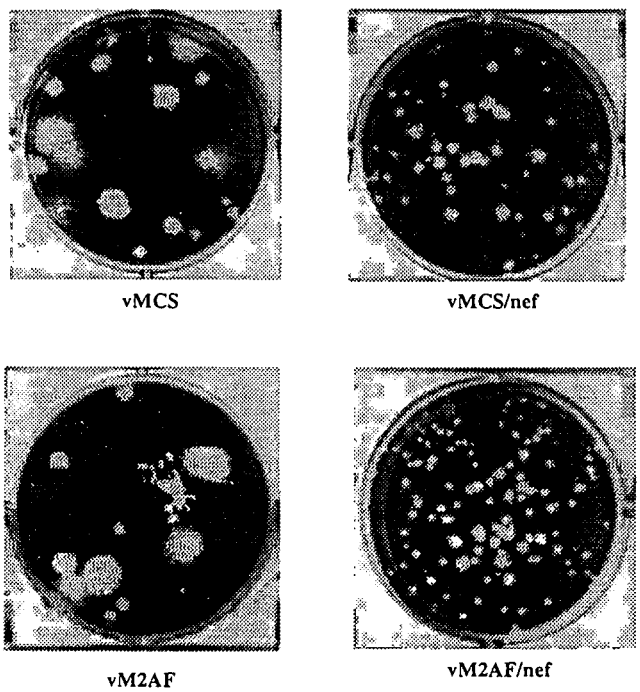

Figure 4. Plaque size of the HIV-1 Nef recombinant viruses compared to that of the parental viruses.

RT-PCR analysis of cellular RNAs obtained from infected HeLa cells as described[41], and using Mengovirus-specific oligonucleotide primers M-609 and M-1212, demonstrated that the recombinant viruses were genetically stable *in vitro* at least to passage 5 (Fig. 5).

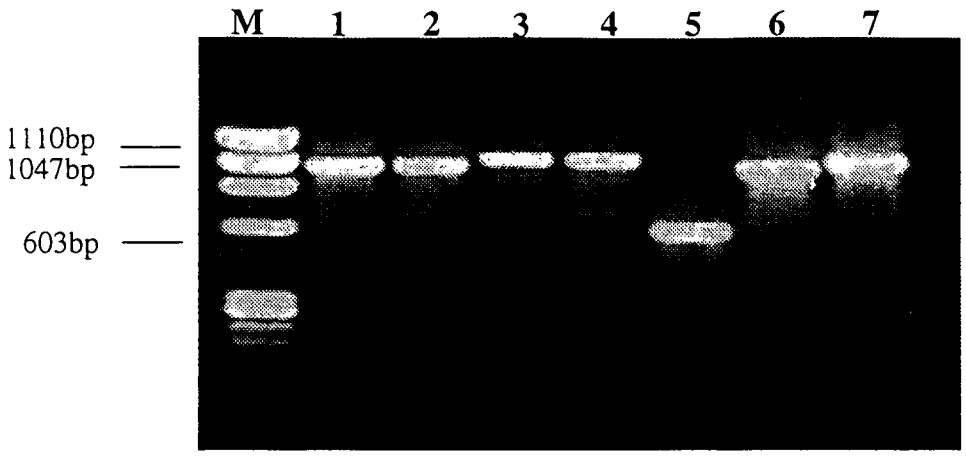

Figure 5. Genetic stability of the HIV-1 Nef recombinant viruses.

Genetic stability of the recombinant viruses was determined by analysing sequential passages of the virus stock for the continued presence of the inserted sequences by RT-PCR using the Mengovirus-specific primers M-609 and M1212. HeLa cell monolayers were infected with the indicated viruses at an moi of 10 pfu/cell and cytoplasmic RNAs were extracted using NP-40 buffer and purified as described.[42] RT-PCR reactions were programmed with total cell mRNAs corresponding to 10^5 cells (lanes 1-4) or with 0.1 μg of the parental plasmid DNAs as controls (lanes 5-7). Lane M: marker; lane 1: vMCS/nef passage (p)1; lane 2: vMCS/nef p5; lane 3: vM2AF/nef p1; lane 4: vM2AF/nef p5; lane 5: pMCS; lane 6: pMCS/nef; lane 7: pM2AF-nef

Despite several attempts at transfection of RNA transcripts obtained from the bicistronic HIV-1 Nef recombinant plasmid, no viable virus could be recovered from the transfection supernatant at any time. The most likely explanation for this is that the insert size (1090bp) was too large to allow for encapsidation of the genome. It has previously been demonstrated that the maximum insert size (fused in frame to L) tolerated by Mengovirus is between 525 and 738bp.[5]

SDS-PAGE analysis of *in vitro* translation products prepared as described, as well as cytoplasmic extracts obtained from vMCS/nef and vM2AF/nef-infected HeLa cells using NP-40 lysis buffer[36], demonstrated that the heterologous proteins were expressed in addition to

Mengovirus proteins and that insertion of the 2AF site allowed cleavage of approximately 85% of the heterologous proteins from L as determined by scanning of the autoradiography films and calculation of the relative band intensities, taking into account methionine content, using NIH Image software.

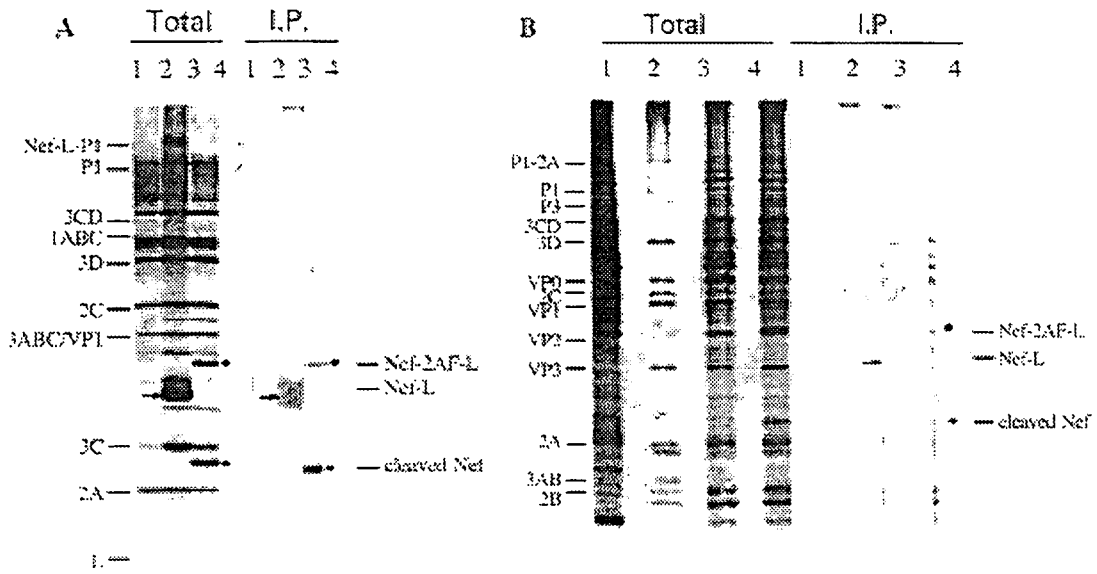

Figure 6. SDS-PAGE analysis of the proteins expressed by the HIV-1 Nef recombinant Mengoviruses.

A: In vitro translation reactions were (directly or following immunoprecipitation using anti-Nef serum) analysed on a 20% SDS-PAGE gel. mRNAs (40μg/ml final concentration) derived from lane 1: pMCS; lane 2: pMCS/nef; lane 3: pM2AF/nef; and lane 4: no RNA.

B: ^{35}[S]-labelled total cell cytoplasmic protein extracts were prepared as described from HeLa cells infected with either vM16, vMCS/nef or vMCS/nef-2AF at an moi of 50 - 60 pfu/cell. Extracts were (directly or following immunoprecipitation using anti-Nef serum) analysed on 20% SDS-PAGE gel. Lane 1: mock infected cells; lane 2: vM16; lane3: vMCS/nef; and lane4: vM2AF/nef.

The identity of the additional proteins (L-Nef and Nef, with sizes of 32 and 25 kD, respectively) expressed by vMCS/nef and vM2AF/nef was confirmed by immunoprecipitation of the ^{35}S-labelled proteins using a polyclonal anti-Nef serum coated onto protein G-sepharose beads (British MRC AIDS Reagent Research Program) (Fig. 6).

In vitro translation of RNA transcripts obtained from the bicistronic HIV-1 Nef recombinant plasmid, performed as described, demonstrated that the ECHO virus 25 IRES led to efficient translation of Mengovirus proteins and that high levels of HIV-1 Nef was expressed (Fig. 7).

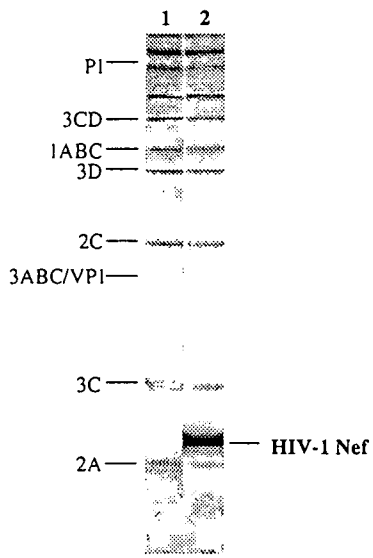

Figure 7. SDS-PAGE analysis of in vitro translation of RNAs derived from the bicistronic recombinant plasmid expressing HIV-1 Nef.
In vitro translation reactions were analysed on a 20% SDS-PAGE gel. RNAs derived from lane 1: pMCS; lane 2: pMCS/nef bicistronic

To determine whether the two Mengovirus recombinants expressing HIV-1 Nef could induce anti-HIV-1 Nef immune responses, BALB/c mice were immunised once ip with 10^6 pfu of either the parental virus vM16 or the recombinant viruses, vMCS/nef or vM2AF/nef. A control group was immunised with a mixture of two recombinant vaccinia viruses expressing HIV-1 Nef aa 73 - 147 and 148 - 206. The mice were euthanised three weeks after inoculation.

Anti-Mengovirus neutralising antibodies developed in mice immunised with vM16 and vM2AF/nef, but not in mice immunised with vMCS/nef, suggesting that vMCS/nef was unable to multiply in the animals (Table 2). None of the mice developed antibodies to the HIV-1 Nef protein, or to the individual peptides, as determined by EIA.

The CTL response to HIV-1 Nef was analysed using an in vitro peptide restimulation assay as described by Sauzet *et al.*[39] Three effector to target (E:T) ratios, 100:1, 50:1 and 25:1, were used. The recombinant Mengovirus vM2AF/nef induced a weak CTL response against an HIV-1 Nef CTL epitope at position 182 - 198.[42] In contrast, the combined recombinant vaccinia viruses expressing the same region of HIV-1 Nef not only induced a stronger CTL response against the 182 - 198 epitope, but also induced a response directed to a second epitope at position 73 - 81.[43] Neither the vaccinia virus, nor the Mengovirus recombinants induced any CTL targeted to the epitope located at position 132 - 147 (Table 2).[42,43]

Table 2. Immunogenicity of HIV-1 Nef recombinant viruses in mice.

Immunogen	Neutr. Ab titer	CTL								
		73 - 81[a]			132 – 147			182 – 198		
		100:1	50:1	25:1[b]	100:1	50:1	25:1	100:1	50:1	25:1
VM16	320	0	0.5	0	0	0	0	0	0	0
Vaccinia/Nef	<20	15.6	10.5	8.6	8.2	6	4.4	26.7	18.3	12.4
VMCS/nef	<20	0	0	0	0	0.2	0	0	0	0.9
VM2AF/nef	80	11.1	3.2	0	0	0	0	11.1	10.1	9.3

CTL results are given as % specific lysis of P815 target cells infected with recombinant vaccinia viruses expressing HIV-1 Nef. Background, defined as % lysis of cells infected with wild-type vaccinia virus (Copenhagen strain), was subtracted. A significant CTL response was defined as ≥10% specific lysis (above background) at two or more effector:target (E:T) ratios.

[a] *Nef amino acid residues in the peptides used for in vitro amplification of the CTL population*

[b] *Effector to target ratio.*

No proliferative responses were induced by either the Mengovirus or the vaccinia virus HIV-1 Nef recombinants as analysed by either direct measurement of [^{3}H]-thymidine incorporation into splenocytes from immunised mice in reponse to stimulation by HIV-1 Nef-specific peptides. This was confirmed by the lack of Il-2 production as measured by [^{3}H]-thymidine incorporation into CTL.L2 cells cultured in the presence of supernatant obtained from peptide-stimulated splenocytes.

2.3.2. Characterisation of recombinant Mengoviruses expressing SIV Pol, Gag and Nef CTL epitopes and immunogenicity in macaques and mice

Recombinant Mengovirus cDNA plasmids encoding several SIV CTL epitopes were constructed. Infectious RNA transcripts were prepared and transfected into HeLa cells as described, to obtain the recombinant viruses vMC0S-Δgag/nef, vMCS-Δpol/gag/nef and vMCS-Δpol/gag/nef-2AF. Similar to what was seen for the HIV-1 Nef recombinants, all of the recombinant viruses had a very small plaque phenotype (≤25% that of vM16) (Fig. 8).

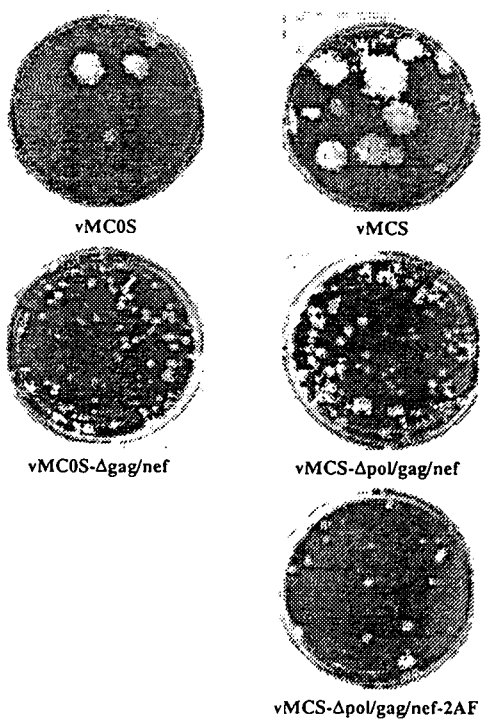

Figure 8. Plaque size of the SIV CTL recombinant viruses compared to that of the parental viruses.

RT-PCR analysis of cellular RNAs obtained from infected HeLa cells as described[41], and using Mengovirus-specific oligonucleotide primers M-609 and M-1212, demonstrated that the recombinant viruses were genetically stable *in vitro* at least to passage 10 (Fig. 9).

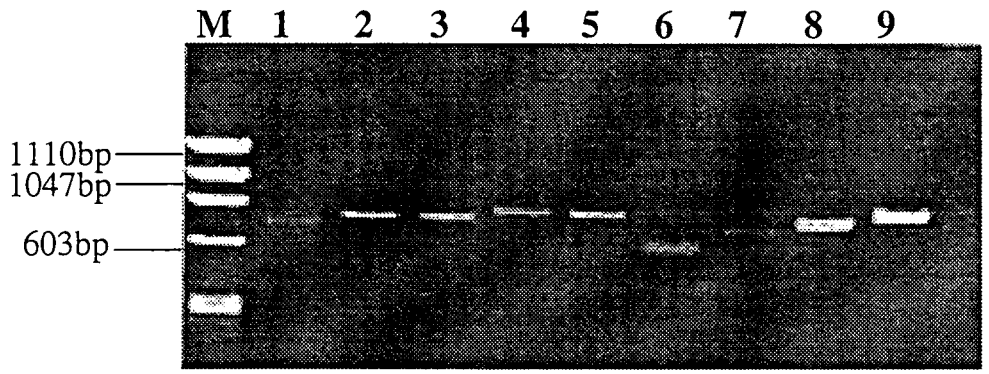

Figure 9. Genetic stability of the SIV CTL recombinant viruses.

Genetic stability of the recombinant viruses was determined by analysing sequential passages of the virus stock for the continued presence of the inserted sequences by RT-PCR using the Mengovirus-specific primers M-609 and M1212 as descibed for the HIV-1 Nef recombinant viruses. Lane M: marker; lane 1: vMC0S-Δgag/nef p10; lane 2: vMCS-Δpol/gag/nef p1; lane 3: vMCS-Δpol/gag/nef p10; lane 4: vMCS-Δpol/gag/nef-2AF p1; lane 5: vMCS-Δpol/gag/nef-2AF p10; lane 6: pMCS; lane 7: pMC0S; lane 8: pMCS-Δpol/gag/nef; lane 9: pMCS-Δpol/gag/nef-2AF.

In vitro translation analysis of RNAs transcribed from the pMCS-Δpol/gag/nef and pMCS-Δpol/gag/nef-2AF cDNAs confirmed the synthesis of additional non-viral proteins with molecular weights compatible with the Δpol/gag/nef-L and Δpol/gag/nef-2AF proteins. As described for the HIV-1 Nef recombinants, the 2AF sequence allowed efficient separation (>70%) of the heterologous protein from L (Fig. 10).

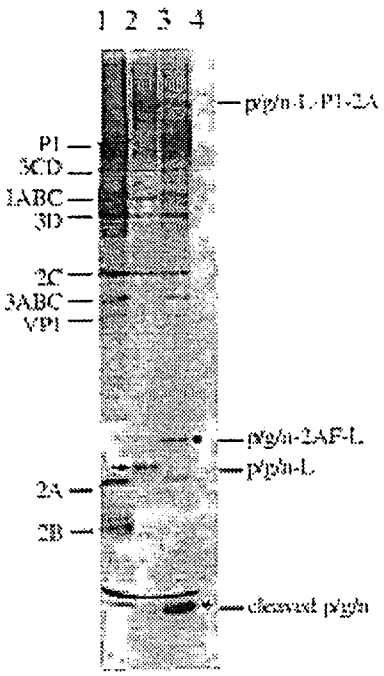

Figure 10. SDS-PAGE analysis of expression of the SIV CTL epitopes as demonstrated by in vitro translation.

Reactions were programmed with mRNAs (40μg/ml final concentration) derived from lane 1: pMCS; lane 2: pMCS-Δpol/gag/nef; lane 3: pMCS-Δpol/gag/nef-2AF; and lane 4: no RNA.

In a first experiment to evaluate the immunogenicity of these SIV CTL recombinants, three rhesus macaques were immunised twice, one month apart, by the im route with 10^6 pfu of vMC0S-Δgag/nef. A control animal was immunized with vM16. All of the animals became infected, as demonstrated by the development of anti-Mengovirus neutralising antibodies (Table 3), but no CTL activity directed to SIV Gag (182 - 190) or Nef (155 - 178) epitopes could be detected using an *in vitro* peptide restimulation assay and autologous peptide-pulsed *Herpesvirus papio*-transformed B-lymphocytes as target cells as described, even though this method could detect significant anti-Nef CTL responses (specific lysis of >10% at E:T ratios of 70:1 and 23:1 in several experiments) in a positive control animal which had previously been immunised with lipopeptides corresponding to amino acids 155 – 178 of SIV Nef.[44]

Table 3. Immunogenicity of recombinant Mengovirus expressing SIV Gag and Nef CTL epitopes in macaques.

Macaque	Immunogen	Neutr. Ab titer after 1st immunisation	Neutr. Ab titer after 2nd immunisation
92194	vM16	5500	5500
92193	vMC0S-Δgag/nef	700	1800
92195	VMC0S-Δgag/nef	2500	6500
92196	VMC0S-Δgag/nef	2500	6500

The animals received two immunisations one month apart of 10^6 pfu each, of the indicated viruses via the im route. Anti-Mengovirus neutralising antibody titers 30 days after each immunisation were determined as described.[38]

In a second experiment, four rhesus macaques were immunised via either the im or iv routes with 10^6 pfu of the recombinant virus vMCS-Δpol/gag/nef, while two control animals received vM16. Due to the fact that the SIV Gag CTL epitope (182 - 190) is Mamu A01 restricted, only animals that had a Mamu A01-like haplotype were included in the study. As the haplotypes of the animals were determined using a serological assay, discrimination between Mamu A01 and Mamu A01-like could not be made.[45] Surprisingly, none of the animals that received the recombinant were infected, not even those inoculated by the iv route (Table 4), while both controls were readily infected. A second immunisation with 10^7 pfu via the on or iv routes was, therefore given. After this second immunisation, two of the animals developed very low titers of neutralising antibodies to Mengovirus, while the other two again resisted infection (Table 4). Three months later the animals were again immunised, but this time with $2x10^7$ pfu of vMCS-Δpol/gag/nef-2AF. The rationale for this third immunisation was to determine whether the presence of a cleavage site allowing expression of the heterologous protein separate from L would increase infectivity of the recombinant *in vivo*. All 4 animals developed high titers of anti-Mengovirus neutralising antibodies (Table 4) in

response to this high-dose inoculation. but no CTL or antibody responses to the expressed epitopes were detectable.

Table 4. Immunogenicity in macaques of recombinant Mengoviruses expressing SIV Pol, Gag and Nef CTL epitopes.

Macaque	Immunogen	Route	Dose	Neutr. Ab	Immunogen	Route	Neutr. Ab
0140	vM16	iv/on	$10^6/10^7$	2500/2500	vM16	iv	8000
0142	vM16	im/iv	$10^6/10^7$	11000/11000	vM16	im	12000
0143	VMCS-PGN	iv/on	$10^6/10^7$	<100/400	VMCS-PGN-2AF	iv	>16000
0144	VMCS-PGN	iv/on	$10^6/10^7$	<100/200	VMCS-PGN-2AF	Iv	6000
0146	VMCS-PGN	im/iv	$10^6/10^7$	<100/<100	VMCS-PGN-2AF	Im	4000
0150	VMCS-PGN	im/iv	$10^6/10^7$	<100/<100	VMCS-PGN-2AF	Im	2500

Animals were immunised a total of 3 times with different viruses at different doses and via different routes. The first 2 immunisations were with recombinant virus expressing the Pol, Gag and Nef CTL epitopes fused with L and were given 2 months apart. The third immunisation was with recombinant virus expressing the Pol, Gag and Nef CTL epitopes cleaved from L and was given 3 months after the second immunisation. Neutralising antibody titers were determined 30 days after each immunisation as described.[38]

The apparent lack of infectivity of some of these recombinants for macaques, in contrast to what was previously shown[6,13], prompted us to investigate the infectivity of these recombinants for mice. BALB/c mice were immunised ip with 10^6 pfu of either the recombinant viruses or the parental poly(C) truncated or deleted Mengovirus vectors vM16[10] or vMC0.[11] All of the animals were infected, but the neutralising antibody titers in the animals which received the recombinant viruses were substantially lower than those found in animals immunised with the parental vector virus (Table 5).

Table 5. Neutralising antibody titers of BALB/c mice immunised with the Mengovirus SIV CTL recombinants.

Immunogen	Neutralising antibody titer
VM16	320
VMC0S	140
VMC0S-Δgag/nef	30
VMCS-Δpol/gag/nef	100
VMCS-Δpol/gag/nef-2AF	80

Mice were inoculated once via the ip route with 10^6 pfu of the indicated virus preparations.

Furthermore, no antibodies, as determined by EIA using SIV peptides corresponding to: Nef 155-178, Gag 182-190 and Pol 590-598 and HIV-1: Nef 66-100, 93-120, 115-146, 137-168, 155-185 and 182-206) (Neosystems, Strasbourg) at 100ng/well to coat plates, and anti-human IgG as secondary antibody, could be demonstrated. Moreover, no CTL responses targeted to SIV Nef 155 - 178 could be demonstrated in the animals. However, absence of an anti-Nef CTL response in this experiment is perhaps not surprising since Winter *et al.*[40] recently failed to demonstrate CTL epitopes in this region of SIV Nef in BALB/c mice. The lack of antibodies to the expressed epitopes in both mice and macaques can possibly be explained by the fact that these short epitopes might not include any complete B-cell epitopes.

2.4. Discussion

The immune mechanisms involved in protection from HIV and SIV infections remain unclear, but it is likely that a successful vaccine will need to induce both neutralising antibody and CTL responses. A strong CTL response against the regulatory viral proteins Nef, Tat and Rev, which are expressed early in the viral replication cycle, could allow elimination of

infected cells before release of new particles could occur.[40,46] The Nef protein is an attractive target because: i) Nef mRNA represents the most abundant viral transcript in the first hours of viral replication[24] and ii) Nef is highly immunogenic in HIV-infected individuals[25,46,47] and SIV-infected macaques.[29,44,48] Although the exact role of Nef in HIV and SIV infection has not been determined, there is good evidence that it is required for maintaining high virus loads in SIV-infected macaques.[18] Furthermore, it has been demonstrated that some HIV-1-infected long-term non-progressors carry viruses containing truncated *nef* sequences.[17,19] Additionally, in the SIV macaque model, it has been shown that inoculation of macaques with a live attenuated *nef*-deleted SIV can induce protection from subsequent challenge with virulent SIV.[49] Gallimore *et al.* also demonstrated that a vaccinia virus SIV Nef recombinant could induce protection from SIV challenge in macaques if the CTL precursor frequency is $\geq$ 1: 10000.[50] Similarly, the *gag* gene products, which are also present in large amounts in infected cells, should prove useful targets for induction of anti-HIV-1 or -SIV CTL responses.[27,48]

Recombinant Mengoviruses expressing HIV-1 Nef (aa 65 - 206) or SIV Pol (590 - 598), Gag (182 - 190) and Nef (155 - 178) CTL epitopes were constructed. The N-terminal 64 aa of the HIV-1 Nef protein were deleted as it was previously demonstrated that the maximum insert size tolerated by Mengovirus was 525-738 nucleotides[5], and to overcome potential problems due to the oncogenicity of intact HIV-1 Nef.[20] Furthermore, the regions known to contain CTL epitopes are located in the central and C-terminal regions of the protein.[42,51] The recombinant viruses vMCS/nef and vM2AF/nef were viable and genetically stable *in vitro* for several passages, but both had extremely small plaque phenotypes. The heterologous sequences were expressed either as a fusion protein with the Mengovirus L protein, or in cleaved form through introduction of the FMDV 2A protein, which was shown to undergo autocatalytic cleavage.[30] The rationale for the induction of a cleavage site was to reduce

potential interference between the foreign proteins and the viral polyprotein, in order to increase infectivity of the resulting recombinant viruses and possibly allow for a larger insertion. Cleavage of the heterologous proteins as mediated by the 2AF sequence seemed to be efficient, at least *in vitro*, with approximately 85% cleavage as demonstrated using *in vitro* translation. The cleavage of the heterologous sequences from L, however, did not seem to influence the plaque size of the recombinants, as both types of recombinant viruses had a similar plaque phenotype. Although the Mengovirus HIV-1 Nef recombinants both had a similar small plaque phenotype *in vitro* (Fig. 4), the recombinant Mengovirus vMCS/nef, that expressed HIV-1 Nef fused to L, failed to infect BALB/c mice, while the recombinant that expressed HIV-1 Nef cleaved from L (vM2AF/nef) was infectious *in vivo*. Thus it appears that for certain of these recombinants, infectivity for animals could not be correlated with *in vitro* viability. However, the neutralising antibody titers in mice immunised with the recombinant vM2AF/nef was significantly lower than that of mice immunised with the parental virus vM16, and this together with the small plaque phenotype of both HIV-1 Nef recombinants, would indicate that these viruses are severely attenuated, both *in vitro* and *in vivo*.

Similar results were obtained in macaques for the recombinant Mengoviruses expressing SIV Pol, Gag and Nef CTL epitopes arranged as a string of beads upstream of the Mengovirus polyprotein. In mice, neutralising antibody titers to these recombinants were significantly lower than those obtained in mice immunised with the parental vM16 (Table 5). Moreover, the recombinant virus vMCS-Δpol/gag/nef, that expressed the CTL epitopes fused to L, failed to infect rhesus macaques, while the recombinant that expressed the CTL epitopes cleaved from L (vMCS-Δpol/gag/nef-2AF) was infectious for the animals. It, therefore, appears that some Mengovirus recombinants that express heterologous proteins fused to L are severely

attenuated *in vivo*, leading to loss of infectivity for animals. It has recently been reported that the Mengovirus L protein is involved in shut-off of host cell protein synthesis.[52] Indeed, extracts from HeLa cells infected with the HIV-1 Nef recombinant viruses show that cells infected with these recombinants showed very little shut-off of host-cell protein synthesis (Fig. 5). It is, therefore, possible that insertion of the foreign genes into L has inhibited its function sufficiently to prevent infection *in vivo*, although still allowing infection *in vitro*. This inhibition seems to depend on the nature of the inserted sequence, as Mengovirus recombinants expressing a 450 bp region of HIV-1 gp120 or a CTL epitope from LCMV fused to L, was not only infectious *in vitro*, but also infectious and immunogenic *in vivo*.[6,16] Infectivity *in vivo* for mice and macaques was not correlated, as the recombinant vMCS-Δpol/gag/nef that failed to infect macaques, retained at least some infectivity for BALB/c mice (Tables 4 and 5). Zoll *et al.* demonstrated that deletion of L resulted in host cell-restricted virus growth, and it is possible that this phenomenon could explain the results obtained with certain of these Mengovirus recombinants.[52] Most puzzling is the failure of vMCS-Δpol/gag/nef to infect macaques, given that the theoretically more attenuated vMC0S-Δgag/nef apparently retained significant infectivity for these animals. At the present time, we have no concrete explanation for this discrepancy. The vMC0s recombinant has a totally deleted poly(C) tract, a deletion which has previously been shown to severely attenuate Mengovirus infectivity for mice, and which would thus be expected to result in a less infectious virus than the vMCS recombinant which still retains about 50% of its poly(C) tract.[11] Whether the additional Pol CTL epitope (9 aa) in vMCS-Δpol/gag/nef is able to alter virus infectivity for rhesus macaques so drastically is not known.

The failure of the HIV-1 Nef recombinants to induce antibody responses to the Nef protein is disappointing as a Mengovirus recombinant expressing 450 bp from the V3-C4 region of

HIV-1 gp120 induced strong humoral immune responses in both mice and macaques.[6] However, it remains possible that a booster immunisation three weeks to one month later might have led to the development of an anamnestic antibody response in the mice. In the case of the SIV CTL recombinants, the lack of antibodies to the expressed epitopes could be explained by the fact that the short epitopes expressed by the recombinants possibly did not contain complete B-cell epitopes.

These results indicate that although attenuated Mengovirus has proven to be a good vector for induction of CTL to a short linear epitope of LCMV,[6] and also to an epitope in HIV-1 gp120[16], it was only weakly immunogenic for HIV-1 Nef, and also failed to induce CTL directed to selected epitopes of SIV Pol, Gag and Nef. In the case of the SIV CTL recombinants, the possibility that the "string of beads" approach using the 3Gly linkers might not have allowed the correct processing of the epitopes by the proteosome, and subsequent presentation of the epitopes in combination with MHC class I molecules on the cell-surface, cannot be dismissed. However, this approach was successfully used by Oldstone *et al.*[53] Furthermore, the 24 aa from SIV Nef contains 3 overlapping CTL epitopes[44] which were expressed without the presence of 3Gly linkers. Alternatively, in the light of the recent results which suggests that the Nef protein causes down-modulation of MHC class I receptors on the cell surface, thereby providing a mechanism for immune escape[54], it could be argued that the approach used here might have been successful if the Nef epitope had not been included. However, Nef-induced immunosuppression is unlikely to be the reason for this failure, as immunogenic SIV[40,50] and HIV-1[51] Nef have successfully been expressed in several viral and bacterial vectors. Similarly, Bourgault *et al.* have demonstrated that immunization of macaques with synthetic lipopeptides could induce Nef-specific CTL.[44] Furthermore, in the study presented here, recombinant vaccinia viruses expressing HIV-1 Nef, and to a lesser

extent the vMCS/Nef-2AF recombinant were able to induce CTL targeted to HIV-1 Nef in BALB/c mice. Asakura *et al.* have recently demonstrated that a Nef-expressing DNA vaccine induced strong Nef-specific CTL in BALB/c mice.[42] In conclusion, these results demonstrate that the nature of the insert plays an important role in the immunogenicity of live recombinant Mengovirus vectors, and this may also be true for other vectors. Altogether, in contrast to the promising results obtained in earlier studies, the results of this study provide a cautionary note on the potential utility of Mengovirus as a vector for live recombinant vaccines.

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2.6. Papers relating to study (see appendix)

1. **van der Ryst E**, Nakasone T, Habel A, Venet A, Gomard E, Altmeyer R, Girard M, Borman AM. Study of the immunogenicity of different recombinant Mengo viruses expressing HIV-1 and SIV epitopes. *Res Virol* 1998; **149**: 5 – 20.

Note

The candidate cloned all the Mengovirus HIV-1 Nef recombinants and performed all experiments to characterise these clones and recombinant viruses, including transcription, transfection, plaque assays, in vitro translation, expression of viral proteins in infected cells, genetic stability and preparation of titrated stocks. The mice inoculation studies with these viruses were performed by the candidate, as well as the subsequent neutralisation, EIA, CTL and T-cell proliferation assays. The candidate established and validated the HIV-1 Nef and peptide EIAs. The candidate was also responsible for the planning of, and preparing the protocols for (together with T Nakasone), the animal studies with the SIV CTL recombinants,

as well as helping with the inoculations and the CTL, EIA and neutralising antibody assays. The candidate also analysed and interpreted the results, and prepared the paper related to the study.

Contributions of other authors:

T Nakasone – cloning and characterisation of the SIV CTL recombinants, and animal work related to them (together with the candidate)

A Habel – provided the pM2AF parental vector

R Altmeyer – provided the pMCS parental vector

E Gomard and A Venet - valuable advice for establishing the CTL assays

M Girard - initiated the Mengovirus experiments and provided valuable scientific advice

A Borman – supervision of the research

CHAPTER 3

EVALUATION OF CANARYPOX-HIV-1 RECOMBINANT VACCINE CANDIDATES IN THE CHIMPANZEE MODEL

3.1. Introduction

Primate models for AIDS vaccine development include the SIV/macaque model, the HIV-1/chimpanzee model, the HIV-2/macaque model, and the SHIV/macaque model.[1] The chimpanzee is the only animal that can be reliably infected with HIV-1[2], but pigtail macaques (*Macaca nemestrina*) can be infected under certain conditions.[3] Chimpanzees can be infected with some T-cell line-adapted stains of HIV-1 via both the iv and intravaginal routes, but although the animals become persistently infected, they do not develop AIDS.[2] However, one case of AIDS developing in a HIV-1 infected chimpanzee was recently reported.[4] The chimpanzee model has been widely used for the study of vaccine-induced protection from HIV-1 challenge, and to date more than 25 vaccine strategies, (both active and passive vaccines) have been tested in chimpanzees.[5] Several groups have shown that gp120- or gp140-based vaccines that induce neutralising antibodies provide protection against experimental infection with either cell-free or cell-associated HIV-$1_{IIIB/LAI}$.[6,7,8,9] These studies demonstrated a strong correlation between protection against challenge and the titer of neutralising antibodies directed to the V3 loop of the HIV-1 gp120. Antibody-dependent cytotoxicity was also detected in the immunised animals.[10] Passive immunisation of a chimpanzee with a V3-targeted, HIV-1_{LAI}-specific neutralising monoclonal antibody protected the animal fully from challenge with cell-free HIV-$1_{IIIB/LAI}$, even when the antibody was administered after virus inoculation.[11,12] Altogether, these studies established neutralising antibody as a reliable surrogate marker for HIV-1 vaccine efficacy. However, the neutralisation sensitivity of primary isolates of HIV-1 differ from that of T-cell line adapted

strains, and it is possible that neutralising antibody might be less important in protection from primary isolates of HIV-1.[13]

The use of live recombinant vaccines for HIV-1 is an atttractive option, as they have the ability to stimulate both humoral and cell-mediated immunity. Poxviruses are attractive as candidate viral vectors for the following reasons: i) the double-stranded viral DNA encodes all the enzymes necessary for transcription, ii) they enter cells in a non-specific manner, iii) the viral genome is expressed exclusively in the cytoplasm of infected cells, and do not undergo reverse transcription, and iv) isolated viral DNA is non-infectious.[14] The successful vaccinia immunisation program against smallpox, moreover, demonstrated that poxviruses are good immunogens.[15] It has, furthermore, been demonstrated that poxviruses can tolerate insertions of at least 25000bp in size and retain viability.[16] This, together with the ability to manipulate the genome of poxviruses to simultaneously express multiple heterologous antigens have raised the prospect of using these viruses to produce multivalent vaccines.[17] Vaccinia virus vectors expressing a variety of HIV and SIV genes have been tested in both animals and humans.[18,19,20] In most cases HIV-1-specific cellular responses, including CTL, were induced, but humoral responses were much weaker. Altogether, this has raised interest in vaccinia virus as a vector for live recombinant HIV vaccine candidates. Unfortunately, there are safety concerns with the use of vaccinia virus as a live vector. This is due to its broad host range and the rare, but severe, complications that occurred during the smallpox vaccination program. In general the incidence of complications were associated with the immunological status and age of the vaccinee, as well as the virus strain. There are particular safety concerns in using vaccinia virus in immunocompromised hosts.[17,21,22] Furthermore, large numbers of the current population have pre-existing immunity to vaccinia virus, due to former widespread immunisation against smallpox. These issues have led to the development

of new attenuated vaccinia strains such as the NYVAC strain (derived from the Copenhagen strain of vaccinia virus) and modified vaccinia virus Ankara that should be safer.[23,24] In order to overcome the problem of pre-existing immunity to vaccinia, poxviruses from other genera are also being considered.

In contrast to vaccinia virus and other members of the genus orthopoxvirus, members of the genus avipox virus are highly host range-restricted, and productive replication can only take place in avian species.[25] Initial interest in avipoxviruses as viral vectors was focussed on applications in the poultry industry, but results from studies with a fowlpox recombinant virus expressing the rabies viral glycoprotein G demonstrated that the recombinant not only expressed the rabies glycoprotein in mammalian cells in tissue culture, but also elicited a strong anti-rabies immune response in several non-avian species. This ability of avipoxviruses to elicit protective immune responses in non-avian species, together with their inability to productively replicate in these species (which provides a significant safety advantage), led to considerable interest in avipoxviruses as live viral vectors for human and animal vaccines.[17] Subsequent studies with a canarypox rabies recombinant demonstrated it to be superior to a fowlpox recombinant. Following these results a plaque cloned isolate of canarypox virus was derived from the vaccine strain KANAPOX, and designated ALVAC.[17] Although canarypox-based viral vectors shows promise as HIV vaccine candidates, they are likely to be less immunognic than vaccinia virus that can replicate in mammalian cells, and several approaches to improve their efficacy as immunisation vehicles are being explored. These include modulating virus/host cell interctions and co-expression of cytokines as immunoadjuvants.[22]

In the studies desribed here protection of chimpanzees from iv HIV-1 challenge by immunisation with a recombinant canarypox-HIV-1 virus was studied in a first series of experiments. In a second series of experiments, vaginal HIV-1 infection of female chimpanzees, and protection from vaginal challenge following immunisation with a recombinant canarypox-HIV-1 virus, were studied.

3.2. Study of protection from intravenous HIV-1 challenge in chimpanzees immunised with a recombinant canarypox-HIV-1 virus

3.2.1. Introduction

Recombinant canarypox virus vectors induce both humoral and T cell-mediated immunity against diverse pathogens in animals.[26,27] Furthermore, a recombinant canarypox-rabies G protein virus proved to be safe and immunogenic in humans.[28] In a recent study ALVAC-HIV-1 vCP125, a recombinant canarypox virus expressing the gp160 *env* gene product of HIV-1_{MN}, induced HIV-1-specific neutralising antibody and CTL responses in 90% and 40% of human volunteers, respectively, when combined with a subunit rgp160 vaccine in a prime-boost regimen.[29] A similar regimen, however, failed to protect chimpanzees from a heterologous challenge with cell-free HIV-1_{SF2}.[30] A possible explanation for this failure was that the dose ($10^{6.1}$ $TCID_{50}$) of canarypox virus used to immunise the chimpanzees was too low. In this study chimpanzees were immunised with a higher dose of a recombinant canarypox-HIV-1 virus, and challenged with homologous and heterologous HIV-1 strains.

3.2.2. Materials and Methods

3.2.2.1. Animals

Adult male chimpanzees (*Pan troglodytes*) were housed at the Laboratory for Experimental Medicine and Surgery in Primates (LEMSIP), New York University, in biosafety level 2

facilities in accordance with institutional guidelines and standard practices for the containment of infectious diseases and the humane care and use of chimpanzees in biomedical research.[31] Before all procedures the chimpanzees were anaesthetized by im injection of ketamine hydrochloride (10 mg/kg).

3.2.2.2. Study design

Two naive chimpanzees (C-401 and C-451), were immunised via the im route with $4x10^8$ pfu of ALVAC-HIV-1 vCP250, a recombinant canarypox virus that expresses not only the HIV-$1_{IIIB/LAI}$ gp120 and the transmembrane segment from gp41 (gp120/TM), but also the *gag* and protease gene products. The genomic organisation of vCP250 is shown in figure 1. The ALVAC recombinants were contructed by the Virogenetics corporation.

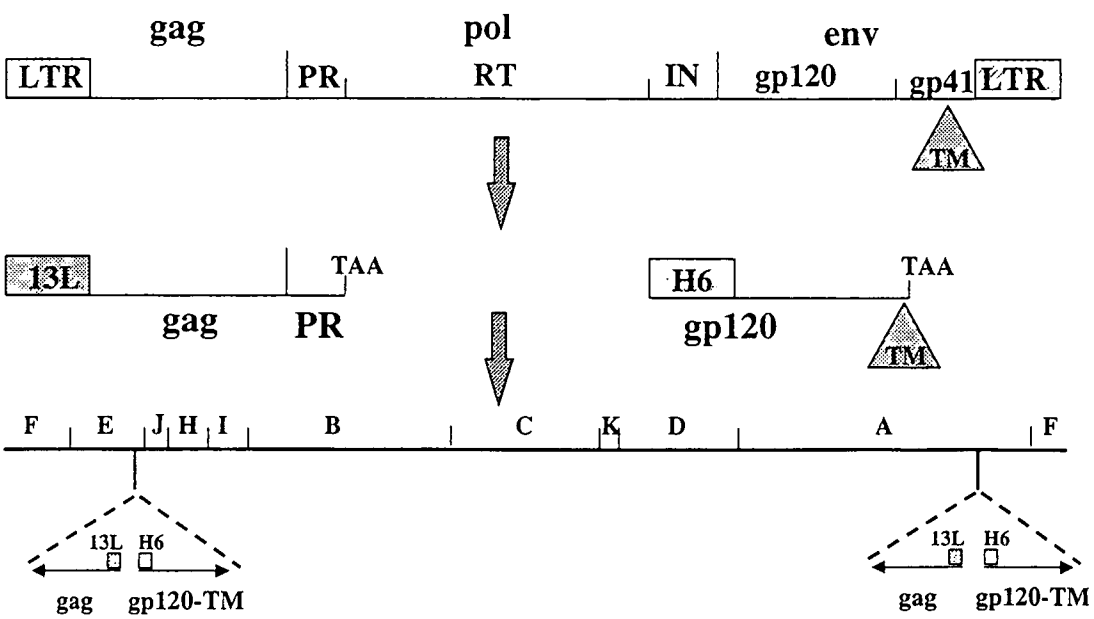

Figure 1. Genomic organisation of ALVAC vCP250

Insertion of the HIV-1 genes is displayed on a schematic representation of the ALVAC genome. The HIV-1 genes are placed under control of the poxvirus H6 and 13L promoter elements. Arrows indicate transcription orientation (J Tartaglia, personal communication).

The animals received a total of five doses of vCP250 at months 0, 1, 5, 9 and 11. No subunit booster inoculations were given (Figure 2). One month after the last booster inoculation, the immunised animals and a naive control (C-353) were challenged via the iv route with $6x10^5$ viable PBMC (equivalent to 20 infectious doses) from an HIV-$1_{IIIB/LAI}$ infected chimpanzee (C-435) (see section 3.2.2.3) (Figure 2).

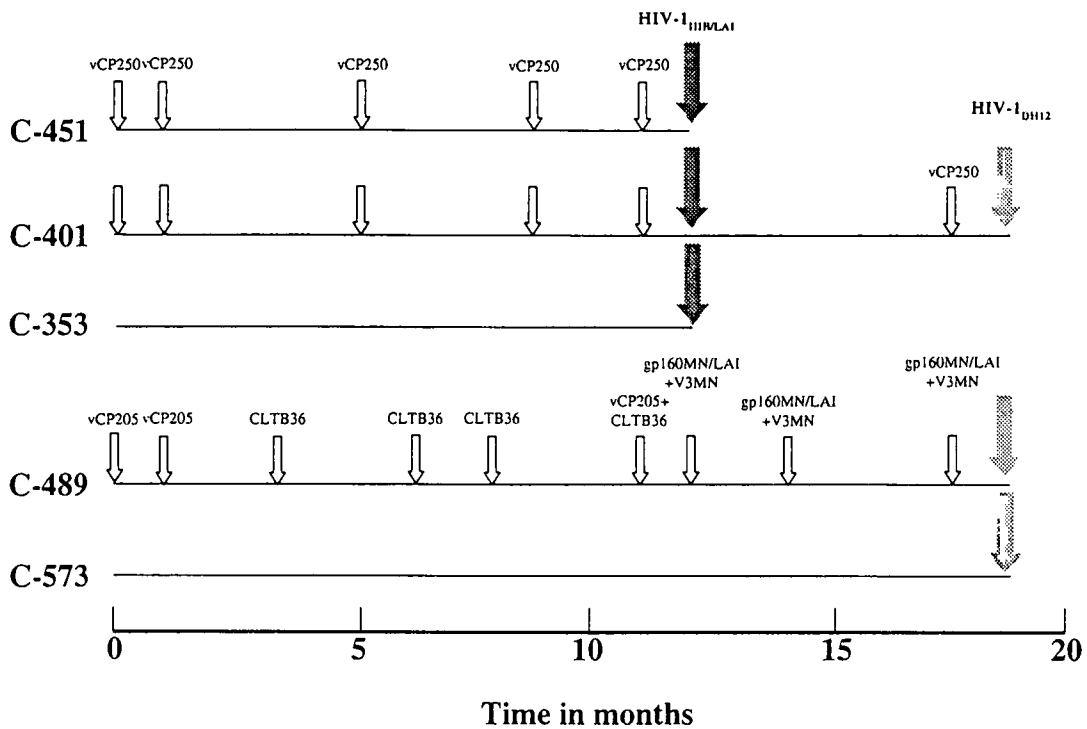

Figure 2. Study design for iv HIV-1 challenge study

Immunisations are shown as open arrows and challenges as closed arrows.

3.2.2.3. Challenge viruses

For preparation of the cell-associated HIV-$1_{IIIB/LAI}$ challenge stock, a chimpanzee (C-087) was inoculated via the iv route with 100 $TCID_{50}$ of the NIH HIV-$1_{IIIB/LAI}$ stock.[32] The animal became readily infected and a PBMC stock obtained 3 months after infection was used to infect a second naive chimpanzee (C-435). This animal also became infected as demonstrated by persistent isolation of virus from its PBMC.[8] At 4 months after inoculation, a stock of its

PBMC was prepared, aliquoted and cryopreserved. The titer of this stock is 30 infectious cells (IC) per 10^6 PBMC.

HIV-1_{DH12} is a clinical isolate that readily infects chimpanzees and multiplies in their cells *in vitro*.[33] A chimpanzee challenge stock of HIV-1_{DH12} was obtained from Malcolm Martin and Alan Schultz at the NIH. The chimpanzee infectious dose (CID) of this stock is between 6 and 16 $TCID_{50}$.[34]

3.2.2.4. Virus isolation and serology

The presence of virus in PBMC was monitored by cocultivation of each animal's PBMC with phytohemagglutinin-stimulated human indicator PBMC and periodic assays of culture supernatants for reverse transcriptase activity[35] or p24*gag* antigen.[36] Freshly stimulated human PBMC were added every 10 days.

Serial two-fold dilutions of serum samples were tested for total anti-HIV-1 antibodies with a commercially available EIA kit (Diagnostics Pasteur, Marnes-la-Coquette, France). Titers were defined as the reciprocal of the last serum dilution to give an optical density reading above the cut-off recommended by the manufacturer. Antibodies to HIV-1 strains IIIB/LAI and DH12 V3-loop peptides were also determined by EIA, as previously described.[30] WB assays were done using a commercially available kit (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) and a serum dilution of 1:100. Neutralising antibodies to HIV-$1_{IIIB/LAI}$ were measured using a quantitative syncytium inhibition assay on CEM-SS cells.[37]

Anti-gp120 antibody titers and avidity were determined using an EIA. Briefly, duplicate microtiter plates (Maxisorp, Nunc) were coated with concanavalin-A at 2.5µg/well for 1h at

room temperature, after which they were washed with PBS containing 1% Tween-20 and coated with 50 ng/well of rgp120 (British MRC AIDS Reagent Research Project) which was derived from a clinical isolate of HIV-1 (HIV-1_{GB8}).[38,39] Sequential two-fold dilutions of sera in PBS containing 2% BSA were added and incubated for two hours at 37°C. Binding antibodies were detected by standard methods with alkaline phosphatase-conjugated anti-human IgG and p-nitrophenyl phosphate substrate using one of the duplicate plates, whereas the other plate was first soaked with PBS containing 8M urea and then processed. The anti-gp120 antibody avidity index (AI) was then calculated as previously described from the difference in OD values at 405 nm in corresponding wells.[40,41] The AI was determined over a range of serum dilutions in order to control for variations in antibody concentration.

3.2.2.5. CTL assays

$CD8^+$-specific CTL responses in the animals were evaluated using a peptide-restimulation assay and autologous Epstein-Barr virus-transformed B-lymphoblastoid cell lines as target cells.[42] PBMC were stimulated *in vitro* with pools of overlapping 20-mer peptides corresponding to amino acids 30 to 510 in Env and 139 to 369 in p24*gag* of HIV-1_{SF2}; culture medium was RPMI-1640 containing 10% FBS, 5% human IL-2 (Schiaparelli), 100 IU/ml rIL-2 (Cetus Corporation), antibiotics, and 10μg/ml of each peptide. After 8 days in culture, $CD8^+$ cells were purified using magnetic beads coated with anti-CD8 antibodies (Dynal, Lake Success, New York) and tested for cytolytic activity in a standard ^{51}Cr release assay against autologous target cells pulsed with the homologous peptide pool (10μg/ml of each peptide).[43] Control targets were pulsed with a heterologous peptide pool.

3.2.3. Results and discussion

Two chimpanzees were immunised with ALVAC-HIV-1 vCP250; animals were bled regularly and their immune responses to HIV-1 monitored. Anti-V3 IIIB/LAI antibody titers remained at low levels until after the fourth immunisation (9 months) when an increase in titers was observed in serum from C-401 (Table 1); C-451 had an increase in anti-V3 antibody titers only after the fifth immunisation at 11 months. Total anti-HIV-1 antibody titers, however, remained at low levels (Fig. 3).

Table 1. Anti-V3 antibody titers of chimpanzees immunised with ALVAC-HIV-1 vCP250.

	C-451	C401	
Months[a]	**V3-IIIB/LAI**	**V3-IIIB/LAI**	**V3-DH12**
0	589	330	ND[b]
6	710	2 100	ND
9[c]	330	760	ND
10	850	8 900	ND
11[c]	340	3 600	ND
12[d]	7 900	22 000	ND
17[c]	ND	8 800	<6
18[e]	ND	32 000	<6
19	ND	38 000	22
20	ND	20 000	140
21	ND	15 000	170

[a] *Months after first immunisation.*

[b] *ND, not done.*

[c] *Booster inoculation with vCP250.*

[d] *Challenge with cell-associated HIV-$1_{IIIB/LAI}$.*

[e] *Challenge with cell-free HIV-1_{DH12}.*

WB assays done at the time of challenge (12 months) demonstrated that although a good anti-p24*gag* antibody response was seen in both immunised animals, p17*gag* antibodies were observed only in C-401; and that the anti-Env response was weak in both chimpanzees (Fig. 4; lanes BC, first challenge). Neutralising antibodies to HIV-$1_{IIIB/LAI}$ were detected only after the fourth and fifth immunisations for C-401 and C-451, respectively (Table 2). In addition, no neutralising antibodies to a clinical isolate of HIV-1 (BZ167) were detected using a resting cell assay, as described by Zolla-Pazner *et al.*[44]

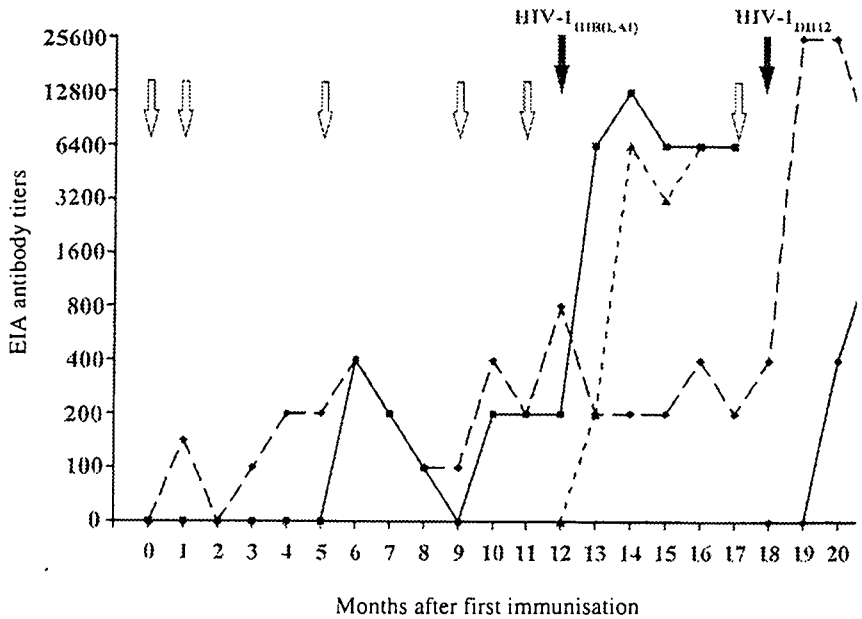

Figure 3. Anti-HIV-1 antibody responses in chimpanzees.

Animals C-451 and C-401 were immunised with ALVAC-HIV-1 vCP250 (open arrows) and challenged with HIV-$1_{IIIB/LAI}$ and/or HIV-1_{DH12} (closed arrows). Animals C-353 and C-573 were included as naive unimmunised controls. Antibody titers were determined by EIA using serial two-fold dilutions of chimpanzee serum samples; titers are defined as the reciprocal of the last dilution of serum that gave an OD reading above the cut-off value recommended by the manufacturer.

Symbols: C-451 (■; solid line), C-401 (◆; dashed line), C-353 (▲; dotted line) and C-573 (●; solid line).

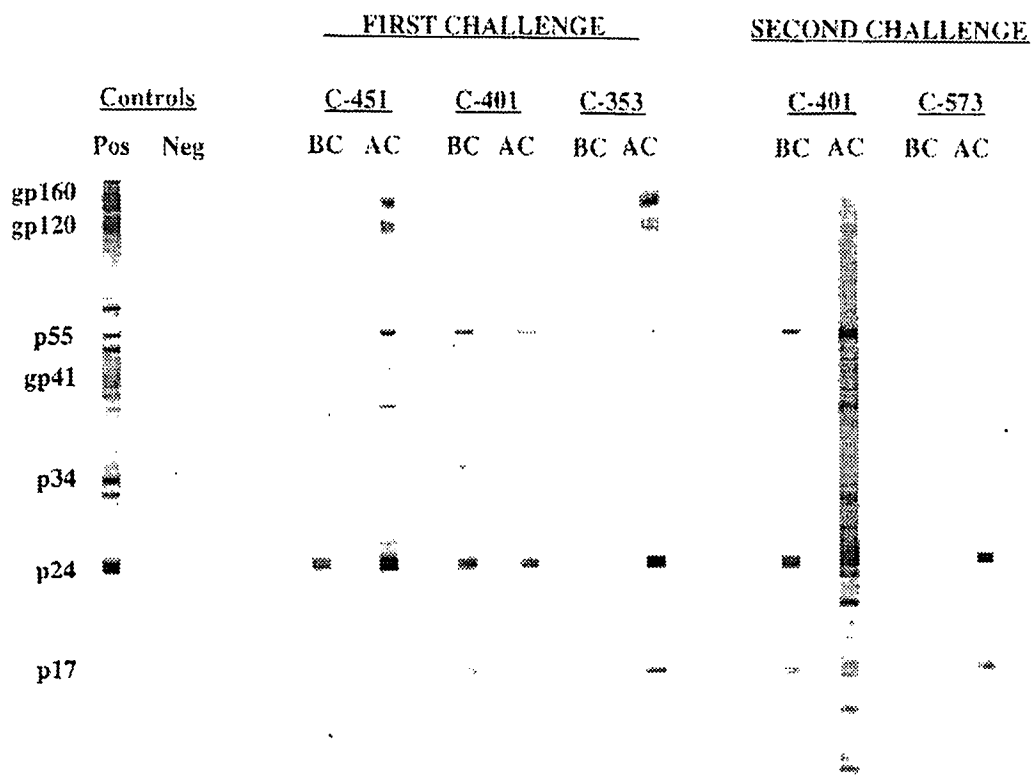

Figure 4. HIV-1 antigen-specific responses of chimpanzees at time of challenge (BC) and 8 weeks after challenge (AC).

WB assays were performed using a serum dilution of 1:100. First challenge: HIV-$1_{IIIB(LAI)}$, second challenge: HIV-1_{DH12}.

One month after the last booster inoculation, the immunised animals and a naive control (C-353) were challenged via the iv route with $6x10^5$ viable PBMC (equivalent to 20 infectious doses) from an HIV-$1_{IIIB/LAI}$ infected chimpanzee (C-435). In three previous experiments, this dose of cell-associated virus from the same stock of cryopreserved PBMC, readily infected three of three naive chimpanzees.[8,45] The total anti-HIV-1, anti-V3 IIIB/LAI and neutralising antibody titers of C-401 were 2 to 4-fold higher than those of C-451 at time of challenge (Fig. 3, Tables 1 and 2). The anti-canarypox antibody levels of C-401 and C-451 as determined by EIA were 160 and 60 EU/ml, respectively, indicating a strong immune response to the virus vector (a cut-off value of 0.6 EU/ml is considered positive in human volunteers). C-401 was,

therefore, in all aspects, a better responder than C-451 to the canarypox virus-HIV-1 vaccine candidate.

Table 2. HIV-1$_{IIIB/LAI}$ neutralising antibody titers.

	Chimpanzee	
Months[a]	**C-451**	**C-401**
6	4	4
9[c]	0	0
10	4	32
11[c]	0	16
12[d]	32	128
13	>512	64
14	>512	32
18[e]	ND[b]	64

Neutralising antibody titers were expressed as the reciprocal of the dilution of serum that resulted in 90% reduction of the number of syncytia formed on CEM-SS cells by HIV-1$_{IIIB/LAI}$.[37]

[a]*Months after first immunisation.*

[b]*ND, not done.*

[c]*Booster inoculation with vCP250.*

[d]*Challenge with cell-associated HIV-1$_{IIIB/LAI}$.*

[e]*Challenge with cell-free HIV-1$_{DH12}$.*

No CTL activity directed to either Env or p24*gag* epitopes were, however, detected in either of the chimpanzees at any of the time points tested, including the day of challenge. The lack of a CTL response following five immunisations with a live recombinant canarypox virus is surprising, but it should be noted that a significant CTL reponse, using a different recombinant canarypox-HIV-1 virus (vCP125), was seen in only 40% of human volunteers,[29] while chimpanzees immunised with this recombinant failed to develop a CTL response.[30] Another explanation for this lack of CTL activity might be that the assay was not sensitive

enough. However, CTL activity could be demonstrated in HIV-1_{SF2}-infected chimpanzees, starting at 6 - 8 weeks post-inoculation, using this assay method (C Walker, personal communication).

After challenge, the chimpanzees were bled every 2 weeks for 8 weeks and then at monthly intervals. PBMC from the control animal and C-451 were initially virus positive at 4 and 2 weeks after challenge, respectively. Chimpanzee C-451 also showed a strong anamnestic antibody response by both EIA (Fig. 3) and neutralising antibody assays (Table 2). Consistent with these results, WB analysis showed not only that bands representing Env and Gag antibody reactivity had intensified, but also that novel serum reactivities had appeared (Fig. 4, lane AC, first challenge). In contrast, C-401 remained virus negative during 5 months of follow-up, and its antibody titers decreased slowly by all assays (Fig. 3, Fig. 4, Table 2). Of interest was the finding that the neutralising antibody titer of the unprotected animal (C-451) at time of challenge was 32, whereas that of the protected animal (C-401) was 128. Although no firm conclusions can be drawn from a study involving only two animals, this observation agrees with previous results that suggested an association between neutralising antibody titers and protection from HIV-1 infection in the chimpanzee model.[6,7,8,9,30,46] Similar results were obtained for HIV-2[47,48], while Van Rompay *et al.*[49] demonstrated that protection from oral SIV infection of neonatal macaques born from immunised mothers correlated with the titers of neutralising antibodies of the immunised females. Furthermore, it was recently demonstrated that the protection from SIV infection conferred by live attenuated SIVΔ*nef* vaccines is associated with the levels of high-avidity neutralising antibodies in the immunised animals.[50] This result also suggests that recombinant canarypox-HIV-1 viruses can induce protective neutralising antibody titers in some chimpanzees when used at a high dose in repeated immunisations.

After 5 months of follow-up, C-401 was again inoculated im with $4x10^8$ pfu of vCP250, and 1 month later it was challenged with 20 $TCID_{50}$ of cell-free HIV-1_{DH12} (1 ml of a 1:400 dilution of the virus stock provided by Malcolm Martin) together with a naive control (C-573). The gp120 gene of HIV-1_{DH12} has only 80% homology with that of HIV-$1_{IIIB/LAI}$, making this a heterologous intraclade challenge.[34] Both C-401 and the control animal readily became infected, as shown by virus isolation from PBMC at 2 weeks after challenge and thereafter. C-401 developed a strong anamnestic response to HIV-1 (Figs. 3 and 4), and infection of both animals elicited antibodies to a V3 peptide from HIV-1_{DH12} (Table 1). At the times of the last vCP250 inoculation and subsequent HIV-1_{DH12} challenge, a third animal (C-489) was also included. This chimpanzee had been immunised previously with a variety of HIV-1 immunogens, including vCP205 (a recombinant canarypox virus expressing the *env* gp120 with transmembrane region, *gag* and protease genes of HIV-1_{MN}), a HIV-1_{MN} T- and B-cell chimeric peptide, recombinant gp140 MN/LAI and a V3 MN peptide. Like C-401, chimpanzee C-489 was not protected from HIV-1_{DH12} challenge; virus was isolated from its PBMC and its antibody titer to HIV-1 increased (not shown).

At time of challenge, C-489 had an anti-HIV-1_{MN} neutralising antibody titer of 8, but no neutralising antibodies to HIV-1_{DH12} (not shown) as determined using a quantitative immunohistochemically based infectivity reduction assay on PBMC.[51] Similarly, C-401 had an anti-HIV-$1_{IIIB/LAI}$ neutralising antibody titer of 64, but no neutralising antibodies to HIV-1_{DH12}. The lack of protection from HIV-1_{DH12} challenge, therefore, was associated with the absence of neutralising antibodies to the challenge virus. However, we previously showed that two chimpanzees immunised with a recombinant gp140 MN/LAI molecule and V3 MN peptide were protected from a heterologous intraclade challenge with HIV-1_{SF2}, despite the

absence of demonstrable neutralising antibodies to HIV-1_{SF2}.[30] Berman *et al.* also demonstrated that three chimpanzees immunised with recombinant gp120 MN were protected from heterologous challenge with HIV-1_{SF2}.[52] Sera from the protected animals were unable to neutralise HIV-1_{SF2} infectivity for PBMC, but they did neutralise infectivity for AA5 cells of a T-cell line-adapted HIV-1_{SF2} variant. Since HIV-1_{SF2} replicates poorly in chimpanzees[30], it is possible that protection of chimpanzees from infection with this virus is easier to achieve than it is from other subtype B strains. This possibility is supported by the fact that chimpanzees infected with HIV-1_{SF2} could readily be superinfected with HIV-$1_{IIIB/LAI}$ or HIV-1_{DH12}.[34,53,54] However, at least one chimpanzee infected with HIV-$1_{IIIB/LAI}$ was superinfected with HIV-1_{DH12}.[53] In contrast, Shibata *et al.* could not demonstrate HIV-1_{DH12} superinfection of HIV-$1_{IIIB/LAI}$-infected chimpanzees.[34] Considerable evidence suggests that the neutralisation sensitivity of primary isolates of HIV-1 differs from that of T-cell line-adapted viruses.[13,55,56] Antibodies directed to epitopes outside of the V3 loop are more important in the neutralisation of primary isolates, while V3-targeted neutralising antibodies are important for neutralisation of T-cell line-adapted strains.[57] The lack of neutralising antibodies could, therefore, be explained by the fact that HIV-1_{DH12} is a primary isolate, whereas the IIIB/LAI strain is T-cell adapted.

The presence of high avidity anti-gp120 antibodies has also been implicated as an important correlate for protective antibody responses against HIV-1 and SIV infection.[41,58] The sera from the immunised chimpanzees were, therefore, tested for such antibodies by EIA. Sera from chimpanzees immunised with rgp140 MN/LAI combined with either a recombinant canarypox virus expressing the gp120/TM of HIV-1_{MN} (ALVAC-HIV-1 vCP125), or a V3 MN peptide, and challenged with HIV-1_{SF2} were also included in this experiment.[30] The results demonstrated that the two chimpanzees immunised with vCP250 (C-401 and C-451)

had low anti-gp120 antibody titers with low avidity, while the chimpanzees that had immunisation regimens which included envelope subunits had higher antibody titers with high avidity (Table 3).

Table 3: Lack of correlation between anti-gp120 antibody titers, avidity of antibodies to gp120, and protection from challenge.

Experiment	Chimpanzee	Immunogens	Anti-gp120 titer[a]	Avidity index[b]	Challenge Virus	Protection
1	C-451	VCP250	50	19	IIIB (cells)	No
	C-401	VCP250	100	23	IIIB (cells)	Yes
2	C-401	VCP250	100	25	DH12	No
	C-489	VCP205 + gp140 MN/LAI + V3-MN peptide + MN-T/B peptide	400	80	DH12	No
3[c]	C-477	VCP125 + gp140 MN/LAI	800	91	SF2	No
	C-641	VCP125 + gp140 MN/LAI	200	90	SF2	No
	C-483	Gp140 MN/LAI + V3 MN peptide	3200	96	SF2	Yes
	C-323	Gp140 MN/LAI + V3 MN peptide	1600	89	SF2	Yes

[a]*Titers expressed as the reciprocal of the last serum dilution to give an OD reading of •0.1 at 405 nm.*

[b]*Avidity index = (OD urea treated well/OD untreated well) x 100; AI values <30% are considered to indicate low avidity antibody.*[40]

[c]*Described in Girard et al.*[30]

However, no correlation between anti-gp120 antibody avidity and protection from challenge was noted in any of these chimpanzees. Although the HIV-1_{GB8} gp160 *env* gene shows only approximately 84% overall homology with the gp160 *env* genes from IIIB/LAI, MN and SF2, Vella *et al.* demonstrated that the rgp120 GB8 was recognised by all sera from HIV-1 infected patients tested.[59] Furthermore, hyperimmune sera raised against rgp120 derived from HIV-1 strains IIIB/LAI, MN, SF2 or GB8 showed comparable recognition of the HIV-1_{GB8} gp120 by EIA.

Altogether, these results leave the question of immune correlates of protection from HIV-1 in the chimpanzee model unanswered, and suggest that intraclade cross-protection among HIV-1 isolates will not be easily achieved and might differ from isolate to isolate. The induction of antibodies with broad spectrum neutralising activity against multiple primary isolates remains at this time one of the major goals in the development of an efficacious HIV-1 vaccine.

3.3. The development of a vaginal HIV-1 challenge model in chimpanzees

3.3.1. Introduction

Worldwide, epidemiological data demonstrate that approximately 80% of HIV-1 infections are transmitted via heterosexual contact.[60,61,62,63,64,65] However, little is known regarding the precise mechanism of, and immune reponses involved in protection from, infection acquired via the vaginal route. HIV-1 in semen is present either associated with lymphoid cells, or as free virions. The virus can be detected in the semen of 70% - 80% of seropositive men using PCR. The HIV-1 proviral DNA copy number ranges from <10/ml to >10000/ml.[66,67] HIV-1 has most often been detected in semen fractions enriched for white blood cells and studies have shown that lymphocytes and macrophages in semen can be infected with HIV-1.[68,69] Cell-free HIV-1 can directly infect Langerhans cells and macrophages on the epithelial surface of genital tract mucosal membranes.[63,70,71,72,73,74,75] It has, furthermore, been demonstrated that HIV-1 infected lymphocytes can attach to cervical epithelial cells *in vitro* and transmit virus directly[76]; this phenomenon is, moreover, enhanced by the presence of human seminal plasma (hsp).[77] Bomsell recently demonstrated that trancytosis of infectious HIV-1 across a tight human epithelial barrier can occur and lead to productive infection of mononuclear cells located at the basolateral side of the epithelial barrier.[78]

As demonstrated above, mechanisms of transmission of HIV across mucosal surfaces are not well understood. This makes it difficult to define specific immune responses that might be involved in protection from infection, and should, therefore, be elicited by a vaccine that is designed to protect from HIV infection via the female genital tract. Although models of mucosal infection of macaques with various SHIV strains are in development, these models are not as ideal as HIV infection of chimpanzees.[79,80] However, only one study on genital infection of a female chimpanzee, using a high-dose of cell-free HIV-$1_{IIIB/LAI}$, has been published.[81] A series of experiments to develop a chimpanzee challenge model for the study of protection from mucosal infection by HIV candidate vaccines was therefore initiated.

*In a first attempt at cervico-vaginal infection, two female chimpanzees, C-382 and C-120, were inoculated at the *os cervix* with $1.2x10^6$ and $1x10^7$ viable cells containing 35 and 300 IC, respectively, from the C-435 cell-associated HIV-$1_{IIIB/LAI}$ stock (see 3.3.2.2). Animal C-120 became readily infected as shown by virus isolation from PBMC from 2 weeks after infection onwards and seroconversion to HIV-1 antigens, as determined by EIA and immunoblot, at 4 weeks. In contrast, C-382 was only virus positive in PBMC on three occasions between weeks 2 and 6 after inoculation; the animal, furthermore, never seroconverted to HIV-1 antigens. PCR analysis of a lymph node biopsy done at 33 weeks was positive for HIV-1 proviral DNA. At one time point (6 weeks post-inoculation), low titer (20) HIV-1-specific sIgA was detected in vaginal wash fluid from the animal.[82] These results would indicate that the animal was infected, but maintained an extremely low level of replication of HIV-1. After a nine month period, during which the animal remained seronegative, C-382 was again inoculated via the cervico-vaginal route, but this time with 300 IC ($1x10^7$ PBMC) from the C-435 stock, a ten-fold greater inoculum than was used for the first attempt.

* Although the inoculations described in this section were performed before the candidate joined the team, the data was collated, databased and prepared for publication by the candidate.

The animal still did not seroconvert and no virus was recovered from her PBMC during the 3 months following inoculation, suggesting that the animal might be resistant to infection with HIV-1 via the cervico-vaginal route. To determine whether this also applied to systemic infection, a third inoculation with 30 IC ($1x10^6$ PBMC) from the C-435 stock was done, but this time via the iv route. The animal became virus positive in PBMC from 2 weeks after inoculation onwards and also seroconverted at 4 weeks post-infection, demonstrating that in spite of apparent resistance to infection by the vaginal route, C-382 was not protected from iv challenge with a small dose of cell-associated HIV-$1_{IIIB/LAI}$.

In a second experiment, two additional females, C-454 and C-562, were inoculated with 100 IC ($3.5x10^6$ PBMC) from the C-435 stock, on the vaginal mucosa and at the *os cervix*, respectively. Neither animal seroconverted, and both remained virus negative in PBMC, lymph nodes and vaginal wash fluid during 34 weeks of follow-up. However, repeated PCR analyses on PBMCs from C454 were positive for HIV-1 proviral DNA and vaginal washes of C-562 were consistently positive for HIV-1 specific sIgA.[82] Because neither animal was overtly infected, both together with a naïve animal C-460, were again inoculated with the C-435 stock via the cervico-vaginal route, but this time with 420 IC ($1.4x10^7$ PBMC). No evidence of infection of C-454 and C-562 could be obtained, but a single co-culture of PBMC taken from C460 at week 4 was positive for HIV-1 proviral DNA by PCR and for p24 antigen.

In a final attempt a further animal, C-304, was inoculated with 300 IC ($1x10^7$ PBMC) of the C-435 stock, but also failed to become infected. Cervico-vaginal inoculation of female chimpanzees using cell-associated HIV-$1_{IIIB/LAI}$, therefore resulted in overt persistent infection

in only one (C-120) of nine attempts (in six animals), with another chimpanzee (C-382, first inoculation) showing unequivocal signs of low-grade persistent infection.

Because of the limited success of the previous experiments, a second series of experiments, based on the fact that the low vaginal pH (3.5 - 6.5) might be detrimental to the survival of both the inoculated PBMC and the virus[83], was performed. It was shown in the SIV/macaque model that buffering the virus inoculum with human seminal plasma (hsp) enhanced survival of cell-free virus and infectivity by the vaginal route.[84] The effect of hsp on the viability of PBMC from the C-435 stock was investigated. It was found that the addition of 25% hsp resulted in a higher percentage of viable cells when measured after a 3h incubation *in vitro* (Table 4).

Table 4. Effect of human seminal plasma on short-term viability of chimpanzee PBMC.

	Incubation time (hours)			
% hsp	**Half**	**One**	**Two**	**Three**
0	98	95	69	59
25	97	97	96	94
50	97	91	90	92

HIV-1 infected PBMC from the C-435 stock were incubated at 20°C in RPMI-1640 medium supplemented with 10% FBS and the indicated percentage of hsp. Cell viability was estimated at the indicated times by trypan blue staining.

Chimpanzee C-460, which previously appeared to experience transient infection following inoculation of 400 IC, was subsequently inoculated with 300 IC from the C-435 stock ($1x10^7$ PBMC) in the presence of 25% hsp. An additional naïve female (C-90) was similarly inoculated. Both animals became readily infected as shown by virus isolation from PBMC from 2 weeks after infection onwards and seroconversion to HIV-1 antigens. Two sequential

attempts were then made to infect C-454 (which had already resisted two cell-associated and one cell-free genital challenge) using this procedure, but neither attempt resulted in infection. Infection of C-304 (which was not infected by one previous cell-associated inoculation) was also not achieved using 300 IC ($1x10^7$ PBMC) of the C-435 stock in the presence of 25% hsp. Therefore, cell-associated cervico-vaginal challenge using 25% hsp as buffer resulted in persistent infection following two of five attempts (in four animals) only.

In a first attempt at genital infection of chimpanzees using cell-free virus, two animals, C-454 and C-562, which had both resisted two previous attempts at cell-associated infection, were inoculated, respectively, with 2000 (500 CID_{50}) and 200 (50 CID_{50}) $TCID_{50}$ of the HIV-$1_{IIIB/LAI}$ chimpanzee challenge stock obtained from Larry Arthur.[32] Virus was isolated from PBMC of C-562 from weeks 6 and 8 post-inoculation and the animal also seroconverted; C-454, on the other hand, showed no evidence of infection. A further attempt was made to infect C-454 using this virus stock, this time using 1250 $TCID_{50}$ (250 CID_{50}) in the presence of 25% hsp. The animal, however, once again resisted infection. For subsequent experiments using cell-free virus, a chimpanzee-passaged virus stock (HIV-$1_{IIIB/LAI}$ C-90) was used (see 3.3.2.). In a first attempt, C-304, which had resisted two inoculations with cell-associated virus, was inoculated with 500 $TCID_{50}$ of the C-90 stock in the presence of 25% hsp via the cervico-vaginal route. The animal became readily infected as evidenced by virus isolation from PBMC and seroconversion. An attempt was then made to infect two further animals, C-454 and C-446, by cervico-vaginal inoculation with 125 $TCID_{50}$ of the HIV-$1_{IIIB/LAI}$ C90 stock in the presence of 25% hsp. Neither animal became infected. A second attempt was made to infect C-446, but this time using 500 $TCID_{50}$ of the C-90 stock in the presence of 25% hsp. The animal once again resisted infection.

The current study was undertaken to allow further determination of comparative virus doses necessary for genital infection, and to study the vaginal transmission of clinical isolates of HIV-1. There is some evidence non-subtype B HIV-1 strains (such as subtypes C and E) are more efficiently transmitted via the genital route than subtype B strains.[75,85,86] This, together with the need to develop HIV-1 challenge stocks for cross-protection experiments, have prompted us to also study vaginal infection with primary isolates from subtypes A and E.

3.3.2. Materials and Methods

3.3.2.1. Animals

Adult female chimpanzees (*Pan troglodytes*) were housed at the LEMSIP, as described in 3.2.2.1. As far as possible, the animals were inoculated at or near the peak of oestrus, as evidenced by oedema of the genital area. When necessary, menstrual cycles were synchronised by injection of depoprovera; this treatment did not appear to influence whether a productive infection was established in the animals. Prior to inoculation, the absence of blood in the vagina was ascertained by visual inspection using a speculum.

3.3.2.2. Virus stocks

The initial cell-free HIV-$1_{IIIB/LAI}$ chimpanzee challenge stock was obtained from Larry Arthur. This stock was prepared on H9 cells and has a CID_{50} of about 4 $TCID_{50}$ by the iv route.[32] For preparation of a cell-associated HIV-$1_{IIIB/LAI}$ stock, a chimpanzee (C-87) was inoculated via the iv route with 100 $TCID_{50}$ (25 CID_{50}) of this stock. The animal seroconverted at 4 weeks, and virus was consistently isolated from its PBMC from 2 weeks after inoculation onwards. A sample of PBMC obtained from C-87 3 months after infection was used to inoculate a second chimpanzee (C-435) via the iv route. This animal became persistently infected, as shown by virus isolation from PBMC from 2 weeks after infection onwards and

seroconversion to HIV-1 antigens at 4 weeks.[8] At 4 months after inoculation, a stock of its PBMC was prepared, aliquoted and cryopreserved. The titer of this 2nd passage cell-associated virus stock is 30 IC per 10^6 PBMC, as determined by *in vitro* co-cultivation with mitogen-activated human or chimpanzee PBMC. The infectivity of the C-435 stock was ascertained by injecting naive chimpanzees with $7x10^5$ PBMC (20-25 IC) by the iv route in three independent experiments. All three animals became readily infected and virus was repeatedly isolated from their PBMC starting from 2 weeks after inoculation.[8,45] The V3 loop sequence of 10 PCR clones obtained from the virus inoculum was determined, confirming that the virus was HIV-$1_{IIIB/LAI}$.[87] In an attempt to obtain a challenge virus stock with characteristics as close as possible to that of a primary isolate, a cell-free HIV-$1_{IIIB/LAI}$ stock was prepared from the PBMC of a female chimpanzee (C-90) that had been infected via the cervico-vaginal route with the C-435 cell-associated virus stock. The titer of this chimpanzee-passaged cell-free virus stock (designated HIV-$1_{IIIB/LAI}$C-90) is 10000 $TCID_{50}$/ml (PN Fultz, unpublished data).

A chimpanzee challenge stock of HIV-1_{DH12}, a subtype B primary isolate which readily infects chimpanzees, was obtained from Alan Schultz and Malcolm Martin at the NIH. The titer of this stock is 8000 $TCID_{50}$/ml and the CID (iv route) is between 6 and 16 $TCID_{50}$.[33,34]

A subtype E HIV-1 isolate, HIV-$1_{E/90CR402}$ was adapted to grow on chimpanzee PBMC and a chimpanzee challenge stock prepared.[88] The titer of the stock is 4000 $TCID_{50}$/ml and the CID (iv route) between 2 and 5 $TCID_{50}$.

A subtype A primary isolate (HIV-$1_{A/92UG029}$) was obtained from S Osmanov (WHO/UNAIDS) and a chimpanzee stock with a titer of 1200 $TCID_{50}$/ml on chimpanzee

PBMC prepared. The CID of this stock has not been determined, but 100 $TCID_{50}$ given by the iv route readily infected a naïve chimpanzee (C-631) (unpublished data).

3.3.2.3. Cervico-vaginal inoculation

The virus inoculum was diluted to a total volume of 0.25 ml in RPMI-1640 medium and taken into a 1 ml syringe mounted on a rigid metal catheter to which was attached a short piece of soft flexible rubber tubing. A sterile speculum lubricated with a water-based lubricating jelly (KY jelly, Johnson Pharmaceuticals) was inserted into the vagina, and the tip of the rubber tube gently inserted no more than 1 - 2 mm into the endocervical canal under direct vision. The contents of the syringe were then slowly injected into the cervical canal. At times, some of the inoculum was seen oozing back into the vagina, but no quantitation was made of what proportion remained in the endocervix and what proportion leaked out. Great care was taken to prevent trauma and colposcopic examination was done to confirm the absence of visible bleeding. The animals were positioned in the ventral decubitus position for the procedure and were left in this position with their hindquarters slightly elevated for approximately 30 min afterwards.

3.3.2.4. Virus isolation and serology

Blood samples were obtained from chimpanzees on the day of virus inoculation and then every other week for 8 weeks and at monthly intervals thereafter. Virus isolation was performed as described in 3.2.4. In some cases cultures were enriched for $CD4^+$ lymphocytes by removal of $CD8^+$ cells with magnetic beads coated with anti-CD8 monoclonal antibodies (Dynabeads, Dynal) in order to improve isolation efficiency. EIAs and WB analysis were performed as desribed in 3.2.2.4.

3.3.2.5. PCR and DNA sequence analyses

Oligonucleotide primers that flank the V3 loop (from C2 to V5) and recognise HIV-1 isolates from multiple subtypes were used to detect proviral DNA by nested PCR, using primers and conditions as described previously.[30,89] Two different sets of external and internal primers were used: set 1, 587S/588 (external) and 589/590 (internal);[30] and set 2, C/H (external) and D/F (internal).[89] Multiple PCR analyses were done using 0.5 to 1.5μg of genomic DNA from PBMCs, bone marrow and lymph node biopsies. Great care was taken to avoid contamination during PCR analysis, and all reactions were performed in a sterile biosafety hood, using positive displacement pipettes and filtered tips. DNA sequencing was performed using an automated sequencer as described.[87]

3.3.3. Results

3.3.3.1. Inoculation of female chimpanzees with cell-free HIV-$1_{IIIB/LAI}$

Two female chimpanzees, C-92 and C-498, were inoculated with 1250 $TCID_{50}$ in 25% hsp via the cervico-vaginal route. C-92 became infected as indicated by virus isolation from PBMC from 6 weeks after infection onwards and seroconversion; C-498, however, resisted infection and was again inoculated with the same dose of virus, after which it became virus positive in PBMC at 6 weeks after infection and seroconverted. Cervico-vaginal inoculation with the cell-free HIV-$1_{IIIB/LAI}$ C-90 stock, therefore, resulted in infection of three of five chimpanzees (three of seven attempts). A dose of 125 $TCID_{50}$ appeared to be insufficient, that of 500 $TCID_{50}$ was effective in one of two attempts, and that of 1250 $TCID_{50}$ was effective in two of three attempts (Table 5).

Table 5. Inoculation of female chimpanzees with the cell-free HIV-$1_{IIIB/LAI}$ C-90 stock.

Chimpanzee	Dose ($TCID_{50}$)	Infection[a]
C-454	125	No
C-446	125	No
C-446	500	No
C-304	500	Yes
C-92[c]	1250	Yes
C-498	1250	No
C-498	1250	Yes
C-454	10	Yes

Female chimpanzees were inoculated with the indicated dose of the chimpanzee-passaged cell-free HIV-$1_{IIIB/LAI}$ C-90 in 25% hsp at the os cervix, except for the second inoculation of C-454[b] which was done by the iv route.

[a]Overt infection as indicated by virus isolation from PBMC and seroconversion to HIV-1 antigens.

In a final attempt to infect C-454, the animal that had resisted a total of 7 inoculations via the genital route, was inoculated with 10 $TCID_{50}$ of the C-90 stock via the iv route. The chimpanzee immediately became infected and was virus positive in PBMC from 2 weeks after inoculation onwards and also seroconverted to HIV-1 antigens as determined by EIA and immunoblot assays (Table 6). This result demonstrates that resistance to HIV-1 infection in this animal was limited to the genital port of entry and did not extend to systemic infection. This is similar to what was seen for the female chimpanzee C-382, which also showed resistance to infection by the cervico-vaginal route, but not by the iv route.

Table 6. Attempts to infect chimpanzee C-454 via the genital and iv routes.

Virus stock		Dose		Route	hsp	Infection
Cell-associated	Cell-free	IC	$TCID_{50}$			
C-435 PBMC		100		vag	-	No
C-435 PBMC		420		cx	-	No
	IIIB/LAI[a]		2000	cx	-	No
	IIIB/LAI[a]		1250	cx	25%	No
C-435 PBMC		300		cx	25%	No
C-435 PBMC		300		cx	25%	No
	IIIB/LAI C-90		125	cx	25%	No
	E/90CR402		500	cx	12.5%	No
	IIIB/LAI C-90		10	iv	-	Yes

Female C-454 was repeatedly inoculated with the indicated virus stocks via the vaginal (vag) or the endocervical (cx) and then by the iv route. Inoculations are listed in chronological order.

[a]Original cell-free HIV-$1_{IIIB/LAI}$ stock grown on H9 cells[32]

3.3.3.2. Inoculation of female chimpanzees with HIV-1_{DH12}

There is considerable evidence that the neutralisation sensitivity of T-cell line-adapted HIV-1 strains (such as IIIB/LAI) and those of primary isolates differ.[13,55,56] It was, therefore, felt important to establish a model of HIV-1 genital infection using a primary isolate. The only titrated stock of a subtype B primary isolate of HIV-1 that was demonstrated to be infectious for chimpanzees is that of HIV-1_{DH12}.[33,34] In a first attempt at genital infection with HIV-1_{DH12}, a female chimpanzee (C-534) was inoculated with 500 $TCID_{50}$ of the cell-free virus stock in the presence of 12.5% hsp via the cervico-vaginal route. The animal did not become infected. A second female, C-110 was then inoculated with 1000 $TCID_{50}$ in 25% hsp. The

animal became virus positive at week 4 after inoculation and seroconverted, demonstrating that genital infection with HIV-1_{DH12} could be established using a dose of 1000 $TCID_{50}$.

3.3.3.3. Inoculation of female chimpanzees with HIV-$1_{E/90CR402}$

There is some evidence that interclade cross-protection among HIV-1 subtypes may be difficult to achieve,[90,91,92] and that some HIV-1 subtypes (such as subtypes C and E) are more efficiently transmitted via the genital route than subtype B.[75,85,86] We, therefore undertook to study genital infection of female chimpanzees with HIV-1 subtypes other than B. A subtype E isolate from the Central African Republic (HIV-$1_{E/90CR402}$) was adapted to grow in chimpanzee PBMC.[93] Titration of the virus in chimpanzees showed that the CID by the i.v. route is between 2 and 5 $TCID_{50}$.[88] A female chimpanzee (C-380) was inoculated via the cervico-vaginal route with 500 $TCID_{50}$ of cell-free HIV-$1_{E/90CR402}$ in the presence of 25% hsp. The animal readily became virus positive in PBMC and seroconverted to HIV-1 antigens. We next inoculated C-454, that had already resisted six genital challenges with various HIV-$1_{IIIB(LAI)}$ virus preparations, again using 500 $TCID_{50}$ of the HIV-$1_{E/90CR402}$ stock. The animal, however, also resisted the subtype E genital challenge (Table 6). A third female, C-366, was subsequently inoculated at the *os cervix* with 500 $TCID_{50}$ of the HIV-$1_{E/90CR402}$ stock in 25% hsp. She became readily infected as shown by virus isolation from PBMC and seroconversion to HIV-1 antigens. Therefore, cervico-vaginal inoculation of only 500 $TCID_{50}$ of HIV-$1_{E90/CR402}$ resulted in infection of two of three animals.

To investigate the possibility that HIV-1 subtype E isolates are transmitted more easily via the genital route than subtype B isolates, a female chimpanzee, C-370, was inoculated at the *os cervix* with a mixture of 500 $TCID_{50}$ each of the HIV-$1_{IIIB/LAI}$ C-90 and the HIV-$1_{E/90CR402}$ stocks in 25% hsp. The animal became readily infected and virus was isolated from its

PBMC from 2 weeks after challenge onwards. At 2, 6 and 16 weeks, DNA was extracted from PBMC or lymph node cells, amplified by PCR, cloned and sequenced or analysed using HMA. As shown in Table 7, all clones isolated at any of the three time points belonged to HIV-$1_{E/90CR402}$. No evidence for infection with HIV-$1_{IIIB(LAI)}$ could be detected, suggesting preferential susceptibility of the animal to mucosal infection by the subtype E virus.

Table 7. Identification of virus strains in chimpanzee C-370.

		Number of clones	
Time[a]	**Sample**	**HIV-$1_{IIIB/LAI}$**	**HIV-$1_{E/90CR402}$**
2	PBMC	0	24
6	Lymph node	0	52
16	PBMC	0	34

C-370 was inoculated with 500 $TCID_{50}$ each of the HIV-$1_{E90/CR402}$ and HIV-$1_{IIIB/LAI}$ C-90 stocks. At the indicated times, DNA was extracted from PBMC or lymph node cells, PCR amplified using HIV-1 env specific primers and cloned. Clones were typed by sequencing.
[a]Weeks after inoculation.

3.3.3.4. Inoculation of female chimpanzees with HIV-$1_{A/92UG029}$

In order to have another non-B subtype stock available for cross-challenge experiments, a female chimpanzee, C-110, was inoculated with 500 $TCID_{50}$ of HIV-$1_{A/92UG029}$, a subtype A primary isolate, in 12.5% hsp, via the cervico-vaginal route. The animal, however, failed to become infected. A further attempt was made to infect female C-534, which had resisted previous inoculation with HIV-1_{DH12}. The animal was inoculated on two occasions with 1000 $TCID_{50}$ of the HIV-$1_{A/92UG029}$ stock in 25% hsp via the genital route. Neither of the 2 attempts led to infection, as evidenced by failure to isolate virus from PBMC and lack of seroconversion. However, the infectivity of the HIV-$1_{A/92UG029}$ stock for chimpanzees was demonstrated by inoculating a chimpanzee (C-631) with 100 $TCID_{50}$ by the iv route. The

animal became readily infected as demonstrated by virus isolation from PBMC and seroconversion to HIV-1 antigens.

3.3.4. Discussion

This study demonstrates that genital infection of chimpanzees can be achieved with a relatively low frequency using either cell-associated (3 of 14 attempts) or cell free (4 of 10 attempts) HIV-$1_{IIIB/LAI}$ (Table 8). The inoculum was deposited in the cervix rather than the vagina. The reason for this is that the vaginal epithelium is stratified, while the cervical canal has a single layer epithelium, which should allow for easier entry by the virus.[87,94] Addition of hsp to the inoculum to act as a buffer did not appear to considerably increase the probability of infection, although the number of animals in the study was too low to allow for meaningful comparisons. The fact that infection was obtained with either cell-free or cell-associated virus is important, as it has been demonstrated that HIV-1 is present in semen both as infected lymphoid cells, and as cell-free virus.[95]

However, HIV-$1_{IIIB/LAI}$ is a T-cell line-adapted HIV-1 strain, and its cellular tropism and neutralisation sensitivity differ from that of primary isolates.[13,55,56] Most primary isolates of HIV-1 do not replicate in chimpanzees but HIV-1_{DH12}, a dual-tropic primary subtype B isolate, was recently shown to infect chimpanzees and to replicate to high levels in their PBMC following iv inoculation.[33,34,96] A female chimpanzee inoculated with 1000 $TCID_{50}$ of a cell-free HIV-1_{DH12} stock via the cervico-vaginal route became readily infected, demonstrating that genital infection of chimpanzees could be obtained using this primary isolate of HIV-1.

Table 8. Persistent genital infection of female chimpanzees with different HIV-1 isolates.

Subtype	Virus strain	Inoculum	Infections/Attempts
A	A/92UG029	Cell-free	0/3
B	IIIB(LAI)	Cell-associated[a]	3/14
		H9 stock[b]	1/3
		Chimpanzee-passaged[c]	3/7
	DH12	Cell-free	1/2
E	E/90CR402	Cell-free	2/3

Female chimpanzees were inoculated at the os cervix with the indicated virus stocks and the number of persistent infections monitored.

[a]*HIV-1-infected PBMC from chimpanzee C435.*

[b]*Original cell-free HIV-$1_{IIIB/LAI}$ stock grown on H9 cells.*[32]

[c]*HIV-$1_{IIIB/LAI}$ C-90 stock.*

It was not possible to ascertain a true 100% infectious dose for genital infection, using either cell-free or cell-associated virus, in the presence or absence of hsp. A true statistical analysis is precluded by the fact that the number of animals involved in the study remains low due to the high cost of these experiments. Nevertheless, it is clear that even the highest dose of virus used did not lead to infection in 100% of the animals. Inoculation of 1250 $TCID_{50}$ of the $HIV_{IIIB/LAI}$ C-90 stock resulted in infection in two of three attempts, while inoculation with $\geq 10^7$ PBMC from C-435 led to infection in only one of six attempts. Two of three females inoculated by the cervico vaginal route with 500 $TCID_{50}$ of the HIV-$1_{E/90CR402}$ stock became infected. This is reminescent of what was found in the SIV macaque model. Miller *et al.* demonstrated that several inoculations of rhesus macaques with cell-free SIVmac were necessary to obtain a 100% transmission rate.[97]

The majority of HIV-1 infections worldwide, particularly in the developing world, are transmitted via vaginal intercourse.[64] However, while viruses belonging to subtype B predominate in North America and Western Europe, different combinations of subtypes are found in Africa, Asia and other parts of the developing world. For example, virus isolates belonging to subtype A predominate in western and central Africa, while subtypes A, C, D and E have been recovered from patients in the Central African Republic[93] as well as in Russia.[98,99] Subtype E predominates in south-east Asia,[85,100,101] and the majority of isolates from India[102,103] and southern Africa[104,105] are from subtype C. Results of a study in Thailand by Kunanusont *et al.*, indicated that HIV-1 transmission in heterosexual couples was more efficient when the index case harboured a subtype E virus as opposed to a subtype B virus.[85] This seems to imply that the subtypes most prevalent in the developing world (subtypes A, C, D and E) are more easily transmitted via the genital route than subtype B viruses. Indeed, it was recently shown that subtype C and E strains replicated more efficiently and to higher levels in dendritic Langerhans cells from skin than subtype B viruses.[75,86] Langerhans cells are thought to be the primary target cells in mucosal infection.[94] The study of genital infection with the E subtype HIV-$1_{E/90CR402}$ showed that two of three female chimpanzees could be infected using only 500 $TCID_{50}$ of the virus stock (Table 5), as opposed to the 1000 - 1250 $TCID_{50}$ of cell-free virus that was needed to infect animals by the same route using HIV-1_{DH12} or HIV-$1_{IIIB/LAI}$ (both subtype B isolates). Furthermore, only HIV-$1_{E/90CR402}$ was recovered from a female (C-370) that was simultaneously inoculated by the cervico-vaginal route with an equal amount of HIV-$1_{IIIB/LAI}$ and HIV-$1_{E/90CR402}$, whereas both viruses were recovered after mixed inoculation by the iv route (personal communication PN Fulz). Altogether, these results suggest that HIV-$1_{E90/CR402}$ (a subtype E isolate) is transmitted more easily via the genital route than HIV-$1_{IIIB/LAI}$ (a subtype B strain) in the chimpanzee model. Surprisingly, genital infection with a subtype A isolate (HIV-$1_{A/92UG029}$) could not be

established (Table 8), but this might be explained by the fact that this particular virus strain did not replicate to high levels in chimpanzees (Fultz, unpublished data).

Cervico-vaginal inoculation of female chimpanzees with HIV-$1_{IIIB/LAI}$ resulted in three possible outcomes: i) no apparent infection of the animal, as evidenced by repeated failure to detect virus by PCR or co-cultivation, and lack of seroconversion; ii) transient infection, as evidenced by detection of virus in PBMC on only a few occasions at 2 - 6 weeks after inoculation and lack of seroconversion; and iii) overt infection with persistent isolation of virus from PBMC and seroconversion. One of the females (C-382) that experienced transient infection following a low dose of the cell-associated C-435 virus stock, resisted a second attempt at infection using a ten-fold higher dose, but was readily infected after receiving a low dose of the same virus stock via the iv route. In similar fashion, female C-454 resisted a total of eight attempts at infection via the genital route (Table 6), but became infected by a low dose of cell-free virus inoculated by the iv route. It appears, therefore, that a mechanism exists that can render a female chimpanzee refractory to cervico-vaginal, but not systemic, infection with HIV-1. These results are reminiscent of what was shown in the SIV macaque model following mucosal inoculation of cell-free SIV. Miller *et al.* demonstrated that some macaques inoculated with cell-free SIV via the vaginal route developed a transient viremia in the absence of an antibody response,[84] while Pauza *et al.* showed that rectal inoculation of a subinfectious dose of SIV_{mac251} protected against subsequent challenge by the same route with a higher dose of virus in spite of the absence of seroconversion.[106] A role for $CD8^+$ T-lymphocytes was suggested when depletion of these cells from PBMC led to a significant increase in SIV replication *in vitro*. The mechanism of protection, however, did not appear to be mediated by cytotoxic T-lymphocytes, but rather by soluble factors.[107] The presence of CD8 suppressor factors was first described by Walker *et al.*[108] Some of these factors were

identified as the β-chemokines RANTES, MIP-1α and MIP-1β.[109] Lehner *et al.* recently demonstrated that protection from rectal SIV challenge in macaques correlated with production of β-chemokines by activated CD4-depleted T-lymphocytes.[110] It was, furthermore, demonstrated that inoculation of macaques with a sub-infectious dose of SIV induced T-cell proliferative responses which correlated with protection from subsequent challenge with a higher dose of virus.[111] In two of the animals that became transiently infected (C-382 and C-562) and resisted further vaginal challenges with HIV-1, low levels of sIgA were demonstrated in vaginal washes and it is possible that this might have played a role in protection from subsequent challenges. However, demonstration of sIgA was only attempted in three animals (C-382, C-562 and C-120) making it difficult to draw any conclusions regarding the potential importance of secretory antibodies in the resistance to mucosal infection seen in some animals.

The mechanism of protection in the female chimpanzees used in this study was limited to the mucosal route, as infection was readily achieved via the iv route. This might be due to individual genetic factors. On the other hand, some females resisted one or two attempts at genital infection, but were eventually infected via this route, which might indicate that resistance in some cases is only temporary. A possible explanation for this phenomenon might be the presence of undetected concommitant infections that resulted in higher levels of β-chemokines, or hormonal factors. Whatever the reason, vaccine protection studies involving single genital challenge inoculations using this model will be difficult to interpret.

3.4. Study of protection from genital challenge in chimpanzees immunised with a recombinant canarypox-HIV-1 virus

3.4.1. Introduction

Although more than 90% of HIV-1 infections in the developing world are transmitted via the vaginal route,[64] little is known regarding the precise mechanism of, and immune responses involved in protection from, this route of infection. Several studies have demonstrated that protection from diseases transmitted via the mucosal membranes of the respiratory and gastro-intestinal tracts correlate better with the levels of antibodies in the corresponding secretions than in serum.[63,74] There is considerable evidence that the immune system can, in fact, be divided into two functionally independent compartments; systemic (represented by the bone marrow, spleen and lymph nodes), and mucosal (represented by mucosa-associated lymphoid tissues and external secretory glands),[63,112,113,114] and induction of an immune response in one of these compartments may not necessary be reflected in the other.[74,115,116] It is, therefore, likely that the correlates of protection from mucosal immunity will be different from those for intravenous infection. However, all of the HIV-1 vaccine protection studies in the chimpanzee model thus far have used the iv route for challenge of the animals, and the only correlate of protection that could be identified in the immunised animals was the V3-targeted neutralising antibody titer.[6,7,8,9,30,32,52,96] It is, however not known whether these antibodies will afford protection from mucosal infection. Only one previous study demonstrating cervico-vaginal infection of a female chimpanzee has been published.[81] Using the model described in 3.3, genital infection of female chimpanzees with both cell-free and cell-associated HIV-$1_{IIIB/LAI}$, as well as cell-free HIV-1_{DH12} and HIV-$1_{E/90CR402}$ could be obtained.[117] In this part of the study, protection of female chimpanzees from vaginal challenge by immunisation with a recombinant canarypox-HIV-1 virus is evaluated.

3.4.2. Materials and Methods

3.4.2.1. Study design

Adult female chimpanzees (*Pan troglodytes*) were housed at the LEMSIP as described. Cervicovaginal inoculation of immunogen, or challenge virus, was performed as described in 3.3.2.3. Five naive female chimpanzees were immunized with ALVAC-HIV-1 vCP250, a recombinant canarypox virus that expresses not only the HIV-$1_{IIIB/LAI}$ gp120 fused to the transmembrane anchor segment from gp41 (gp120/TM), but also the HIV-$1_{IIIB/LAI}$ *gag* and protease gene products (Figure 1). The animals received a total of five doses of $4x10^8$ pfu of vCP250 at weeks 0, 2, 5, 13 and 16. Two animals (C-420 and C-440) were immunised via the im, vaginal and rectal routes (1/3 dose each); two (C-422 and C-424) via the im, oral and nasal routes, while a fifth animal (C-452) received the whole dose via the im route. No subunit booster inoculations were given (Figure 5). The HIV-$1_{IIIB/LAI}$C-90 virus stock (see 3.3.2.2.), at a dose of 1250 $TCID_{50}$, was used to challenge the animals (Figure 5).

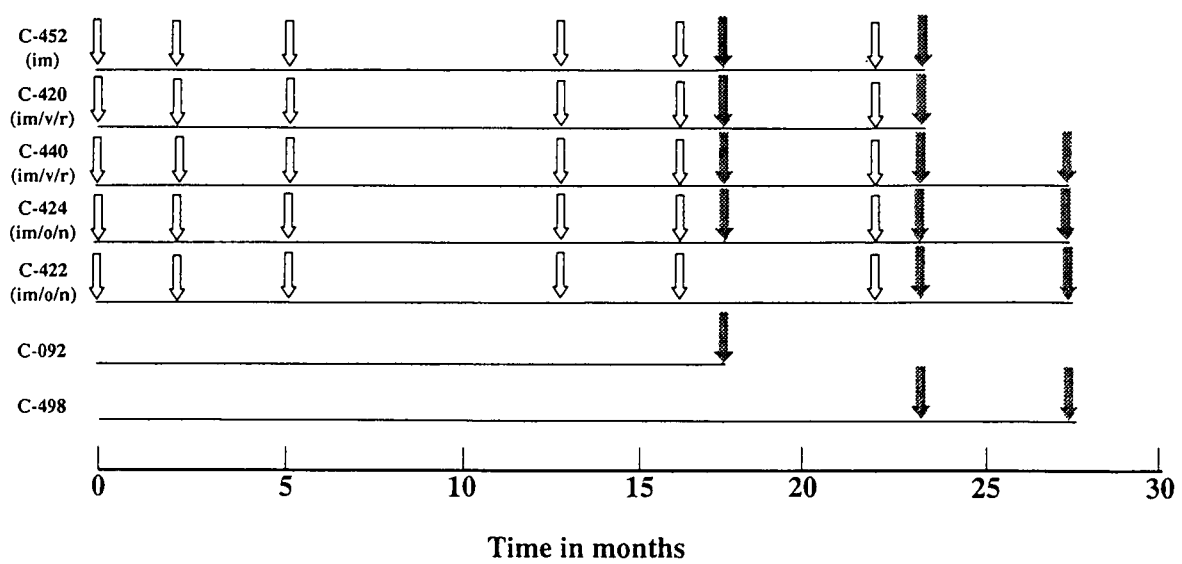

Figure 5. Study design for genital HIV-1 challenge

Open arrows indicate times of immunisation with vCP250 and closed arrows indicate times of cervico-vaginal challenge with HIV-$1_{IIIB/LAI}$C-90.

3.4.2.2. Virus isolation

Blood samples were obtained from chimpanzees on the day of virus inoculation and then every other week for 8 weeks and at monthly intervals thereafter. Virus isolation was performed as described in 3.2.2.4. In some cases cultures were enriched for $CD4^+$ lymphocytes by removal of $CD8^+$ cells with magnetic beads coated with anti-CD8 monoclonal antibodies (Dynabeads, Dynal) in order to improve isolation efficiency. Single cell suspensions of lymph node biopsy tissues and cells from bone marrow aspirates were also evaluated for the presence of virus by co-cultivation with human PBMC.

3.4.2.3. Serologic assays

WB and EIAs were performed as decribed in 3.2.2.4. Antibodies to a HIV-$1_{IIIB/LAI}$ V3 loop peptide were determined by EIA as previously described.[30] Neutralising antibodies to HIV-$1_{IIIB/LAI}$ were measured using a quantitative syncytium inhibition assay on CEM-SS cells.[37] Vaginal, rectal and nasal wash specimens collected in 10mM LiCl, as well as saliva, were obtained from the immunised animals at several timepoints including time of first challenge. The dilution factors of the washing specimens were calculated by measuring the Li concentration in the supernatants as described by Bélec *et al.*[118] The mucosal secretions and serum samples were then tested for anti-gp160 IgG and IgA using a sandwich EIA as described.[119] Serum samples were also tested for the presence of anti-gp120 antibodies, and the avidity of anti-gp120 antibodies were determined as described.[96]

3.4.3. Results

3.4.3.1. Serologic response to immunisation

Serum antibodies to HIV first appeared in one animal after the second immunisation (week 7), and in three other animals after the third (month 5) immunisation. However, these responses

were transient, except in C-440, which maintained relatively stable antibody levels. After the fourth immunisation (month 13), increases in EIA antibody titer was seen in all 5 animals (Fig. 6).

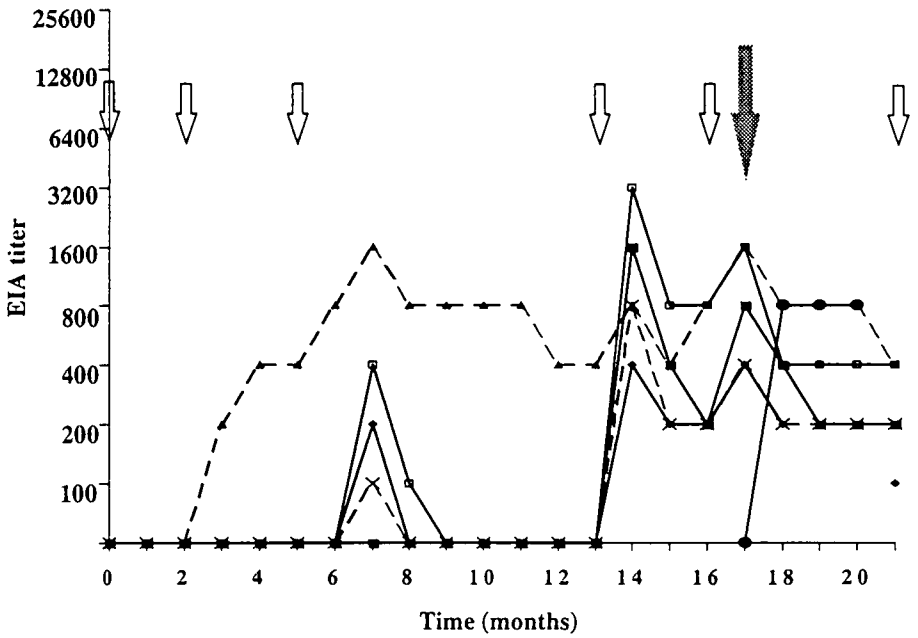

Figure 6. HIV-1-specific antibodies in serum samples from chimpanzees immunised with recombinant ALVAC vCP250 at time of first challenge.

Open arrows indicate times of immunisation. The closed arrow indicates the time at which all chimpanzees, except C-422, were inoculated cervically with HIV-1$_{IIIB/LAI}$C-90.

Symbols: C-452 (□; solid line), C-420 (×; dashed line), C-440 (▲; dashed line), C-422 (♦; solid line), C-424 (■; solid line), C-92 (●; solid line).

WB assays done at time of challenge (month 17), demonstrated that although a good anti-p24*gag* antibody reponse had developed in all of the animals, only three of them (C-420, C440 and C-452) had developed anti-p17*gag* antibodies, while none of the animals showed an anti-Env response (Fig. 7)

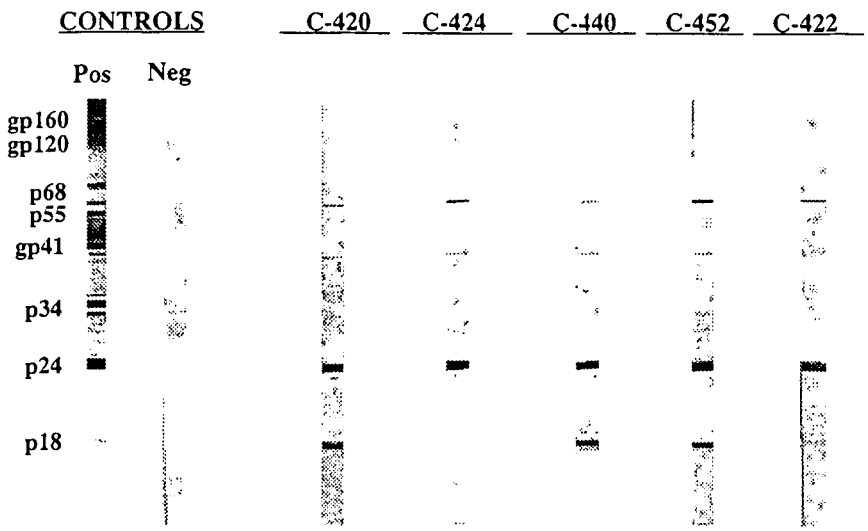

Figure 7. Western blot assays of immunised animals at time of first challenge

Anti-V3 HIV-$1_{IIIB/LAI}$ antibody titers increased after the fourth immunisation in C-440 and C-452, while the others showed an increase only after later immunisations (Table 9). A significant anti-HIV-$1_{IIIB/LAI}$ neutralising antibody response (titer ≥32) developed in only two animals (C-440 and C-452) (Table 10). The anti-gp120 antibody titers of the animals were very low, ranging from <25 to 100, and all of them had antibodies with a low avidity (AI <30%). Moderate titers (ranging from 250 [C-424] to 8000 [C-440] at time of first challenge) of anti-gp160 IgG was detected in the sera of the animals. The anti-canarypox antibody levels of the animals as determined by EIA ranged from 16 (C-420) to 116 (C-440) EU/ml, indicating that all of the animals developed an immune response to the virus vector (a cut-off value of 0.6 EU/ml is considered positive in human volunteers). Of interest were the large differences in the levels of the immune responses mounted by the individual animals (Fig. 6; Tables 9 and 10), indicating that chimpanzees, like humans, can be divided into good and bad responders. This also demonstrates the problem of interpretation of immunological data from studies on chimpanzees, as only small numbers of animals are normally used due to the high cost of this type of study.

Table 9. Anti-V3 antibody titers of chimpanzees immunised with ALVAC-HIV-1 vCP250.

	im/vaginal /rectal[a]		im/oral/nasal		im		
Months[b]	**C420**	**C-440**	**C-422**	**C-424**	**C-452**	**C-092**	**C-498**
6	530	1250	440	710	650	ND	ND
13[c]	500	780	430	390	640	ND	ND
16[c]	500	4000	560	670	2040	ND	ND
17[d]	770	38000	1550	4460	8600	112	ND
22[c]	214	18869	1426	1297	1397	12228	ND
23[e]	843	45433	7041	16594	26983	ND	246
24	15721	17976	7245	4816	109742	ND	177
27[f]	ND	12239	4825	2565	ND	598	<50
28	ND	13626	3176	70314	ND	ND	254
30	ND	9952	2522	ND	ND	ND	ND

[a] *Route of immunisation.*

[b] *Months after first immunisation.*

[c] *Booster inoculation with vCP250.*

[d] *First challenge with HIV-$1_{IIIB/LAI}$C-90.*

[e] *Second challenge.*

[f] *Third challenge.*

ND = not done

Table 10. HIV-$1_{IIIB/LAI}$ neutralising antibody titers.

	im/vaginal /rectal[a]		im/oral/nasal		im
Months[b]	**C-420**	**C-440**	**C-422**	**C-424**	**C-452**
17[c]	8	64	4	16	32
18	8	64	4	8	16
22[d]	<4	<4	<4	<4	<4
23[e]	>8	64	16	32	32
27[f]	32	16	<4	<4	64

[a]*Route of immunisation.*

[b]*Months after first immunisation.*

[c]*First challenge with HIV-$1_{IIIB/LAI}$C-90.*

[d]*Booster inoculation with vCP250.*

[e]*Second challenge.*

[f]*Third challenge.*

None of the animals had anti-gp160 IgA antibodies in their mucosal secretions at any of the timepoints tested. Chimpanzee C-440 had low levels of anti-gp160 IgG antibodies in mucosal secretions; at a dilution of 1:2 the optical density measurements were: 0.2 for saliva, 0.6 for nasal washings, 1.1 for vaginal washings and >2 for rectal washings. None of the other animals had any evidence for anti-gp160 IgG antibodies in their mucosal secretions at any of the time points tested.

3.4.3.2. Cervicovaginal challenge

One month after the last booster inoculation, four of the immunised animals (C-452, C-440, C-420 and C-424) and a naïve control (C-92) were challenged via the cervico-vaginal route

with 1250 $TCID_{50}$ of the cell-free HIV-$1_{IIIB/LAI}$C-90 stock. Chimpanzee C-422 was not challenged due to an unforeseen vaginal bleed on day of challenge. The control animal (C-92) became virus positive in PBMC at 6 weeks after challenge and seroconverted between 6 and 8 weeks after challenge (Fig. 6; Table 11). In contrast, the four immunised animals remained virus negative at all times during 5 months of follow-up, and their antibody titers decreased slowly (Fig.6; Tables 9, 10 and 11).

Table 11. Virus isolation from PBMC of the immunised animals.

		First challenge					Second challenge					Third challenge				
Chimpanzee	**Route[a]**	**0[b]**	**2**	**4**	**6**	**8**	**0**	**2**	**4**	**6**	**8**	**0**	**2**	**4**	**6**	**8**
C-452	Im	-	-	-	-	-	-	-	-	+	+					
C-422	Im/oral/nasal						-	-	-	-	-	-	-	-	-	-
C-424	Im/oral/nasal	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
C-420	Im/vaginal/rectal	-	-	-	-	-	-	-	-	+	+					
C-440	Im/vaginal/rectal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C-092		-	-	-	+	+										
C-498							-	-	-	-	-	-	-	-	-	+

[a] *Route of immunisation.*

[b] *Weeks after challenge*

After 5 months of follow-up, the immunised animals were again boosted with $4x10^8$ pfu of vCP250, and three weeks later all five females, together with a naïve control (C-498), were challenged via the cervico-vaginal route with 1250 $TCID_{50}$ of HIV-$1_{IIIB/LAI}$C-90. Two of the immunised animals (C-452 and C-420) became virus positive in PBMC from 6 weeks post-infection onwards (Table 11) and developed a strong anamnestic antibody reponse by all assays (Fig. 8; Tables 9 and 10). The other three immunised animals appeared to remain uninfected as demonstrated by the inability to isolate virus from their PBMC (Table 11) and a decrease in antibody titers (Fig. 8). However, the control animal (C-498) also failed to

become infected as indicated by its lack of seroconversion (Fig. 7) and the fact that no virus was isolated from its PBMC (Table 11). This is not surprising as we could never achieve 100% infection with this dose of the IIIB C-90 challenge virus when given to naïve animals via the cervico-vaginal route.[117]

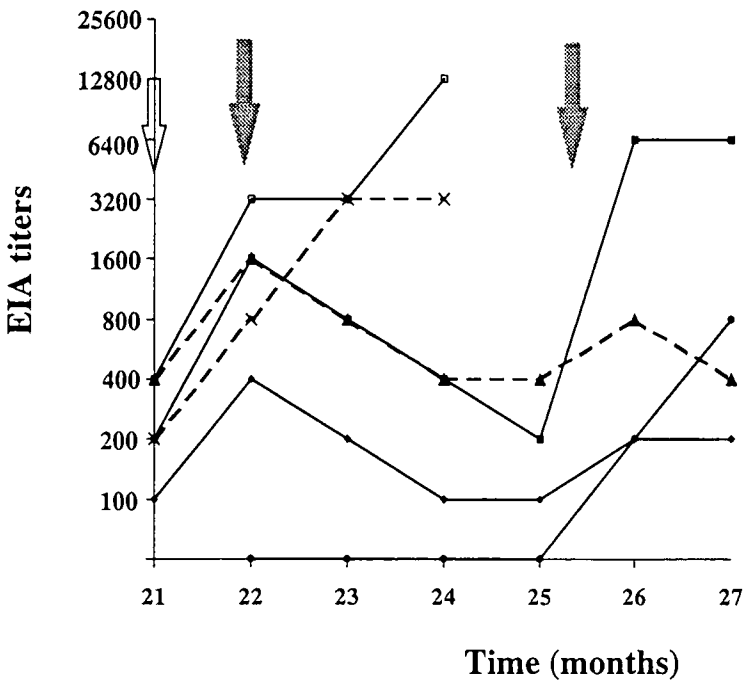

Figure 8. HIV-1-specific antibodies in serum samples of immunised chimpanzees at times of second and third challenge.

Open arrows indicate times of immunisation. The closed arrows indicate the times at which the chimpanzees were inoculated cervically with HIV-$1_{IIIB/LAI}$C-90. Month 21 is the same month 21 as in figure 6.

Symbols: C-452 (□; solid line), C-420 (×; dashed line), C-440 (▲; dashed line), C-422 (♦; solid line), C-424 (■; solid line), C-498 (●; solid line).

At the time of second challenge the protected animals (C-422, C424 and C-440) had an anti-IIIB/LAI neutralising antibody titer of 16, 32 and 64, respectively. The unprotected animals (C-420 and C-452) had titers of >8 and 32, indicating once more no apparent association between protection and neutralising antibody titer.

After four months of follow-up, the three immunised and protected animals, as well as the uninfected control animal, were again challenged via the cervico-vaginal route with 1250 $TCID_{50}$ of the HIV-$1_{IIIB/LAI}$ C-090 stock. The immunised female C-424 and the control animal (C-498) became virus positive in their PBMC at weeks 4 and 8 after challenge, respectively (Table 11). Chimpanzee C-424 also showed a strong anamnestic antibody response by EIA (Fig. 8) and WB (not shown). In contrast, the two other immunised females, C-422 and C-440 remained virus negative in PBMC (Table 11) and showed no anamnestic antibody response following the third challenge (Fig. 8).

3.4.4. Discussion

In this study it was demonstrated that im and/or mucosal immunisation of female chimpanzees with a recombinant canarypox-HIV-1 virus (vCP250) can afford protection from cervico-vaginal challenge with cell-free HIV-$1_{IIIB/LAI}$. However, in contrast to what was demonstrated in previous chimpanzee studies where the iv route of challenge was used,[6,7,8,930,32,52,96] no correlation with neutralising antibodies was noted (Table 10). All four of the immunised animals were protected from the first challenge, even though only two of the animals had significant levels of neutralising antibodies (≥32) (Table 10) to HIV-$1_{IIIB/LAI}$. At time of second challenge the results were even more puzzling, as one of the two animals that became infected (C-452) had a neutralising antibody titer of 64 (Table 10). Neutralising antibody titers determined on day of third challenge demonstrated that, at this time, all three animals had only low levels of neutralising antibodies (Table 10), yet two of them (C-422 and C-440) were again protected from challenge. These low levels of neutralising antibodies are not surprising, as the animals were immunised with a non-replicating recombinant immunogen only. In general, a boost with a purified protein is required to elicit high titers of

antibodies, as was demonstrated in studies with non-human primates and in phase I/II studies in humans with related canarypox-HIV-1 vectors.[29,120,121,122,123,124] However, taken together, these results suggest that neutralising antibodies are not important in protection from mucosal challenge.

In spite of the immunogen being delivered at mucosal sites in four animals, only one (C-440, that received vCP250 via the im, vaginal and rectal routes) developed detectable, but low titer, levels of IgG antibodies in mucosal secretions. No IgA antibodies were detected in mucosal secretions from any of the animals. Interestingly, this animal is the female that resisted a total of 3 challenges. However, no firm conclusions can be drawn from this result as it is based on only one animal. Unfortunately, no attempt was made to quantify levels of mucosal antibodies following the different cervico-vaginal challenges.

The possibility that CTL might have played a role in the protection from challenge of these animals should be considered. However, although we did not test for CTL in this study, we failed to demonstrate any CTL in two chimpanzees immunised with vCP250 via the im route in a previous study.[96] Furthermore, in humans and non-human primates immunised with various ALVAC constructs, CTL activity has been limited.[121,123,125,126] It is, therefore, unlikely that CTL contributed significantly to the protection observed in this study.

The fact that one of the naïve control animals (C-498) used in this study resisted one challenge and only became infected when challenged a second time, makes the results difficult to interpret. It is possible that the results are just a reflection of challenge with a less than 100% infectious dose (1250 $TCID_{50}$ is the maximum dose that can be given in a volume small enough to allow cervicovaginal inoculation), and that vaccination with ALVAC had no

influence on the outcome. In fact, as demonstrated in section 3.3., a 100% rate of infection was never obtained using the chimpanzee vaginal inoculation model. Furthermore, like humans, chimpanzees probably vary in their susceptibility to HIV-1 infection across a mucosal surface. Several factors can influence the cervicovaginal milieu and, therefore, transmission of HIV-1 in any one episode of vaginal sex. These factors include variation in normal vaginal flora and infections with a variety of pathogens; both of which can result in changes in vaginal pH and levels of inflammatory cytokines.[127,128,129,130]

In summary, 1 (C-440) of 5 chimpanzees appeared to resist vaginal challenge with HIV-$1_{IIIB/LAI}$ a total of three times, while 2 further animals (C-422 and C-424) resisted two challenges, but whether this protection was vaccine-mediated is unclear. However, the immune responses induced by vaccination with a recombinant canarypox vector alone were low (but comparable to those seen in human volunteers immunised with related ALVAC constructs), and it appears that, as in humans, boosting with other immunogens, such as purified proteins, will be required in order to optimise humoral and cellular immune responses. Finally, this study demonstrates the problems that might be associated with phase III HIV vaccine trials in humans. Decisions will have to be made as to whether vaccines are protective, and to the contributing role of non-specific factors in the vaginal milieu that might have prevented infection.

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3.6. Papers relating to study (see appendix)

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Genital infection of female chimpanzees with human immunodeficiency virus type 1. *AIDS Res Hum Retroviruses* 1998; **14**: 1357 – 1367.

6. Girard M, **van der Ryst E**, Barré-Sinoussi F, Tartaglia J, Mahoney J, Nara P, Pillot J, Meignier B, Gallo C, Fultz PN. Genital HIV-1 challenge of female chimpanzees immunised with a recombinant canarypox-HIV-1 virus. *AIDS Res Hum Retroviruses* (submitted).

Note:

The candidate played a coordinating role in the chimpanzee immunisation programme and made an important contribution to the planning and execution of studies and protocols (under supervision of Prof M Girard), and was responsible for the day to day running of the various studies (liasing between teams, ensuring that the correct samples and reagents are shipped at appropriate times for inoculation and assay purposes, and resolving of day-to-day problems). The candidate created a database of all results, and was instrumental in analysis and interpretation of results (including preparation of graphs, figures and tables). The candidate also prepared the cells for, and performed all CTL assays. The HIV-1_{GB8} gp120 antibody assay was established and validated by the candidate; and sera from immunised animals were tested for anti-gp120 antibodies and antibody avidity using this assay. The candidate also played an important part in the work on the *in vivo* titration of the HIV-$1_{E/90CR402}$ chimpanzee challenge stock that was used for cross-challenge studies. The candidate prepared the data for publication, and was responsible for preparing all the papers (at least up to pre-final draft stage).

Contributions of other principal authors:

J Tartaglia, E Paoletti – provided the canarypox virus recombinants

E Muchmore, J Mahoney, and M Georges-Courbot – directed work at the different primate centers

B Meignier – provided valuable scientific advice

P Nara – some neutralisation assays were performed in his laboratory

L Yue – Sequencing and cloning of HIV-1 proviral DNA from chimpanzee PBMC for the B/E cross-challenge study (not discussed here)

Q Wei – PCR analysis of sera and tissue samples in the genital infection study

M Girard – initiated the chimpanzee immunisation program and supervised it throughout

PN Fultz – supervised performance of virus isolation, sequencing and PCR assays, and contributed greatly to the planning of the studies and final editing of papers

F Barré-Sinoussi – supervised performance of neutralisation assays and preparation of several virus stocks, and provided valuable scientific advice throughout

CHAPTER 4

STUDY OF THE IMMUNE RESPONSE INDUCED IN RHESUS MACAQUES IMMUNISED WITH A PRIMARY HIV-1 ISOLATE

4.1. Introduction

The development of a vaccine against HIV-1 remains a formidable challenge. One of the most important challenges is the identification of a reliable immune correlate of protection. However, the evolution of immune responses following natural HIV-1 infection remains poorly understood.[1,2] Infection with HIV-1 elicits in most individuals, at least during the early stages of infection, a comprehensive cell-mediated immune response that includes NK cells and CTL responses targeted to cells expressing a variety of HIV-1 antigens.[3] In contrast, neutralising antibodies to the autologous virus strain develop slowly in patients, appearing on average only about 1 year after seroconversion. Antibodies able to neutralise heterologous primary isolates appear even later.[4] It is not known why such a long maturation period is required for the appropriate type of antibodies to be produced by the immune system, or why the titers remain so low. Moreover, virus entry into cells can be enhanced by non-neutralising antibodies. At least 2 different types of enhancing antibodies have been described in infected individuals. Certain antibodies cover the virus particles and interact with the Fc region of the immunoglobulins on the surface of T-lymphocytes, NK cells and macrophages.[5,6] Other antibodies enhance virus entry via complement by fixation of antibody/virion/complement complexes on the CR3 complement receptor on the cell surface.[7,8]

Neutralisation of T-cell line-adapted HIV-1 strains are mediated by antibodies to the hypervariable V3-loop of the HIV-1 gp120.[9] On the other hand, primary HIV-1 isolates are relatively resistant to neutralisation by both sCD4 and and V3-targeted antibodies. The

reason for this might be the three-dimensional envelope protein conformation of these isolates, that results in shielding of the critical epitopes from neutralising antibodies.[10,11]

It is clear that antibodies induced by a vaccine will need to neutralise primary isolates in order to be effective. To evaluate the evolution of neutralising antibodies to a primary isolate of HIV-1, macaques were repeatedly immunised with a preparation of intact HIV obtained from a primary isolate (HIV-1_{BX08}).

4.2. Materials and Methods

4.2.1. Virus stock

The HIV-1_{BX08} isolate was isolated from an HIV seropositive patient in Bordeaux, France.[12] The gp120 V3–loop sequence of this isolate is very close to the concensus sequence for HIV-1 strains isolated in France in the early 1990's.[13,14] The virus was never passaged in T-cell lines, and only cultivated on PBMC from healthy donors. The virus stock used in this study was cultured on human PBMC as previously described[15], purified by centrifugation through saccharose and stored at -80°C. In total five lots of virus were prepared. Half of each lot was inactivated with either β-propiolactone (1/400 for 1h at 4°C followed by 37°C overnight) or formaldehyde (1/1000 for 16h at 4°C). The p24 antigen concentration of each preparation was quantified using a commercial p24 antigen detection kit (Dupont).

4.2.2. Immunisation and follow-up of macaques

Adult male rhesus macaques (*Macaca mulatta*) of Chinese origin were housed at the Rennemoulin Primate Centre in biosafety level 2 facilities in accordance with institutional guidelines and standard practices for the containment of infectious diseases and humane care of experimental animals. Before all procedures the animals were anaesthetised by im

injection of ketamine hydrochloride (10mg/kg). All animals were seronegative for SIV, STLV-I, type D retroviruses and herpes B virus.

Six animals were immunised a total of 12 times with different amounts of virus. The first nine doses were given via the im route, while the final 3 doses were given sc. Three different adjuvants were used; the saponin derivative QS21 (Pasteur Merieux Serums et Vaccins, Marnes la Coquette, France), monophosphoryl lipid A (MPL-A) (Ribi Immunochem Research Inc, Hamilton, MO) and incomplete Freund's adjuvant (IFA) (ISA 51, Seppic, France) (Table 1). Macaques 92205, 92253 and 92303 received a live virus preparation, while 92055, 92087 and 92121 received whole inactivated virus.

Table 1. Immunisation schedule

Time (months)	**Virus dose (μg p24)**	**Immunisation route**	**Adjuvant**	**Inactivation**
0,1,3	20	im	QS21	BPL
6,7,9	175	im	QS21	BPL
14, 15, 17	250	im	MPL-A	Formaldehyde
20, 21, 22	250	sc	IFA	Formaldehyde

The animals were bled monthly and their antibody responses determined. Serial two-fold dilutions of serum samples were tested for total anti-HIV-1 antibodies with a commercially available EIA kit (Diagnostics Pasteur, Marnes-la-Coquette, France). Titers were defined as the reciprocal of the last serum dilution to give an OD reading above the cut-off recommended by the manufacturer. WB assays were done using a commercially available kit (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) and a serum dilution of 1:100.

4.2.3. Neutralisation assays

The ability of sera, taken from the immunised animals at several timepoints, to neutralise 3 HIV-1 strains was determined. Neutralisation of HIV-$1_{IIIB(LAI)}$ was tested on MT4 and CEM-SS cells, that of HIV-1_{MN} on MT4 cells, and that of HIV-1_{BX08} in phytohemagglutinin-stimulated human PBMC as previously described.[16] Briefly, 4-fold dilutions of virus were tested against 2-fold dilutions of sera in a matrix. For each virus and serum dilution, four wells were used. Fifty µl of each virus dilution was incubated for 60 min at 37°C with 50µl of each serum dilution, and 10^5 cells added. Following a further 2h incubation at 37°C, the cells were washed 3 times with RPMI-1640, and resuspended in 2ml of RPMI-1640 containing 10% FBS and 20U of Il-2/ml (Boehringer-Mannheim). After 7 days of culture at 37°C, viral replication was demonstrated by detecting p24 antigen in the supernatant fluid by EIA (Dupont). The virus titer was calculated using the Reed and Muench formula and expressed in $TCID_{50}$.[17] The neutralising antibody titer was defined as the reciprocal of the serum dilution for which a 90% reduction in virus titer was demonstrated.

4.2.4. CTL assays

$CD8^+$-specific CTL responses targeted to *env* were evaluated using a peptide-restimulation assay[18] and autologous Herpes papio virus-transformed B-lymphoblastoid cell lines as target cells.[19] PBMC from blood collected two weeks after the final immunisation at month 22 were stimulated *in vitro* with pools of overlapping 20-mer peptides corresponding to amino acids 30 to 510 in Env of HIV-1_{SF2}; culture medium was RPMI-1640 containing 10% FBS, 5% human IL-2 (Schiaparelli), 100 IU/ml rIL-2 (Cetus Corporation), antibiotics, and 10 µg/ml of each peptide. After 8 days in culture, $CD8^+$ cells were purified using magnetic beads coated with anti-CD8 antibodies (Dynal, Lake Success, New York) and tested for cytolytic activity in a standard ^{51}Cr release assay[20] against autologous target cells pulsed with the homologous

peptide pool (10 μg/ml of each peptide). Control targets were pulsed with a heterologous peptide pool.

4.2.5. Anti gp120 antibody avidity assays

Anti-gp120 antibody titers and avidity of sera taken at months 0, 17 and 22 were determined using an EIA. Briefly, duplicate microtiter plates (Maxisorp, Nunc) were coated with concanavalin-A at 2.5 μg/well for 1h at room temperature, after which they were washed with PBS containing 1% Tween-20 and coated with 50 ng/well of rgp120 (British MRC AIDS Reagent Research Project) that was derived from a clinical isolate of HIV-1 ($HIV\text{-}1_{GB8}$).[21,22] Sequential two-fold dilutions of sera in PBS containing 2% BSA were added and incubated for two hours at 37°C. Binding antibodies were detected by standard methods with alkaline phosphatase-conjugated anti-human IgG and p-nitrophenyl phosphate substrate using one of the duplicate plates, whereas the other plate was first soaked with PBS containing 8M urea and then processed. The anti-gp120 antibody AI was then calculated as described from the difference in OD values at 405nm in corresponding wells.[23,24] The AI was determined over a range of serum dilutions in order to control for variations in antibody concentration.

4.3. Results

Two groups of three macaques each were repeatedly immunised with live or inactivated $HIV\text{-}1_{BX08}$. Three of the animals received live virus, in order to control whether the virus inactivation process has any effect on immune responses. As HIV-1 cannot replicate in rhesus macaques, this was similar to an "inactivated" preparation. The animals developed good levels of total anti-HIV-1 antibodies as determined by EIA after the first four immunisations, and thereafter only a limited anamnestic response was observed (Fig. 1).

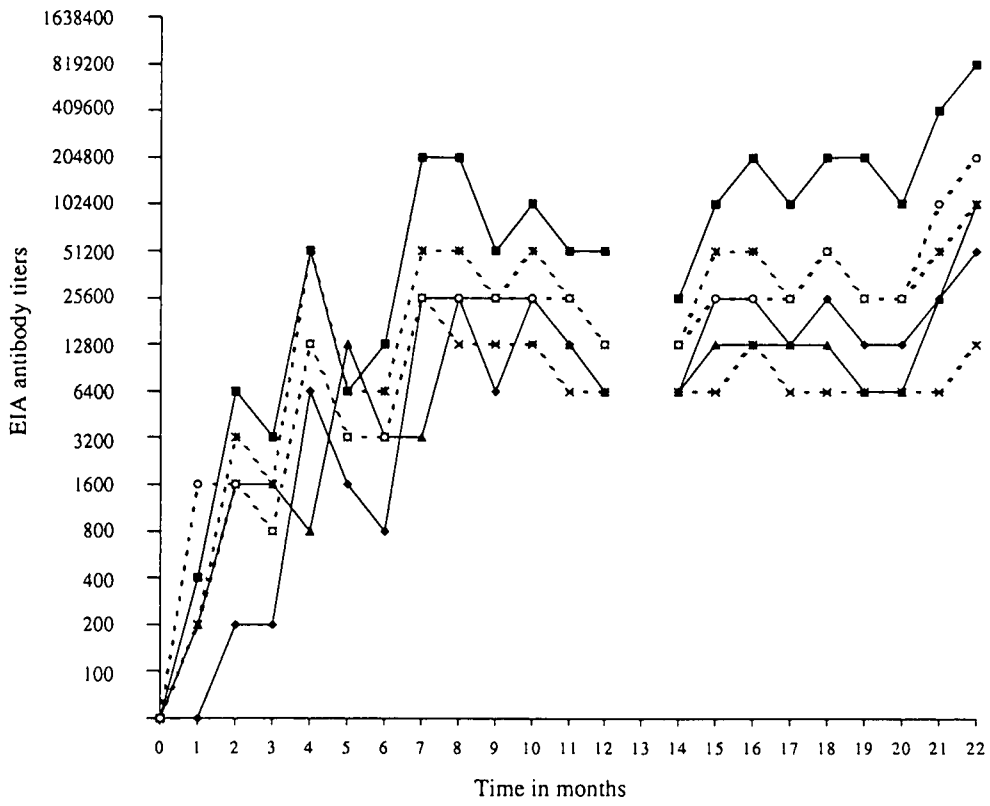

Figure 1: Total anti-HIV antibody responses of macaques immunised with HIV-1_{BX08}

Symbols: 92121 (♦; solid line), 92055 (■; solid line), 92087 (▲; solid line), 92253 (✖; dashed line), 92303 (✳; dashed line), 92205 (○; dashed line)

However, WB analysis demonstrated that the majority of these antibodies were directed to *gag* gene products, and that anti-ENV responses were weak or absent (Fig. 2). Anti-gp120 antibody titers in the serum of all 6 animals were ≤1600, and in only 3 of the macaques an AI of >50, indicating high avidity antibodies, was demonstrated. It appeared that the introduction of IFA as adjuvant for the last 3 immunisations resulted in an increase in the anti-gp120 antibody avidity in 3 of the animals. None of the animals developed significant neutralising antibody titers, except for two animals that developed antibodies that neutralised HIV-$1_{IIIB(LAI)}$ on CEM-SS cells following immunisation with MPL-A as adjuvant.

Surprisingly it was noted that sera from the immunised animals induced strong facilitation of HIV-1_{BX08} replication in PBMC. The infectious titer of virus increased as much as 90 fold in the presence of serum from some of the animals (Table 2). There were no apparent differences in the antibody responses of animals immunised with the live or inactivated preparation.

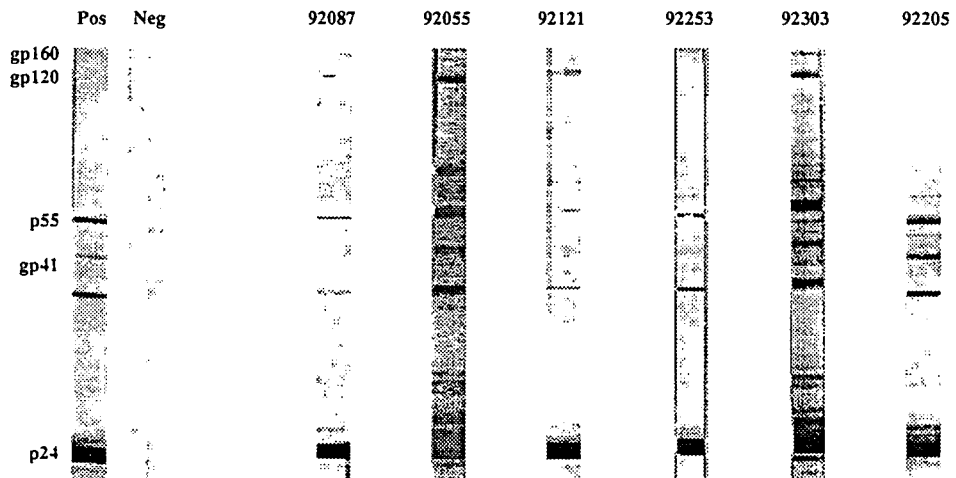

Figure 2: HIV-1 antigen specific responses at month 17 as demonstrated by WB analysis

No *env*-specific CTL could be demonstrated in any of the animals using the peptide restimulation assay as described. However, this assay system has not been validated in the macaque, and there is not a complete sequence match between the gp120 of HIV-1_{BX08} and HIV-1_{SF2}.[25] These results should, therefore be interpreted with caution.

Table 2. Results of neutralising antibody and anti-gp120 antibody assays.

Macaque	Time[a]	Neutralising antibody titer				Enh. Ab[b]	Anti-gp120	AI
		BX08 PBMC	IIIB MT4	IIIB CEM-SS	MN MT4			
92055	0	<10	ND	ND	ND	ND	<10	ND
(inact)	17	<10	<20	**400**	<20	++	1 600	46%
	18	<10	<20	**>640**	<20	++	ND	ND
	22	ND	ND	ND	ND	ND	1 600	46%
92087	0	<10	ND	ND	ND	ND	<10	ND
(inact)	17	<10	<20	40	<20	++	800	39%
	18	<10	<20	40	<20	+++	ND	ND
	22	ND	ND	ND	ND	ND	1 600	55%
92121	0	<10	ND	ND	ND	ND	<10	ND
(inact)	17	<10	ND	ND	ND	++	1 600	38%
	18	<10	<20	40	<20	+++	ND	ND
	22	ND	ND	ND	ND	ND	800	47%
92205	0	<10	ND	ND	ND	ND	<10	ND
(live)	17	<10	ND	ND	ND	++	1 600	36%
	18	<10	20	40	<20	+	ND	ND
	22	ND	ND	ND	ND	ND	1 600	68%
92253	0	<10	ND	ND	ND	ND	<10	ND
(live)	17	<10	<20	<40	<20	+++	400	28%
	18	<10	<20	<40	<20	+++	ND	ND
	22	ND	ND	ND	ND	ND	800	29%
92303	0	<10	ND	ND	ND	ND	<10	ND
(live)	17	<10	<20	**80**	<20	+++	1 600	37%
	18	<10	<20	**80**	<20	++	ND	ND
	22	ND	ND	ND	ND	ND	1 600	69%

ND = not done

a = months of immunisation

b = enhancement of virus titer in presence of serum

\+ *increase in virus titer of 4-7 fold*

++ *increase in virus titer of >7 fold*

+++ *increase in virus titer of >20 fold*

4.4. Discussion

Six rhesus macaques were immunised with an intact primary isolate of HIV-1 in order to study the evolution of the neutralising antibody response over time. Surprisingly, in spite of high levels of total anti-HIV-1 antibodies, none of the animals developed antibodies able to neutralise the primary isolate used for immunisation, and, furthermore, virus replication was enhanced in the presence of serum from the animals. Initially it was assumed that the reason for this might have been denaturation of gp120 caused by the inactivation of the virus preparation, but as a similar phenomenon was observed in the animals immunised with the live virus, this is not likely to be the explanation. Immune enhancement of lentivirus infection has been described in animal lentivirus infections. Increased severity of the disease induced by EIAV was seen in ponies immunised with recombinant protein subunits produced in baculovirus vectors. However, in this model immunisation with whole inactivated virus resulted in protection from challenge with both homologous and heterologous virus.[26] Similar results were obtained in goats immunised with CAEV.[27] Cats immunised with inactivated FIV, recombinant protein, or naked DNA coding for FIV envelope proteins, had an increased FIV viral load (compared to that of control animals) following challenge with FIV.[28] Enhancing antibodies have also been described in HIV-infection.[5,6,7,8] Kostrikis *et al.* demonstrated that serum from HIV-infected persons could be enhancing for some isolates, have no effect on others, and neutralise a third group.[29] These results, together with those of Jiang *et al.* raised the question of whether antibody enhancement or neutralisation of virus might be virus-specific or even serum-specific.[30] Whether the enhancing antibodies observed in the macaques would lead to increased severity of infection is not known, and as no macaque challenge virus with an HIV-1_{BX08} envelope existed at the time of the study, this hypothesis was not tested.

However, enhancing antibodies have been shown to be clinically relevant in dengue virus and RSV infections. Dengue haemorrhagic shock syndrome is caused by the presence of low affinity antibodies on the surface of virus particles, leading to increased adherence of virus to the cell surface, either via the Fc region of immunoglobulin molecules on the cell surface, or via the complement receptor.[31] Children immunised with a formaldehyde-inactivated RSV vaccine developed more severe clinical disease than non-vaccinated children. The apparent reason for this phenomenon is facilitation of virus infection of macrophages by anti-RSV antibodies.[32]

This phenomenon of enhancement of HIV infection of cells by immune sera should be carefully studied, as this would mean that a non-efficacious vaccine could actually be harmful to recipients.

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4.6. Papers related to study (see appendix)

1. Verrier F, Moog C, Barrè-Sinoussi F, **van der Ryst E**, Spenlehauer C, Girard M. Immunisation with whole purified human immunodeficiency virus type 1 particles elicits enhancing antibodies in rhesus macaques. *Bull Acad Natle Med* 2000; **184**: 67 - 87.

Note:

The candidate made a major contribution to protocol planning and execution of the study (under supervision of Prof M Girard). The candidate was responsible for day to day coordination of the study (preparation for inoculations, inoculations at the appropriate times, determining sampling times, deciding which assays to perform on samples taken at specific timepoints, obtaining reagents where required and liasing between groups in different laboratories) and follow-up of the animals. The candidate established and validated the HIV-1_{GB8} gp120 antibody assay, and tested sera from various timepoints for the presence of high-avidity anti-gp120 antibodies using this assay. All CTL assays were performed by the candidate. The candidate was also responsible for establishing a database to collate results, and for analysis and interpretation of the results (including preparation of graphs, figures and tables), as well as for regular preparation of progress reports.

Contributions of other authors:

F Verrier - performed the syncytium assays (not covered in this thesis) and prepared the associated paper

C Moog and C Spenlehauer - performed the neutralisation assays

M Girard – initiated the study and directed it

F Barrè-Sinoussi – preparation of virus stocks and co-direction of the study

CHAPTER 5

SUMMARY

5.1. Immunogenicity of recombinant Mengoviruses expressing HIV-1 Nef or SIV Pol, Gag and Nef CTL epitopes

Recombinant Mengoviruses expressing heterologous genes were proven to be safe and immunogenic in both mice and primates, and to be able to induce both humoral and cellular immune responses. Several recombinant Mengoviruses expressing either a large region (aa 65 - 206) of the HIV-1 Nef gene product, or CTL epitopes from the SIV Gag (aa 182 - 190), Nef (aa 155 - 178) and Pol (aa 587 - 601) gene products were engineered. The heterologous antigens were expressed either as a fusion protein with the Mengovirus L protein, or in cleaved form through autocatalytic cleavage by the FMDV 2A-protein. Rhesus macaques and BALB/c mice inoculated with the Mengovirus-SIV recombinants failed to develop CTL responses against the SIV gene products, while one of the HIV-Nef recombinants induced a weak CTL response directed to an HIV-1 Nef epitope at position 182 - 198. In contrast, BALB/c mice immunized with vaccinia virus recombinants expressing HIV-1 Nef developed a strong CTL response to the epitope at position 182 - 198, and also responded to a second epitope at position 73 - 81. These results indicate that Mengovirus recombinants expressing HIV-1 Nef and SIV CTL epitopes are weak immunogens. One of the fusion recombinants expressing SIV CTL epitopes failed to infect macaques even when used at high doses, while the recombinant expressing HIV-1 Nef as a fusion protein failed to infect BALB/c mice. This shows that the expression of certain heterologous sequences as fusion proteins with L can result in the loss of the ability of the recombinant to infect normally susceptible animals.

5.2. Immunogenicity of canarypox-HIV-1 recombinant viruses in the chimpanzee model

5.2.1. Study of protection from intravenous HIV-1 challenge in chimpanzees immunised with a recombinant canarypox-HIV-1 virus

To evaluate the potential protective efficacy of a live recombinant HIV-1-canarypox virus vaccine candidate, two chimpanzees were immunised five times with ALVAC-HIV-1 vCP250, a recombinant canarypox virus which expresses the HIV-$1_{IIIB/LAI}$ gp120/TM, *gag* and protease gene products. One month after the last booster inoculation, the animals were challenged by intravenous injection of cell-associated virus in the form of PBMC from an HIV-$1_{IIIB/LAI}$-infected chimpanzee. One chimpanzee with a neutralising antibody titer to HIV-$1_{IIIB/LAI}$ of 128 at the time of challenge was protected, whereas both the second animal, with a neutralising antibody titer of 32, and a naive control animal became infected. At 5 months after challenge, the protected chimpanzee and a third animal, previously immunised with various HIV-1_{MN} antigens, were given a booster inoculation. The two animals were challenged intravenously 5 weeks later with 20 $TCID_{50}$ of cell-free HIV-1_{DH12}, a heterologous subtype B isolate. Neither chimpanzee had neutralising antibodies to HIV-1_{DH12}, and neither one was protected from infection with this isolate. The immune responses elicited by vaccination against HIV-$1_{IIIB/LAI}$ or HIV-1_{MN} did not, therefore, protect the animals from challenge with the heterologous cell-free HIV-1_{DH12}. This indicates that even intrasubtype cross-protection might be difficult to achieve, depending on the isolates used for vaccination and challenge.

5.2.2. The development of a vaginal HIV-1 challenge model in chimpanzees

In an attempt to develop an animal model for mucosal HIV-1 infection, adult female chimpanzees were inoculated via the cervico-vaginal route with either cell-associated or cell-free HIV-$1_{IIIB/LAI}$. The inoculum, in a total volume of 0.25 ml, was atraumatically deposited

at the entrance of the cervical canal using a long catheter to which a piece of flexible tubing was attached. Using this procedure, infection could be established with a high dose of cell-associated virus ($2x10^6$ PBMC from an infected chimpanzee) or cell-free HIV-$1_{IIIB/LAI}$ (1250 $TCID_{50}$), in some female chimpanzees. A few animals, however, resisted repeated attempts at infection by the genital route. In contrast, female chimpanzees were readily infected by this route with 500 $TCID_{50}$ of HIV-$1_{E90/CR402}$. To test the relative susceptibilty of female chimpanzees to genital infection with a subtype B virus versus a subtype E virus, a female chimpanzee was simultaneously inoculated with 500 $TCID_{50}$ each of HIV-$1_{IIIB/LAI}$ and HIV-$1_{E/90CR402}$. Only HIV-$1_{E/90CR402}$ was recovered from the PBMC and lymphnode cells of the animal, suggesting that the subtype E virus was transmitted more easily via the genital route. Genital infection could also be established with HIV-1_{DH12}, but not with HIV-$1_{A/92UG02}$. This study paves the way for future studies on vaccine protection from mucosal infection with HIV-1 in the chimpanzee model.

5.2.3. *Study of protection from genital challenge in chimpanzees immunised with a recombinant canarypox-HIV-1 virus*

Vaginal transmission accounts for more than 80% of HIV-1 infections worldwide. An effective vaccine against HIV-1 would, therefore, have to induce immunity to infection acquired via the mucosal route. To study protection from genital HIV-1 challenge, five female chimpanzees were immunised four times via the im and/or mucosal routes with $4x10^8$ pfu of ALVAC-HIV-1 vCP250, a recombinant canarypox virus expressing the HIV-$1_{IIIB/LAI}$ gp120/TM, *gag* and protease genes. Four of the immunised animals and a naive control female were challenged one month after the last boost with 1250 $TCID_{50}$ of a chimpanzee-passaged cell-free HIV-$1_{IIIB/LAI}$ stock inoculated at the *os cervix*. The control animal became infected, while all four of the vaccinated animals were protected. Four months later the 5

animals were again boosted with ALVAC-HIV-1 vCP250, and challenged two months later with the chimpanzee-passaged HIV-$1_{IIIB/LAI}$ at the *os cervix*. This time two of the immunised animals became infected, but the control animal remained uninfected. The uninfected animals were challenged a last time; this time both the control animal and one of the immunised animals became infected. One of the five immunised animals, therefore, appeared to resist a total of 3 challenges, while 2 further animals resisted two cervico-vaginal challenges. These results demonstrate that female chimpanzees can be protected from vaginal challenge with HIV-1 by repeated mucosal and/or systemic immunisation with a live recombinant canarypox virus. However, only low levels of HIV-1-specific serum and secretory antibodies, as well as neutralising antibodies (titers below <64) were present at time of challenge. This suggests that neutralising antibodies may have little importance for protection from mucosal infection in chimpanzees, in contrast with what is seen for intravenous challenge.

5.3. Study of the immune response induced in rhesus macaques immunised with a primary HIV-1 isolate

The immune correlates of protection from infection with HIV remain ill-defined, but it is likely that both a humoral, as well as a cell-mediated immune response will be needed for protective immunity. Humans infected with HIV-1 develop a cell-mediated immune response early on in infection, but the development of a neutralising antibody response is often delayed for more than 6 months following infection. To evaluate the evolution of neutralising antibodies to a primary isolate of HIV-1, six rhesus macaques were repeatedly immunised with a preparation of intact HIV (live or intactivated) obtained from a primary subtype B isolate (HIV-1_{BX08}). All of the animals developed moderate to high titers of total anti-HIV-1 antibodies as measured by EIA. However, Western blot analysis of sera revealed that the response was mainly Gag directed, and that only weak anti-Env responses had developed in

the immunised animals. This was confirmed by the fact that anti-gp120 antibody titers were ≤1600 in all animals, with only low to moderate avidity. Although two animals developed low titer neutralising antibodies to a T-cell line-adapted HIV strain (HIV-$1_{IIIB/LAI}$), the animals developed no antibodies able to neutralise HIV-1_{BX08}. Surprisingly, sera from the animals induced strong facilitation of HIV-1_{BX08} replication in PBMC. The virus infectious titer was increased by as much as 90-fold in the presence of serum from some of the animals. These results raise the concern that whole virus based HIV vaccines might induce facilitating antibodies that can result in facilitation of transmission and/or evolution of disease.

APPENDIX

PAPERS RELATING TO WORK DESCRIBED IN THESIS

1. Girard M, Barré-Sinoussi F, **van der Ryst E**, Fultz P. Vaccination of chimpanzees against HIV-1. *Antibiotics and Chemotherapy* 1996; **48**: 121 –124 (review).

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Vaccination of Chimpanzees against HIV-1

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The development of an effective vaccine against the human immunodeficiency virus type-1 is a formidable task. The only appropriate animal for the study of prototype vaccines is the chimpanzee, which can be infected with only certain strains of HIV-1. Chimpanzees do not develop AIDS but remain persistently infected, therefore allowing one to study protection from infection, not protection from disease. Several groups have succeeded in demonstrating that gp160- or gp120-based HIV-1 vaccines can protect chimpanzees from experimental challenge with cell-free virus, using a variety of HIV-1 antigens, including purified recombinant gp160 and a synthetic peptide of the V3 loop (the principal neutralization determinant of the virus) [1–3], or gp120 but not gp160 [4], or gp160 but not gp120 [5]. It was also shown that immunization can protect chimpanzees from experimental infection with HIV-1-infected lymphocytes [6; unpubl. data]. Remarkably, the common denominator of the immune response in all the protected animals was a high V3-targeted neutralizing antibody titer at the time of challenge. We also recently showed that immunization with purified recombinant gp160 and a V3 peptide from the HIV-1 MN strain could protect chimpanzees from challenge with HIV-1 SF-2 (a heterologous virus strain) [7]. These animals showed high titers of V3-targeted anti-MN neutralizing antibodies. Two chimpanzees immunized in parallel with a combination regimen including a recobinant canarypox virus expressing the gp160 antigen of HIV-1 MN (ALVAC vCP125), followed by purified gp160 MN were not protected from the same challenge. Their titers of anti-MN neutralizing antibodies and anti-V3 MN antibodies at the time of challenge were one order of magnitude lower than that of the protected animals. Surprisingly, none of the animals, irrespective of protection, had neutralizing antibodies to the SF-2 virus used for challenge. The HIV-1 SF-2 stock used for challenge had been passaged only in human peripheral blood mononuclear cells. Thus, it was equivalent to primary isolates of HIV-1, which appear to be refractory to neutralization by antisera from vaccinees [8, 9]. Since primary isolates are sensitive to neutralization by HIV-specific immunoglobulins from infected persons, the question arises as to whether the failure of vaccinees' sera to neutralize primary HIV-1 isolates is due to qualitative differences in the type of antibodies, or to lack of sensitivity of the neutralization assays. Thus, whether the chimpanzees were protected from HIV-1 SF-2 infection by in vivo neutralization of the challenge virus, or by another immune mechanism, is unknown.

Direct evidence that neutralizing antibodies may play a major role in protection of chimpanzees from experimental HIV-1 infection stems from passive protection experiments. Emini et al. [10] showed that a V3-specific monoclonal antibody could prevent HIV-1 infection in chimpanzees when given either before or directly after challenge with the virus. Altogether, the results of vaccine studies in the HIV-chimpanzee model have emphasized the importance of the V3 loop of gp120 in virus neutralization and protection from infection. This is in contrast to results of vaccine studies in the SIV-macaque model where the V3 region of env does not seem to play a particularly important role since antibodies to V3 do not have neutralizing activity. In fact, the importance of neutralizing antibodies in protection from SIV infection is unsure. Recent studies on clinical isolates of HIV-1 suggest that the apparent dominance of V3 may be an artifact of laboratory adaption of virus strains. It is possible that neutralization of primary wild-type HIV-1 strains may be more similar to that observed for SIV, than to that of the T-cell-line-adapted IIIB or MN strains used in chimpanzee studies, and that antibodies targeted to the V2 loop, to the CD4 binding site [11] or to neutralization epitopes in gp41 [12] play a greater role than those targeted to V3. This implies that the relevance of the SIV-macaque model for the development of HIV vaccines may be greater than initially anticipated, but leaves open the question of immune correlates of protection.

The most formidable problem to face in the development of a vaccine against HIV-1 is that of virus variability, particularly the hypervariability of the envelope [13]. Although certain virus clades are found preferentially in certain countries, there does not appear to be a strict localization of clades to precise geographical areas. The F subtype, initially identified in Romania, is also present in Brazil, and the C subtype from South Africa is highly prevalent in India. Virus isolates belonging to clades A, B, C, D and E have been recovered from patients in the Central African Republic [14] as well as in England [15] and Russia [16]. This diversity creates a formidable obstacle to the development of an HIV vaccine. One way to overcome the problem of antigenic variability of the virus would be to develop vaccines capable of inducing antibodies targeted to conserved neutralization epitopes. However, the CD4 binding site in gp120 is a complex three-dimen-

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sional conformational site, which is partly masked on the surface of wild-type virions and is poorly immunogenic. Similarly, it has thus far not been possible to induce significant titers of gp41-targeted neutralizing antibodies with the immunogens currently available. Whether it will be possible to achieve significantly better results using new antigenic formulations, such as pseudo-virus particles, remains unknown at this time. We are left, therefore, with the hope that a cocktail of env and/or V3 sequences might induce a neutralizing antibody response of sufficient diversity to neutralize all virus strains, independent of the subtype. Preliminary evidence suggests that there is no cross-protection between HIV-1 strains from the B and E clades in the chimpanzee model. It is also not known whether there would be cross-protection between all strains within a specific clade, i.e. if a single antigen preparation per clade would be sufficient. In addition, it is not known whether CTL epitopes are conserved among HIV-1 clades, or vary from clade to clade, which would make the induction of broad cell-mediated immunity as difficult as the induction of broadly neutralizing antibodies.

It is obvious that much more basic knowledge is required at the present time. New chimpanzee challenge virus stocks from primary virus isolates, including isolates belonging to subtypes other than B, should be developed. The search for correlates of protective immunity should be continued. A way to induce neutralizing antibodies to the conformation-dependent neutralization epitopes of gp120 is urgently needed. Finally, the variability of the CTL epitopes among HIV-1 clades should be studied.

In the end, it is only from efficacy trials in persons at risk for HIV infection that we will eventually be able to conclude with certainty whether the vaccines under development can be of practical value.

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We wish to express appreciation and thanks to our colleagues who have collaborated in the experiments mentioned in this review; these include Marie-Paule Kieny, Bernard Meignier, Elizabeth Muchmore, Peter Nara and Tom Matthews. We also wish to thank Jean-Paul Levy for continuous encouragement and support.

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Concluding Remarks

An Approach to Vaccines against Human Immunodeficiency Virus

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THE DEVELOPMENT OF an effective vaccine against AIDS is a formidable task. The only appropriate animal for studying prototype human immunodeficiency virus type 1 (HIV-1) vaccines is the chimpanzee, which can be infected with certain strains of HIV-1. Chimpanzees do not develop AIDS but remain persistently infected, therefore allowing the study of protection from infection, but not of protection from disease. Several groups have succeeded in demonstrating that gp160- or gp120-based HIV-1 vaccines can protect chimpanzees from experimental challenge with cell-free virus, using a variety of HIV-1 antigens, including purified recombinant gp160 and a synthetic peptide of the V3 loop (the principal neutralization determinant of the virus)[1–3] or gp120, but not gp160,[4] or gp160, but not gp120.[5] It was also shown that immunization can protect chimpanzees from experimental infection with HIV-1-infected lymphocytes (Ref. 6; and our unpublished data). Remarkably, the common denominator of the immune response in all of the protected animals was a high V3-targeted neutralizing antibody titer at the time of challenge. We have also shown that immunization with purified recombinant gp160 and a V3 peptide from the HIV-1_{MN} strain could protect chimpanzees from challenge with HIV-1_{SF-2} (a heterologous viral strain).[7] Surprisingly, none of the animals showed neutralizing antibody to the challenge virus, in spite of high titers of V3-targeted anti-MN neutralizing antibody. The HIV-1_{SF2} stock used for challenge had been passaged only in human peripheral blood mononuclear cells. Thus, it was equivalent to primary isolates of HIV-1, which appear to be refractory to neutralization by antisera from vaccinees.[8,9] Since primary isolates are sensitive to neutralization by HIV-specific immunoglobulins from infected persons, the question arises as to whether the failure of vaccinees' sera to neutralize primary HIV-1 isolates is due to qualitative differences in the type of antibodies or to lack of sensitivity of the neutralization assays. Thus, whether the chimpanzees were protected from HIV-1_{SF2} infection by neutralization of the challenge virus *in vivo* or by another immune mechanism is unknown.

Direct evidence that neutralizing antibodies play a major role in protecting chimpanzees from experimental HIV-1 infection stems from passive protection experiments. Emini *et al.*[10] showed that a V3-specific monoclonal antibody could prevent HIV-1 infection of chimpanzees when given either before or directly after challenge with the virus. Similar results have been obtained in the case of HIV-2. Putkonen *et al.*[11,12] showed that cynomolgus monkeys could be protected against challenge with HIV-2 by either active or passive immunization; however, to add to the complexity, it has been reported that, in spite of the total absence of anti-HIV-2 neutralizing antibodies, cross-protection from HIV-2 infection could be achieved in rhesus monkeys by vaccination with a recombinant vaccinia virus (NYVAC) expressing HIV-1 Gag, Pol, and Env, followed by purified HIV-1 p24 plus gp160.[13] This result reopens the question of immune correlates of protection and also suggests that broad vaccine protection may be achievable, at least in certain animal models.

In the simian immunodeficiency virus (SIV)–macaque model, the V3 region of *env* does not seem to play a particularly important role, since antibodies to V3 do not have neutralizing activity. In fact, our understanding of the importance of neutralizing antibodies in protection from SIV infection is tentative. This conclusion is in contrast to the results of studies of vaccines in the HIV–chimpanzee model, which demonstrate the importance of the V3 loop of gp120 in viral neutralization and protection from infection. Studies on clinical isolates of HIV-1 suggest that the apparent dominance of V3 may be an artifact. It is possible that neutralization of wild-type HIV-1 strains is more like that observed for SIV than that of the T cell line-adapted IIIB or MN strains used in studies in chimpanzees, implying that the relevance of the SIV–macaque model for the development of HIV vaccines may be greater than initially anticipated.

There is reason to believe that both a strong neutralizing antibody response and a strong cytotoxic T lymphocyte response must be induced if a vaccine for HIV-1 is to be truly effica-

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cious. On the one hand, it is important that the virus be rapidly neutralized and, if possible, prevented from multiplying at the portal of entry. Thus, HIV-1 vaccines should elicit high levels of neutralizing antibodies, including high levels of antibodies in mucosal secretions. On the other hand, the fact that HIV-1-specific helper T cells can be detected in HIV-1-seronegative partners of seropositive individuals[14] has led to the hypothesis that the immune system might be able to clear a low-dose HIV infection via a cell-mediated immune response. HIV-1 vaccines should therefore be able to elicit a cell-mediated immune response—particularly a cytotoxic T cell response—to recognize and destroy virus-infected cells. It is unfortunately difficult for a single vaccine to induce both a strong neutralizing antibody response and a strong cytotoxic T cell response; a combination of vaccines will therefore be required. A clear synergistic effect between two successive vaccines was observed in human volunteers who were primed with a live vaccinia virus/gp160 recombinant[15,16] or a live canarypox virus/gp160 recombinant (ALVAC HIV-1vCP125)[17] and then boosted with a gp160 subunit vaccine. The live canarypox/gp160 regimen induced a sustained neutralizing antibody response to HIV-1_{MN} in all of the vaccinees and a cytotoxic T cell response to Env in 40% of them.[17] Additional live canarypox virus recombinants that express not only gp160 but also cytotoxic T lymphocyte epitopes from *gag*, *nef*, and *pol* have been developed and are under study.

The most formidable problem that we face in the development of a vaccine is viral variability, particularly the hypervariability of the envelope. HIV-1 isolates have been found to form two groups, the M group and the newly identified O group. The M group is divided into at least nine subtypes or clades (designated A–I), on the basis of sequence homologies in the *env* gene.[18] Although certain clades are found preferentially in certain countries, clades do not appear to be strictly localized to precise geographical areas. The F subtype, initially identified in Romania, is also present in Brazil, and the C subtype from South Africa is highly prevalent in India. Viral isolates belonging to clades A, B, C, D, and E have been recovered from patients in the Central African Republic[19] as well as in England.[20] This diversity creates a formidable obstacle to the development of an HIV vaccine. Fortunately, cross-neutralization analyses of different viral isolates suggest that conserved patterns of neutralization may exist across subtypes. For example, some sera from infected patients neutralize all HIV-1 isolates, irrespective of subtype. This is believed to be due to the presence of neutralizing antibodies targeted to conserved neutralization epitopes, such as those present in gp41[21] or corresponding to the CD4-binding site in gp120.[22] One way to overcome the problem of antigenic variability of the virus, therefore, would be to develop vaccines capable of inducing antibodies targeted to such conserved epitopes. The CD4-binding site in gp120, however, is a complex, three-dimensional conformational site, which is partly masked on the surface of wild-type virions and is poorly immunogenic. Similarly, it has not been possible to induce significant titers of gp41-targeted neutralizing antibodies with the immunogens currently available. Whether it will be possible to achieve significantly better results using new antigenic formulations, such as pseudovirus particles, is still unknown. We are left, therefore, with the hope that a cocktail of *env* and/or V3 sequences might induce a neutralizing antibody response of sufficient diversity to neutralize all viral strains, independent of subtype. Preliminary evidence suggests that this will not be easy. In addition, it is not known whether cytotoxic T lymphocyte epitopes are conserved among clades; if they vary from clade to clade, induction of broad cell-mediated immunity would be difficult.

It is obvious that much more basic knowledge is required. New chimpanzee challenge stocks from primary virus isolates, including isolates belonging to subtypes other than B, are badly needed. The search for correlates of protective immunity should be continued in animal models. A way to present the gp120/gp160 molecules from wild-type virus strains in their native three-dimensional conformation is urgently required. Finally, the variability of the cytotoxic T lymphocyte epitopes among HIV-1 clades should be studied.

It is to be hoped that selection of the most appropriate vaccine, or combination of vaccines, will be possible on the basis of phase I clinical trials in human volunteers. These should pave the way to eventual efficacy trials in persons at risk for HIV infection. Only then will we be able to conclude with certainty whether the vaccines under development are of practical value.

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Sous la Direction
du Professeur F. ROUSSELET et
des Docteurs P. CHAPPUIS et J. POUPON

LE BIOLOGISTE FACE AU SIDA

par :

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LA MISE AU POINT D'UN VACCIN CONTRE LE VIH-1*

The development of a vaccine against HIV-1

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RÉSUMÉ

Des progrès essentiels ont été accomplis ces dernières années dans la mise au point de vaccins contre le virus de l'immunodéficience humaine, le VIH-1. Le premier pas important a été fourni par la démonstration que des chimpanzés pouvaient être protégés d'une infection par le VIH-1 LAI grâce à une immunisation par des glycoprotéines d'enveloppe virale, seules (gp 120 et gp160) ou en combinaison avec des peptides synthétiques issus de la région immunodominante de la protéine gp 120, la boucle V3. Il a également été démontré que l'immunisation pouvait protéger les chimpanzés contre une infection expérimentale avec des lymphocytes infectés par le VIH-1 LAI. Un autre pas important fut fourni par la preuve que les chimpanzés immunisés par la protéine gp 120 ou gp 160 et le peptide V3 issus de la souche VIH-1 MN étaient protégés contre l'infection par une souche comprenant un sous-type B hétérologue, la souche VIH-1 SF2. Cette observation montre qu'une protection croisée entre différents virus hétérologues VIH-1 issus de la même classe est possible. Des travaux faisant état d'une protection contre la surinfection de chimpanzés préalablement infectés ont donné des résultats semblables. En revanche, une immunisation ou une préinfection réalisée à l'aide d'une souche de sous-type B ne confère pas de protection contre une infection hétérologue par un virus de sous-type E, ce qui montre que de nombreux antigènes correspondants à divers sous groupes devront être inclus dans un éventuel vaccin contre le VIH-1.

Un autre écueil majeur provient du fait que les vaccins candidats utilisés jusqu'à présent n'ont induit qu'une réponse immune de durée limitée et n'ont pas permis de montrer que les anticorps obtenus pouvaient neutraliser différents isolats cliniques de VIH-1. Ce type d'anticorps, qui n'est que tardivement rencontré au cours de la maladie chez les sujets infectés pourrait, en conséquence, être difficile à induire par vaccination. De nouveaux moyens de présenter l'antigène sont à l'étude pour y remédier. La réponse à ce problème et à d'autres questions comme :

« La réponse de la cellule T cytotoxique est-elle nécessaire pour la protection à long terme ? »
« Une immunité de type muqueuse est elle nécessaire ? »

constituent aussi des prérequis au développement d'un vaccin efficace.

Le développement d'un vaccin efficace contre le SIDA constitue une tâche formidable. L'absence de guérison naturelle authentifiée de cette affection soulève le problème d'une quelconque efficacité d'un éventuel vaccin dirigé contre la transmission naturelle du virus de l'immunodéficience humaine (VIH). On ne sait pas encore quelle type de réponse immune doit être engendrée par un vaccin en vue d'une protection contre l'infection par le VIH. De nombreux obstacles doivent être surmontés, parmi lesquels l'extrême variabilité antigénique du virus, son mode de transmission intracellulaire, sa porte d'entrée par les muqueuses et la nature persistante de l'infection.

Le problème de la variabilité du virus

Le problème le plus redoutable à résoudre pour le développement d'un vaccin anti VIH-1 est celui de la variabilité virale, particulièrement celle de son enveloppe (1). La diversité du virus VIH provient à la fois de la susceptibilité de la reverse transcriptase à la mutation et d'une éventuelle recombinaison entre différents génomes. Les sujets infectés par le VIH-1 hébergent un grand nombre de clones différents de génomes viraux génétiquement apparentés. Au sein de la population, les isolats de VIH-1 peuvent être répartis en 2 groupes, le groupe M et un autre récemment identifié, le groupe O. Si l'on se réfère à des homologies de séquences au sein du gène env (1), le groupe M comprend au moins 8 sous-types ou classes (de A à H). Bien que certaines classes soient préférentiellement retrouvées dans certains pays, ces dernières ne peuvent être strictement localisées à des aires géographiques définies. Le sous-type F, initialement identifié en Roumanie a été retrouvé au Brésil et le sous-type C d'Afrique du Sud est très présent aux Indes et au Brésil. Des isolats viraux appartenant aux classes A, B, C, D et E ont été retrouvés chez des sujets de la république Centrafricaine (2), et

également en Angleterre (3) et en Russie (4). Cette diversité constitue un écueil majeur au développement d'un vaccin. Heureusement, des études de neutralisation croisées menées sur différents isolats de virus suggèrent qu'entre les sous-types existerait des formes de neutralisation qui seraient conservées. Ainsi, on a montré que de nombreux sérums issus de patients infectés d'origine africaine peuvent neutraliser la plupart des isolats VIH-1, quelque soit leur sous-type d'origine. L'hypothèse admise est que les anticorps neutralisants seraient dirigés contre des épitopes de neutralisation conservés, tels ceux rencontrés dans la gp41 (5) ou la gp120 (6) qui constitue le site de fixation du virus sur les cellules CD4. De même, des anticorps à large spectre neutralisant sont retrouvés dans le sérum des sujets porteurs sains à long terme.

L'une des possibilités de surmonter cette variabilité antigénique serait donc de développer des vaccins capables d'induire des anticorps possédants ces épitopes conservés. Malheureusement, le site de fixation à la cellule CD4, situé au sein de la protéine gp120, a la conformation d'un complexe tridimensionnel, qui, partiellement masqué à la surface des virions de la souche sauvage, est peu immunogène. Chez les patients séropositifs, les anticorps pouvant bloquer la fixation au site CD4 n'apparaissent que tardivement et ne sont pas facilement induits lors de l'immunisation d'animaux ou de volontaires humains. De même, en faisant appel à des substances immunogènes les plus courantes, il n'a pas encore été possible d'induire à des taux significatifs des anticorps neutralisants dirigés contre la gp41. Nous ne savons pas encore si de meilleurs résultats seront obtenus en faisant appel à de nouvelles formulations immunogènes comme les liposomes ou des particules pseudo-virales. Pour l'instant, nous sommes contraints d'exploiter des motifs vaccinaux comme la boucle V3 et espérons qu'un cocktail des

séquences env et/ou V3 pourrait potentiellement induire une réponse anticorps suffisamment diversifiée pour neutraliser toutes les souches virales, indépendamment des sous-types. Cependant des études préliminaires montrent que cela ne sera pas facile. De plus, on ne sait pas si oui ou non, les épitopes CTL sont conservés parmi les classes de VIH-1. Si la réponse était négative, obtenir une large immunité cellulaire constituerait un véritable casse-tête.

La nécessité d'induire une réponse cellulaire cytotoxique et des anticorps neutralisants

Aucun vaccin unique n'est capable d'induire à la fois une réponse importante en anticorps neutralisants et une importante réponse cytotoxique cellulaire. Il y a des raisons de croire que les 2 types de réponse immune seront nécessaires à l'efficacité véritable d'un vaccin contre le VIH-1. Il est tout d'abord essentiel que le virus soit rapidement neutralisé, et, que, si possible, sa multiplication à la porte d'entrée soit inhibée, ce qui implique qu'un vaccin contre le VIH-1 devrait engendrer l'apparition d'un haut niveau d'anticorps neutralisants, y compris ceux retrouvés dans les sécrétions muqueuses. De hauts titres en anticorps neutralisants ont été induits à plusieurs reprises chez les chimpanzés et chez des volontaires humains (9) en faisant appel à la gp120 suivie d'une réactivation par des peptides V3. De plus, chez les partenaires sexuels séronégatifs de sujets VIH-1 séropositifs (10), il a été retrouvé des cellules T-auxiliaires spécifiques du VIH-1, si bien qu'une hypothèse a pu être formulée quant à la possibilité pour le système immunitaire d'éliminer par une réponse cellulaire une infection VIH à faible dose. Des lymphocytes cytotoxiques spécifiques contre le VIH-1 ont été détectés chez les nouveau-nés non infectés de mères séropositives et

chez des prostituées séronégatives fréquemment exposées au VIH-1, ce qui indique bien qu'un vaccin VIH-1 devrait engendrer une réponse immune d'ordre cellulaire, faisant intervenir plus particulièrement les lymphocytes cytotoxiques, capables de reconnaître et de détruire les cellules infectées par le virus.

Un effet synergique certain entre 2 vaccinations successives a été obtenu chez des sujets humains volontaires. Une administration initiale d'un vaccin à base de virus vivant/gp160 recombinant (11,12) ou d'un virus vivant de type canarypox/gp160 recombinant (ALVAC HIV-1 v CP125) (13) a été ensuite réactivée par un vaccin comportant la sous-unité gp160. Le vaccin de type canarypox/gp160 a induit une réponse soutenue en anticorps neutralisants vis à vis du VIH-1 MN chez tous les vaccinés et une réponse T cytotoxique cellulaire vis à vis de env chez 40% d'entre eux (13). D'autres virus vivants recombinants de type canarypox ont été développés et sont à l'étude. Ils expriment non seulement la gp160 mais aussi des épitopes issus de gag, nef et pol. Reste à savoir si ces espèces, en combinaison avec des sous-unités vaccinantes, des vaccins comportant des particules pseudovirales ou des vaccins synthétiques seront capables d'induire une réponse cellulaire de longue durée et également une réponse immune.

Les modèles animaux

Le seul modèle animal approprié qui puisse servir à l'étude d'un vaccin candidat est le chimpanzé qui ne peut n'être infecté que par certaines souches du VIH-1. Le chimpanzé ne développe pas de SIDA mais reste infecté de manière persistante, ce qui permet de l'utiliser pour étudier la protection contre l'infection mais non contre la maladie. De nombreuses équipes ont démontré que des vaccins comprenant la

gp160 ou la gp120 peuvent protéger le chimpanzé contre une inoculation expérimentale avec du virus libre, en faisant appel à toute une variété d'antigène VIH-1, comprenant la gp 160 recombinante et un peptide synthétique issu de la boucle V3 (déterminant principal du virus impliqué dans la neutralisation) (7, 14, 15), la gp 120 sans la gp160 (16), ou encore la gp 160 sans la gp 120 (17). Il a également été montré que l'immunisation peut protéger le chimpanzé de l'infection expérimentale avec des lymphocytes infectés par le VIH-1 (18, résultats non publiés). Fait remarquable, le dénominateur commun de la réponse immune chez tous les animaux protégés était le haut titre en anticorps neutralisants dirigés contre le V3 au moment de l'essai. Nous avons récemment montré qu'une immunisation par de la gp 160 recombinante purifiée et un peptide V3 issu de la souche VIH-1 MN pouvait protéger le chimpanzé contre l'infection par le VIH-1 SF-2 (une souche virale hétérologue) (8). Ces animaux possédaient de hauts titres d'anticorps neutralisants anti MN dirigés contre le V3. Deux chimpanzés immunisés en parallèle par une combinaison comprenant du virus canarypox recombinant exprimant l'antigène gp160 du VIH-1 MN (ALVAC vCP 125), suivie d'une administration de gp160 MN purifiée n'ont pas été protégés contre une infection identique. Le titre de leurs anticorps neutralisants anti MN et anti V3 MN, au moment de l'essai, se situait à un ordre de grandeur plus bas que chez les animaux protégés. De façon étonnante, et indépendamment de la protection, aucun des animaux n'avait d'anticorps neutralisants contre le virus SF2 utilisé pour l'infection. La souche SF2 utilisée pour l'épreuve n'avait été cultivée que dans les cellules mononucléaires du sang périphérique humain (PBMC) primaires et non pas dans des lignées continues de lymphocytes T. En conséquence ceci était équivalent à des isolats primaires du VIH-1, qui semblent être réfractaires à la neutralisation par des antisérums issus d'individus vaccinés (19,20). Puisque des isolats primaires sont sensibles à la neutralisation spécifique par des immunoglobulines spécifiques anti-VIH issues de personnes infectées, il reste à savoir si l'impossibilité pour le sérum d'individus vaccinés à neutraliser des isolats primaires du VIH-1 provient de différences qualitatives portant sur le type d'anticorps ou sur le manque de sensibilité des essais de neutralisation. Finalement, on ne sait pas encore si les chimpanzés ont été protégés contre l'infection par le VIH-1 SF2 selon un processus de neutralisation in vivo du virus infectant ou par un autre mécanisme immun.

La preuve directe que des anticorps neutralisants pourraient jouer un rôle important pour la protection des chimpanzés contre l'infection expérimentale à VIH-1 a été apportée par des études de protection passive. EMINI et al (21), ont montré qu'un anticorps monoclonal spécifique antiV3 pouvait prévenir de l'infection à VIH-1 chez le chimpanzé à condition de l'administrer avant ou immédiatement après l'inoculation par le virus. Au total, les résultats des études de vaccination chez le chimpanzé ont montré l'importance de la boucle V3 de la gp120 pour neutraliser le virus et pour assurer la protection contre l'infection. Cette observation est en contradiction avec les résultats d'études de vaccination chez le macaque, sensible au SIV, pour qui la région V3 de env ne semble jouer de rôle particulièrement important puisque les anticorps anti V3 n'ont pas d'activité neutralisante. En réalité, il n'est pas certain que les anticorps neutralisants soient essentiels à la protection contre l'infection par le SIV. Des études récentes portant sur des isolats cliniques du VIH-1 suggèrent que l'apparente prédominance du V3 pourrait n'être qu'un artefact de laboratoire, résultat d'une adaptation de la souche virale. Il est possible que le type de neutralisation de la souche primaire type "sauvage" de VIH-1 s'apparente plus à celui observé pour le SIV, que celui observé pour les souches IIIB ou MN ayant subi un pas-

sage sur des lignées cellulaires T et étudié chez le chimpanzé. Il est possible que les anticorps dirigés contre la boucle V2, contre le site de fixation au CD4 (5) ou contre les épitopes neutralisants de la gp41 (6) jouent un plus grand rôle que ceux dirigés contre la boucle V3. Dans ce cas le modèle macaque pour le SIV serait plus intéressant qu'initialement prévu pour élaborer un vaccin contre le VIH. Cependant, la question de la relation entre la réponse immune et la protection reste entière.

Conclusion

Il est sur qu'à présent des connaissances fondamentales sont encore nécessaires. Ainsi, des inoculations de chimpanzés par des virus issus d'isolats primaires, comprenant des isolats appartenant à des sous-types autres que le sous-type B sont indispensables. Les recherches portant à la fois sur l'immunité et la protection devraient être poursuivies. Il faudra trouver une méthode pour présenter sous leur conformation native oligomérique, les molécules gp120/gp160 issues des souches virales sauvages. Enfin, il faudra étudier la variabilité des épitopes CTL parmi les différentes classes de VIH-1.

Bien que les modèles animaux fournissent d'importantes données pour les études portant sur l'immunisation, la sélection appropriée du vaccin ou de la combinaison de vaccins à utiliser ne sera possible que par des essais cliniques de phase I chez des volontaires humains. Vus sous un angle optimiste, ces travaux constitueront un guide vers des essais d'efficacité menés chez des sujets présentant un risque d'infection par le VIH. Seuls ces essais, conduits de manière totalement cohérente et éthique pourront peut-être permettre d'apprécier la réelle valeur de différents candidats vaccins.

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HIV Vaccines: Where are we now?

Based on the preceding article and your own personal beliefs, you have now had an opportunity to decide whether or not you would participate in HIV studies. Dr Elna van der Ryst, Acting Head of Virology at the University of the Orange Free State now shares her opinion of the true state of HIV vaccine research. After reading this, you may wonder whether we are ethically ready for human studies at all?

The relentless global expansion of the HIV epidemic claims thousands of lives each year, and the financial cost adds to the economic burden of the already poor developing countries. It is estimated that there are currently 30.6 million people living with HIV/AIDS globally. Of these more than 90% live in the developing world, and 20.8 million in sub-Saharan Africa[1]. Efforts at controlling the epidemic through education and behaviour modification have had limited success, and the need for a vaccine is desperate.

However, the development of an effective vaccine against HIV is a formidable task. The extremely rare reported cases of natural clearance of the infection,[2] and the absence of any documented cases of recovery from the disease, even raise the question of whether any vaccine against HIV-1 could possibly be effective in preventing infection with the virus, and the development of such a vaccine presents the greatest challenge vaccinology has ever had to face.

Virus variability

The biggest problem in the development of an HIV-1 vaccine remains that of virus variability, particularly the hypervariability of the envelope. HIV-1 isolates have been found to form two groups, the M group and the recently identified O group. The M group is sub-divided into at least 10 subtypes (designated A through J), on the basis of sequence homologies in the *env* and *gag* genes[3]. Although certain subtypes are found preferentially in certain countries, there does not appear to be a strict localization of subtypes to precise geographical areas. Chimpanzees that were protected from an intra-subtype heterologous challenge (using HIV-1 SF2) by a vaccine regimen consisting of rgp160 MN/LAI and V3 MN peptides were not protected from inter-subtype cross-challenge using a subtype E virus strain *(E90/CR402)*, showing that no cross-protection exists between HIV-1 subtypes B and E.[4] This implies that although intra-subtype cross-protection might be achievable, the induction of antibodies that will provide inter-subtype cross-protection will be more difficult and that a vaccine might have to contain antigens from several strains in order to induce broad cross-protection. More recently, however, it was demonstrated that a chimpanzee which was immunized with a recombinant canarypox-HIV-1 virus expressing the gpl20/TM, gag and protease genes of HIV-1 IIIB/LAI and protected from challenge with IIIB/LAI, was not protected from challenge with the heterologous subtype B strain, HIV-1 DH12[5], demonstrating that even intra-subtype cross-protection might be difficult to achieve.

...a clear synergistic effect between two successive vaccines was observed in human volunteers...

Immune responses to HIV-1 infection

There is reason to believe that both humoral and cell-mediated immune responses will have to be induced if a vaccine for HIV-1 is to be truly efficacious. On the one hand, it is important that the virus should be rapidly neutralized, and if possible, prevented from multiplying at the portal of entry, therefore, an HIV-1 vaccine should induce high levels of neutralizing antibodies, including high levels of antibodies in mucosal secretions. High neutralizing antibody titres were reproducibly induced in chimpanzees and in human volunteers by priming with gpl60 followed by boosting with V3 peptides.[6] On the other hand, the fact that HIV-1-specific T-helper cells could be detected in HIV-1 seronegative sex partners of seropositive individuals has led to the hypothesis that the immune system might be able to successfully clear a low-dose HIV-1 infection via a cell-mediated immune response.[7,8] This implies that HIV-1 vaccines should also elicit a cell-mediated immune response, particularly CTL able to recognise and destroy virus-infected cells Unfortunately, a single vaccine is unlikely to be able to elicit both a strong neutralizing antibody response and a strong CTL response, and it will probably be necessary to use two vaccines in a prime/boost combination. In fact, a clear synergistic effect between two successive vaccines was observed in human volunteers who were primed with live poxvirus/gp 160 recombinants and then boosted with a gpl60 subunit vaccine.[9]

The HIV-1 secondary receptors and mechanisms of genetic resistance to HIV-1 infection

In 1996 it was shown that apart from CD4, HIV-1 needs a second receptor for efficient binding to the host cell. Macrophage-tropic isolates use the CCR-5 and to a lesser extent also the CCR2 and CCR3 receptors, while T-cell tropic strains use the CXR-4 receptor. This might explain the recent observations that HIV-1 neutralization serotypes, that do not directly correspond to genetic subtypes, exist. Antibody neutralization epitopes do not directly depend on the primary sequence, and genetic variation cannot always be assumed to directly influence the sero-reactivity of these epitopes. Furthermore, when cross-neutralization is observed it often extends even to group O, confirming that some neutralization epitopes are independent of primary sequence variation. More evidence for conserved epitopes in HIV-1 gpl20 comes from recent studies on secondary receptor usage by different HIV-1 strains. It has recently been demonstrated that CCR5 is the major co-receptor for all primary macrophage-tropic strains of HIV-1, irrespective of genetic subtype. From these new data it would follow that if the entry mechanism for all HIV-1 strains is essentially the same (binding to CD4, followed by interaction with CCR5 or CXCR4 and fusion), all HIV-1 strains must have conserved binding sites on their gpl20 molecules. It is likely that these sites are three-dimensional envelope glycoprotein complexes that are relatively similar for all strains, irrespective of subtype. These results are very promising and show that the obstacle of HIV-1 genetic diversity might not be as daunting as we initially feared. Unfortunately, it remains extremely difficult to induce effective immune responses to any primary isolate of HIV-1, whatever the genetic subtype.[10, 11]

Of special interest is the fact that certain individuals carry abnormalities in the genes coding for these receptors[12,13] or other natural ligands[14], resulting in partial resistance to infection

or slower disease progression in these individuals. It has also been shown that certain HLA phenotypes confer relative protection from HIV- 1 infection and lead to slower progression to disease in HIV-1 infected people.[15]

Results of vaccine studies in animal models

Some success has been obtained in studies on animal models of HIV infection. Several groups have succeeded in demonstrating that gpl60 or gpl20-based HIV-1 vaccines can protect chimpanzees from experimental challenge with cell-free virus. It was also shown that immunization can protect chimpanzees from experimental infection with HIV-1 LAI-infected lymphocytes. Remarkably, the common denominator of the immune response in all the protected animals was a high V3-targeted neutralizing antibody response at the time of challenge. Direct evidence that neutralizing antibodies might play a major role in protection of chimpanzees from experimental HIV-1 infection stems from passive protection experiments. A V3-specific monoclonal antibody could prevent HIV-1 infection in chimpanzees when given either before or directly after challenge with the virus. Similar results have been obtained in the case of HIV-2. However, it was recently reported that, in spite of the total absence of anti-HIV-2 neutralizing antibodies, cross-protection from HIV-2 infection could be achieved in rhesus macaques by vaccination with a recombinant vaccinia virus (NYVAC) expressing HIV-1 *gag*, *pol* and *env*, followed by HIV-1 p24 plus gp160. This result reopens the question of immune correlates of protection and also suggests that broad vaccine protection might be achievable, at least in certain animal models[16]. This is supported by the study of Travers et al which demonstrated that a group of high-risk HIV-2 seropositive women had a lower risk of becoming HIV-1 seropositive than an HIV-2 seronegative control group.[17]

Macaque monkeys infected with the simian immunodeficiency virus (SIV) are widely used as an animal model for AIDS. Protection from SIV infection in macaques has proved to be difficult thus far and success has only been obtained by vaccination with live non-pathogenic strains of SIV, such as the SIV nef-deletion mutants described by Desrosiers *et al.*[18] Unfortunately, the immune correlates of the protection conferred by these live attenuated viruses remain undefined. In the SIV-macaque model, the V3 region of env does not seem to play a particularly important role since antibodies to V3 do not have neutralizing activity. In fact the importance of neutralizing antibodies in protection from SIV infection is unsure. Recent studies on clinical isolates of HIV-1 suggest that the apparent dominance of V3 might be an artefact caused by adaptation of virus strains to growth on T-cell lines. It is possible that neutralization of wild-type HIV-1 strains may be more similar to that observed for SIV, than to that of the laboratory-adapted IIIB/LAI or MN strains used in chimpanzee studies and that antibodies targeted to the V2 loop, to the CD4 binding site, or to neutralization epitopes in gp41, play a greater role than those targeted to V3. This implies that the relevance of the SIV-macaque model for the development of HIV vaccines may be much greater than initially anticipated.[16] The development of SIV/HIV recombinant viruses (SHIV) has further increased the importance of the macaque as animal model for HIV-infection, as the protective effect of several HIV antigens can now be evaluated in macaques.

To complicate matters even further, it was shown that non-neutralizing antibodies to *env* antigens can have an enhancing effect in ponies challenged with equine infectious anaemia virus[19]. It was also shown that rhesus macaques repeatedly immunized with whole killed HIV-1 BX08 developed no neutralizing antibodies to the BX08 primary isolate, but rather facilitating antibodies.[20]

The need for human trials

Although studies of HIV-1 vaccines in animal models are essential, selection of the most appropriate vaccine, or combination of vaccines will only be possible through phase 1 clinical trials in human volunteers. This should hopefully pave the way to efficacy trials in persons at risk for HIV-infection. Only through such trials, conducted in a fully coherent and ethical manner, will we eventually be able to assess the true value of candidate HIV vaccines. However, the issues surrounding clinical efficacy trials of HIV vaccines are many and complex. Ethical issues include, among other, the issue of true informed consent, lack of coercion, protection of confidentiality, the fact that the patient will test positive for antibodies.

Results of more than 26 phase I/II trials in more than 2600 humans volunteers have shown that the candidate vaccines tested up to now are safe and immunogenic, but that the immunity induced is, in general, of narrow spectrum and short duration. The most promising results have been obtained in a phase 1 trial using a prime/boost regimen consisting of a canarypox-gpl60 recombinant and a gpl60 subunit vaccine. Neutralizing antibodies developed in >90% of the volunteers and CTL in 40%, but perhaps the most encouraging result of this trial is the fact that several volunteers developed broadly-reactive CTL. This has prompted the announcement of a phase II trial using this combination, which will start in the USA in 1998. The first phase III clinical efficacy trial of a candidate HIV vaccine (rgpl20 from subtype E) is projected to start in Thailand sometime in 1998. The trial is designed to be able to show at least 30% efficacy and results should be available by 2003. If the trial is successful (defined by an interpretable result) this will pave the way for future trials.[21]

Recently it was proposed that a trial using a live attenuated HIV-1, based on the SIV nef-deletion mutants described by Desrosiers et al., be conducted in human volunteers. The rationale for this is the promising results obtained using live attenuated SIV Strains in the macaque model. However, the safety concerns when using a live attenuated retrovirus vaccine are daunting. Firstly, the live SIV deletion mutants, although attenuated for adults, can still cause AIDS in neonatal macaques (admittedly when given at relatively high doses).[22] Secondly, the attenuated viruses might be transmitted to other individuals, and could even be pathogenic for them. A case of a female long-term survivor, who appeared to harbour an attenuated virus, but nonetheless transmitted the virus to her baby who subsequently died of AIDS, has been described.[23] However, the most worrying fact remains the integration of HIV into host DNA, leading not only to the likelihood of lifelong persistence of the vaccine virus, but also carries the risk of insertional mutagenesis.[24] Therefore, to consider the testing of nef-deleted live HIV strains in humans at this stage must be considered as premature, as a candidate vaccine must, above all, be safe.

In conclusion, although human trials are essential, basic research into mechanisms to overcome the problems in developing a vaccine against HIV should not be neglected. New ways to

overcome the problem of antigenic variability of the virus must be sought. One solution would be to develop vaccines capable of inducing antibodies targeted to conserved epitopes such as gp41 or the CD4 binding site. Unfortunately, the CD4 binding site in gp120 is a complex 3D conformational Site, which is partly masked on the surface of wild-type virions and is poorly immunogenic. Similarly, it has thus far not been possible to achieve significant titres of gp41-targeted neutralizing antibodies with the immunogens currently available. Whether it will be possible to achieve significantly better results using new antigenic formulations, such as liposomes or pseudo-virus particles, remains unknown at this time.

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Study of the immunogenicity of different recombinant Mengo viruses expressing HIV1 and SIV epitopes

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SUMMARY

Recombinant Mengo viruses expressing heterologous genes have proven to be safe and immunogenic in both mice and primates, and to be able to induce both humoral and cellular immune responses (Altmeyer *et al.*, 1995, 1996). Several recombinant Mengo viruses expressing either a large region (aa 65-206) of the HIV1 *nef* gene product, or cytotoxic T lymphocyte (CTL) epitopic regions from the SIV Gag (aa 182-190), Nef (aa 155-178) and Pol (aa 587-601) gene products were engineered. The heterologous antigens were expressed either as fusion proteins with the Mengo virus leader (L) protein, or in cleaved form through autocatalytic cleavage by the foot-and-mouth disease virus 2A protein. Rhesus macaques and BALB/c mice inoculated with the Mengo virus SIV recombinants failed to develop CTL responses against the SIV gene products, while one of the HIV-Nef recombinants induced a weak CTL response in mice directed to an HIV1 Nef peptide spanning positions 182-198. In contrast, BALB/c mice immunized with vaccinia virus recombinants expressing HIV1 Nef developed a strong CTL response to the 182-198 peptide and also responded to a second peptide spanning positions 73-81. These results indicate that Mengo virus recombinants expressing HIV1 Nef and SIV CTL epitopes are weak immunogens. One of the fusion recombinants expressing SIV CTL epitopes failed to infect macaques even when used at high doses, while the recombinant expressing HIV1 Nef as a fusion protein failed to infect BALB/c mice. These results demonstrate that the expression of certain heterologous sequences as fusion proteins with L can result in the loss of the ability of the recombinant to infect normally susceptible animals.

Key-words: Mengo virus, HIV1, SIV, CTL; Nef, Pol, Gag.

INTRODUCTION

Live replicating viruses are attractive as vaccine vectors, since they permit the presentation of foreign antigens via the major histocompatibility complex (MHC) class I pathway, and the subsequent induction of cytotoxic T lymphocyte (CTL) responses (Zinkernagel, 1993). Mengo virus is a cardiovirus with an extremely wide host range, which includes primates (Palmenberg, 1990). The

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pathogenic potential of Mengo virus is determined by a poly(C) tract in the 5' non-coding region of the genome, and truncation or deletion of the poly(C) tract leads to a reduction in pathogenic potential (Duke *et al.*, 1990). For instance, the wild-type Mengo virus (which contains a poly(C) tract of $C_{50}UC_{10}$) has a 50% lethal dose (LD_{50}) for 4-week-old BALB/c mice of only 9 plaque-forming units (PFU) when inoculated intracranially. In contrast, a virus containing a truncated poly(C) tract (vM16, poly(C) tract of $C_{13}UC_{10}$) has an LD_{50} of 8×10^6 by the same route, while a virus with a deleted poly(C) tract, vMC0, has an LD_{50} of $>10^9$ (Duke and Palmenberg, 1989; Duke *et al.*, 1990). Furthermore, it has been demonstrated that strains of Mengo virus carrying shortened poly(C) tracts can induce lifelong protective immunity against challenge with virulent wild-type Mengo virus or the related encephalomyocarditis virus in mice and pigs (Osario *et al.*, 1996a,b). Moreover, the replicative cycle of Mengo virus and all other picornaviruses is exclusively cytoplasmic, and the viral RNA genome is amplified without recourse to a DNA intermediate (Rueckert, 1990). Altogether, these properties make Mengo virus an attractive candidate for development as a potential vaccine vector.

We have previously shown that a recombinant Mengo virus expressing a CTL epitope from lymphocytic choriomeningitis virus (LCMV) induced strong CTL responses in mice and afforded protection from lethal LCMV challenge (Altmeyer *et al.*, 1995). Furthermore, a recombinant Mengo virus expressing a 450-bp region from the $HIV1_{LAI}$ gp120 gene induced humoral and cellular immune responses in mice and humoral immunity in macaques (Altmeyer *et al.*, 1994). The aim of the current study was to test the ability of recombinant Mengo virus vectors to induce CTL responses to various HIV and SIV proteins. Several recombinant Mengo viruses with differing degrees of attenuation were constructed to express either a large region of HIV1 Nef, or previously described SIV CTL epitopes. The heterologous proteins were either fused in-frame into the N terminus of the L polypeptide of the vectors, or expressed as a cleaved product. The immunogenicity of each different recombinant was then tested in mice and/or macaques.

MATERIALS AND METHODS

Construction of recombinant viruses

All DNA manipulations for construction of recombinant plasmids were performed according to standard procedures (Sambrook *et al.*, 1989). The following parental vector plasmids were used: (i) pMCS (Altmeyer *et al.*, 1995) which contains a mutagenesis cassette containing unique *Xho*I, *Sna*BI and *Nhe*I sites inserted in the *Nco*I site of the infectious Mengo virus cDNA plasmid (pM16, poly(C) tract of $C_{13}UC_{10}$) (Duke and Palmenberg, 1989); (ii) pMC0S which was constructed by cloning the *Pfl*MI/*Bgl*II fragment of pMCS (containing the mutagenesis cassette) into the *Pfl*MI and *Bgl*II sites of the plasmid pMC0 which is totally bereft of its poly(C) tract (Duke *et al.*, 1990); and (iii) pMCS-2AF which contains the foot-and-mouth disease virus (FMDV) 2A-protein-coding region inserted into the *Sna*BI and *Nhe*I sites of pMCS. This FMDV 2A peptide has previously been shown to undergo autocatalytic cleavage (Ryan and Drew, 1994) and was thus included in

aa = amino acid.
CTL = cytotoxic T lymphocyte.
ELISA = enzyme-linked immunoassay.
FMDV = foot-and-mouth disease virus.
3G = 3-glycine.
HIV1 = human immunodeficiency virus type 1.
IL2 = interleukin-2.
i.m. = intramuscular.
i.p. = intraperitoneal(ly).
i.v. = intravenous.
L = leader (protein).
LCMV = lymphocytic choriomeningitis virus.
LD_{50} = 50% lethal dose.
MHC = major histocompatibility complex.
m.o.i. = multiplicity of infection.
o.n. = oronasal.
p = passage.
PBS = phosphate-buffered saline.
PCR = polymerase chain reaction.
PFU = plaque-forming units.
SDS-PAGE = sodium dodecyl sulphate/polyacrylamide gel electrophoresis.
SIV = simian immunodeficiency virus.
$TCID_{50}$ = 50% tissue culture infectious dose.

certain constructs to enable cleavage of the heterologous protein from the Mengo virus L protein (Habel *et al.*, manuscript in preparation).

A 444-bp cDNA fragment from the $HIV1_{LAI}$ *nef* gene (coding for aa 65-206) was amplified by polymerase chain reaction (PCR) from plasmid pTG1147 (Guy *et al.*, 1987), using oligonucleotides Nef-s (5'-ATT AAC TCG AGG GTG GGT TTT CCA GTC ACA CCT-3') and Nef-as (5'-AT TAA AGC TAG CCA GAG CTC GCA GTT CTT GAA GTA CTC CGG-3') to create unique *Xho*I and *Nhe*I sites. The PCR product digested at these sites was introduced into the *Xho*I and *Nhe*I sites of pMCS and pMCS-2AF to construct plasmids pMCS/nef and pMCS/nef-2AF, respectively (fig. 1A).

Three different SIV Mengo virus recombinants carrying SIV Gag, Nef and Pol CTL epitopes, expressed in tandem and separated by a 3-glycine (3G) linker, were constructed. In a first step, plasmid pMC0S-Δnef was constructed by the introduction of the synthetic oligonucleotides +*Sna*BI/SIVnef/*Nhe*I (5'-GTA TAT TCC TGA TTG GCA GGA TTA TAC TTC TGG TCC TGG TCC TGG TAT TAG ATA TCC TAA GAC TTT TGG TTG GTT GTG GAA GTT GGT GCC TGT GAC G-3') and –*Sna*BI/SIVnef/*Nhe*I (5'-CTA GCG TCA CAG GCA CCA ACT TCC ACA ACC AAC CAA AAG TCT TAG GAT ATC TAA TAC CAG GAC CAG GAC CAG AAG TAT AAT CCT GCC AAT CAG GAA TAT AC-3') encoding aa 153-180 from SIVmac251 *nef*, and with unique *Sna*BI and *Nhe*I sites, into the *Sna*BI and *Nhe*I sites of pMC0S. The sequence coding for aa 182-190 of the SIVmac251 *gag* gene product was then cloned into the *Xho*I and *Sna*BI sites of pMC0S-Δnef to construct pMC0S-Δgag,nef, using synthetic oligonucleotides +*Xho*I/*Sac*I-SIVgag/*Sna*BI (5'-TCG AGT GAG CTC ACT CCT TAT GAT ATT AAT CAA ATG TTG GGT TGG TGG TAC-3') and –*Xho*I/*Sac*I-SIVgag/*Sna*BI (5'-GTA CCA CCA ACC CAA CAT TTG ATT AAT ATC ATA AGG AGT GAG CTC AC-3') to create *Xho*I and *Sac*I sites at the 5' terminus and a 3G spacer and a *Sna*BI site at the 3' terminus. Similarly, pMC0S-Δpol/gag/nef was constructed by cloning synthetic oligonucleotides +*Xho*I/SIVpol/*Sac*I (5'-TCG AGT TTT ATT TCT ACT CCT CCT TTG GTG AGA TTG GTG TTT AAT TTG GTG GGT GGT GGT GAG CT-3') and –*Xho*I/SIVpol/*Sac*I (5'-CAC CAC CAC CCA CCA AAT TAA ACA CCA ATC TCA CCA AAG GAG GAG TAG AAA TAA AAC-3'), encoding aa 587-601 from the SIVmac251 *pol* gene product with unique *Sac*I and *Xho*I sites, and with a 3G spacer at the 3' terminus, into the *Xho*I and *Sac*I sites of pMC0S-Δgag/nef. Plasmid pMCS-Δpol/gag/nef was constructed by replacing the *Pfl*MI/*Bgl*II fragment of pMCS with the corresponding fragment of pMC0S-Δpol/gag/nef. Finally, pMCS-Δpol/gag/nef-2AF was constructed by replacing the *Nhe*I/*Bgl*II fragment of pMCS-Δpol/gag/nef with the corresponding fragment from pMCS-2AF (fig. 1B). All plasmids were sequenced using the "T7 sequencing kit" (Pharmacia) to verify the integrity of the inserted fragments.

The recombinant plasmids were linearized with *Bam*HI and infectious RNA transcripts synthesized using the "T7-RNA-polymerase kit" (Promega). The RNA transcripts were transfected into HeLa cells as described by Duke and Palmenberg, (1989) to generate recombinant viruses. Confluent HeLa cell monolayers were then infected with stock preparations of the recombinant viruses, and these passage-1 virus stocks were sequentially passaged and titrated as described (Emini *et al.*, 1982). Genetic stability of the recombinant viruses was assessed by analysis of the sequentially passaged viruses for the presence of the inserted sequences by reverse transcription (RT)-PCR using Mengo virus-specific oligonucleotides M-609 (5'-A TCT GAT CTG GGG CCT CGG T-3') and M-1212 (5'-GT CTT GAG ACA CTC GGT C-3').

On the basis of genetic stability, five constructions were retained for further analysis: pMCS/nef (HIV) and pMC0S-Δgag/nef and pMCS-Δpol/gag/nef (both SIV) which carry the foreign epitopes as fusion proteins with L; and pMCS/nef-2AF (HIV) and pMCS-Δpol/gag/nef-2AF (SIV) in which the foreign epitopes are expressed in cleaved form separated from the L protein.

In vitro translations, expression in infected cells and immunoprecipitation

*Bam*HI-linearized DNAs were transcribed *in vitro* using T7 RNA polymerase (Pharmacia) (Borman *et al.*, 1994) and purified RNAs were used at different dilutions in reticulocyte lysate (Flexi-Retic, Promega) *in vitro* translation reactions essentially as described (Borman and Jackson, 1992). The reactions, with a final volume of 10 µl, contained 80% (by volume) of reticulocyte lysate, final concentrations of added KCl and $MgCl_2$ of 100 mM, and 0.5 mM, respectively, and 2 mM of amino acids (except methionine). *De novo* synthesized proteins were labelled with ^{35}S-methionine. To study Mengo virus-directed expression of HIV1 Nef in infected cells, HeLa cells were infected at a multiplicity of infection (m.o.i.) of 50-60 PFU per cell, and ^{35}S-methionine-labelled cytoplasmic extracts prepared as described (Harber *et al.*, 1991). The labelled proteins were analysed on 20% polyacrylamide gels followed by exposure of dried gels to "Biomax" film (Kodak) for 16-20 h. Densitometric analyses of radiolabelled proteins was performed using a "Sharp JX-330" analysis system linked to NIH Image software. For the calculation of efficiency of polyprotein cleavage of the 2AF sequence, densitometric values were adjusted to take into account the relative

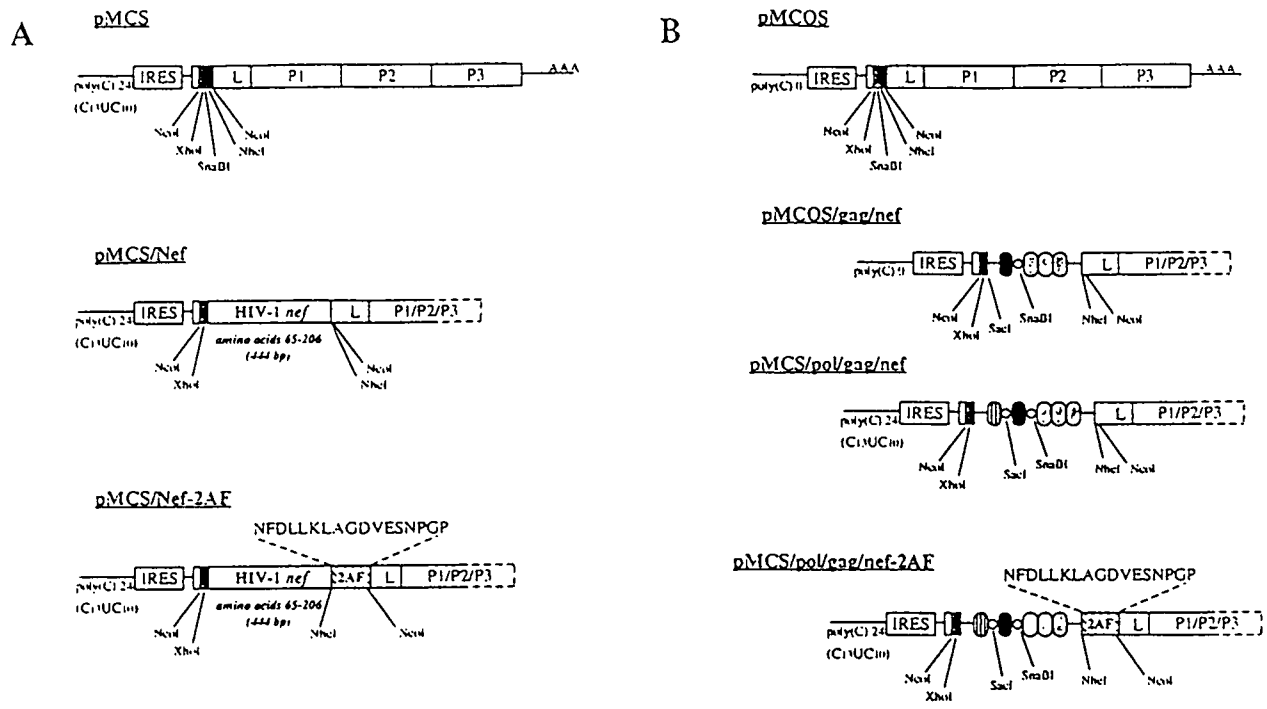

Fig. 1. Organization of recombinant plasmids.

A) Organization of the Mengo virus recombinants pMCS, pMCS/nef and pMCS/nef-2AF. A 444-bp region from the $HIV1_{LAI}$ *nef* gene was generated by PCR and cloned into the *Xho*I and *Nhe*I sites of the parental vectors, pMCS and pMCS-2AF, as described in "Materials and Methods". The amino acid sequence of the FMDV 2A autoproteolytic peptide (2AF) is shown.

B) Organization of the Mengo virus pMC0S, pMC0S-Δgag/nef, pMCS-Δpol/gag/nef and pMCS-Δpol/gag/nef-2AF recombinants. The SIV *pol* (hatched beads) (SIV *pol* aa 590-598, TPPLVRLVF), gag (black beads) (SIV *gag* aa 182-190, TPYDINQML) and *nef* (grey beads) (SIV *nef* aa 155-178, DWQDYTSGPGIRYPKTFGWLWKLV) epitopes were cloned into vectors pMCS, pMC0S and pMCS-2AF as indicated, using the synthetic oligonucleotides described in "Materials and Methods". Where indicated, the different epitopes were separated by spacers of 3G (O).

numbers of methionine residues in the fusion proteins and their cleavage products.

Protein G sepharose beads (Gammabind G, Pharmacia Biotech) labelled with a polyclonal sheep anti-HIV1-Nef serum (MRC AIDS Reagent Research Program) were used for immunoprecipitation (Harlow and Lane, 1988) of the translation products obtained for the Mengo virus recombinants expressing HIV1 Nef.

Immunization of mice and macaques, neutralization and enzyme immunoassays

Adult rhesus macaques (*Macacca mulatta*) of Indian origin were obtained from various primate breeding centres and housed at the Institut Pasteur primate centre under P3 conditions. Animals were housed and cared for according to institutional guidelines for the human care and use of primates in biomedical research; consent for experiments was obtained from the institutional ethics committee. Animals were anaesthetized using ketamine hydrochloride (10 mg/kg via the intramuscular route) prior to all procedures. Animals were immunized with the indicated dose of recombinant virus (see "Results") in a total volume of 1 ml, via the intramuscular (i.m.), intravenous (i.v.) or oronasal (o.n.) route. For o.n. immunization, the virus preparation was slowly dripped into the mouth and nose, and smeared over the buccal areas. Leakage of the inoculum from the mouth and nose did not appear to be significant. The parental virus vM16, obtained from the poly-C-shortened plasmid pM16 (Duke *et al.*, 1990), served as positive control. In a series of separate experi-

ments, seven-week-old BALB/c (H-2^d) mice (4 animals per group) were immunized intraperitoneally (i.p.) with 10^6 PFU (in 200 µl) of either the recombinant viruses, or the parental vectors vM16 and vMC0. A group of mice were also immunized via the i.v. route with 10^7 PFU each of the two recombinant vaccinia viruses which separately express HIV1 Nef aa 73-147 and 148-206 (Transgene, Strasbourg). Virus dilutions for the immunization of the animals were made in Dulbecco's modified Eagle medium (DMEM) supplemented with 50 mM $MgCl_2$. Aliquots of the diluted virus stocks which had served for inoculation of the animals were back-titrated to confirm that the animals had received the correct dose.

Anti-Mengo-virus neutralizing antibody activities were determined by incubating serial two-fold or four-fold dilutions of serum from vaccinated and control animals with a constant amount of virus (100 50% $TCID_{50}$) at 37°C for 1 h. The virus-serum mixture was then added to 10^4 HeLa cells (previously maintained in complete DMEM supplemented with 5% foetal calf serum) in 96-well culture plates and incubated for 6 days at 37°C. Eight wells were used per serum dilution and the neutralizing antibody titre was determined using the Reed and Muench formula (Reed and Muench, 1938).

Antibody responses to the expressed proteins were evaluated by enzyme-linked immunoassay (ELISA). Briefly, 96-well microtitre plates (Nunc Maxisorp) were coated with 100 ng per well of the appropriate peptides (SIV: Nef 155-178, Gag 182-190 and Pol 590-598; or HIV1: Nef 66-100, 93-120, 115-146, 137-168, 155-185 and 182-206) (Neosystems, Strasbourg), or with 100 ng per well of recombinant HIV1 Nef (British MRC AIDS Reagent Research Program). Empty binding sites were blocked by addition of PBS containing 5% bovine serum albumin. Serial two-fold dilutions of sera from the immunized and control animals were then added to the wells. Wells containing only PBS were included as negative controls. After a 2-h incubation at 37°C, the plates were extensively washed and alkaline phosphatase-labelled goat anti-human or anti-mouse IgG (Sigma Immunochemicals; 1:1,000 dilution in PBS) added to the wells and incubated for 1 h. Following extensive washing, *p*-nitrophenyl phosphate substrate was added to each well and the plates were incubated for 30 min in the dark. The optical density (OD) was measured at 405 nm, and the positive cut-off was arbitrarily defined as twice the average OD of the negative control wells.

Cytotoxicity and T-cell proliferation assays

CTL activity in BALB/c mice was evaluated essentially as described by Sauzet *et al.* (1996), except that only 2×10^6 responding cells were mixed with 4×10^6 stimulating cells in 2 ml of culture medium (RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate, 10 mM Hepes, 50 µM 2-mercaptoethanol and 10% fœtal bovine serum), and peptides were used at 10 µg/ml. Target cells were P815 (H-2^d, DBA/2) cells which were either peptide-pulsed (final peptide concentration 10 µg/ml) or were infected with recombinant vaccinia viruses at a m.o.i. of 10 $TCID_{50}$/cell and incubated overnight. Determination of anti-SIV CTL activity in macaques was measured using an *in vitro* peptide restimulation assay as described (Bourgault *et al.*, 1994). Target cells were peptide-pulsed, autologous *Herpesvirus papio* transformed B-lymphocyte cell lines.

T-cell proliferative responses to HIV1 Nef were evaluated using an interleukin-2 (IL2) release assay on CTL.L2 cells as described (Sauzet *et al.*, 1996) and also by measuring direct incorporation of ^{3}H-thymidine into peptide-stimulated splenocytes (Winter *et al.*, 1995) from mice immunized either with the Mengo virus or the vaccinia virus HIV1 Nef recombinants. Six overlapping HIV1 Nef peptides, covering the entire protein from aa 66-206 (66-100, 93-120, 115-146, 137-168, 155-185 and 182-206) (Neosystems, Strasbourg), were used at final concentrations of 3, 10 and 30 µg/ml. Positive controls for proliferation assays used concavalin-A at 2.5 and 5 µg/ml final concentration. Negative controls included incubation with medium alone, or incubation in the presence of equivalent concentrations of an irrelevant peptide corresponding to the V3 loop of HIV1 SF2 gp120.

RESULTS

Study rationale and construction of recombinant viruses

The present study was undertaken to assess the capacity of recombinant Mengo viruses to elicit humoral or cell-mediated (CTL) responses against different HIV1 or SIV epitopes. In the first instance, recombinants were constructed to express a large region of HIV1 Nef. The choice of Nef as a suitable target protein was dictated in part by the fact that it appears to be highly immunogenic in HIV-infected patients (Culmann *et al.*, 1989, 1991; Lahmamedi-Cherradi *et al.*, 1992). However, in order to avoid the complications of the reported oncogenic potential of intact Nef protein (Guy *et al.*, 1987), the recombinants were

designed to express a truncated form of the protein, lacking the first 64 amino acids.

In a second approach to examine the possibility of using this system to induce anti-SIV CTL responses, Mengo virus recombinants containing CTL epitopes from SIV Pol (aa 590-598) (N. Letvin, personal communication), Gag (aa 182-190) (Yamamoto *et al.*, 1990; Miller *et al.*, 1990, 1991) and Nef (aa 155-178) (Bourgault *et al.*, 1994) were constructed. The different epitopes were introduced as a single polypeptide which contains the Pol, Gag and Nef domains in the form of a string of beads in a manner similar to that described by Whitton *et al.* (1993) in a vaccinia virus vector. However, in our constructions, each domain was separated from the others by 3G spacers. The domains from Pol and Gag each contain a single CTL epitope, while the longer 24-aa sequence from Nef contains 3 overlapping CTL epitopes. For both the approaches described above, additional recombinants were also constructed in which the foreign gene insertion and the viral L-coding region were separated by a short sequence encoding the 17-aa 2A peptide from FMDV. This sequence, which has been reported to undergo autocatalytic cleavage (Ryan and Drew, 1994), was used to attain cleavage of the heterologous proteins from L protein with the aim of reducing potential interference between the foreign proteins and the viral polyprotein, and thus increasing the infectivity of the resulting recombinant viruses.

Thus, recombinant Mengo virus cDNA plasmids encoding a 444-bp region coding for aa 65-206 of the HIV1 Nef gene product (fig. 1A), or CTL epitopes from the SIV Pol (aa 590-598), Gag (aa 182-190) and Nef (aa 155-178) gene products (fig. 1B), were constructed as described in "Materials and Methods", using parental vectors pM16 (Duke and Palmenberg, 1989) into which a mutagenesis cassette was inserted (pMCS) (Altmeyer *et al.*, 1995), and pMC0 (Duke *et al.*, 1990). A third vector, pM2AF, was constructed by insertion of the 2A peptide of FMDV (Ryan and Drew, 1994) downstream of the mutagenesis cassette of pMCS (Habel *et al.*, manuscript in preparation) in order to allow cleavage of the heterologous sequence from the Mengo virus polyprotein (fig. 1A, B).

Infectious RNA transcripts were prepared and transfected into HeLa cells as described in "Materials and Methods" to obtain the recombinant viruses vMCS/nef, vMCS/nef-2AF, vMC0S-Δgag/nef, vMCS-Δpol/gag/nef and vMCS-Δpol/gag/nef-2AF. The recombinant viruses were viable, and transfected cells showed a clear cytopathic effect approximately 72 h after transfection, as compared to 48 h for the parental Mengo virus RNA. All of the recombinants had a very small plaque phenotype (≤25% that of vM16) as determined by infection of HeLa cells under an agarose overlay, followed by crystal violet staining of the cell layers (fig. 2). However, RT-PCR analysis of total cytoplasmic RNA isolated from infected HeLa cells as described (Marc *et al.*, 1989) using Mengo virus-specific oligonucleotide primers M-609 and M-1212, demonstrated that the recombinants were genetically stable *in vitro* during at least 5 sequential virus passages (fig. 3).

Next, we analysed the ability of the recombinant viruses to express the heterologous proteins. To this end, genome-length RNA transcripts synthesized from pMCS/nef and pMCS/nef-2AF were used to program reticulocyte lysate *in vitro* translation reactions ("Materials and Methods"). SDS-PAGE analysis of the resulting translation products demonstrated that the heterologous proteins were expressed in addition to Mengo virus proteins and that insertion of the 2AF site allowed cleavage of approximately 85% of the heterologous proteins from L (fig. 4, panel A, total lanes) as determined by densitometric analyses of the autoradiography films. Essentially identical results were obtained upon analysis of radiolabelled proteins present in cytoplasmic extracts prepared as described (Harber *et al.*, 1991) from HeLa cells infected with the two corresponding recombinants (fig. 4B). For both the *in vitro* and *ex vivo* analyses, the identity of the additional proteins (L-Nef and Nef, with sizes of 32 and 25 kD, respectively) expressed by vMCS/nef and vMCS/nef-2AF was confirmed by immunoprecipitation of the ^{35}S-labelled proteins using a polyclonal anti-Nef serum coated onto

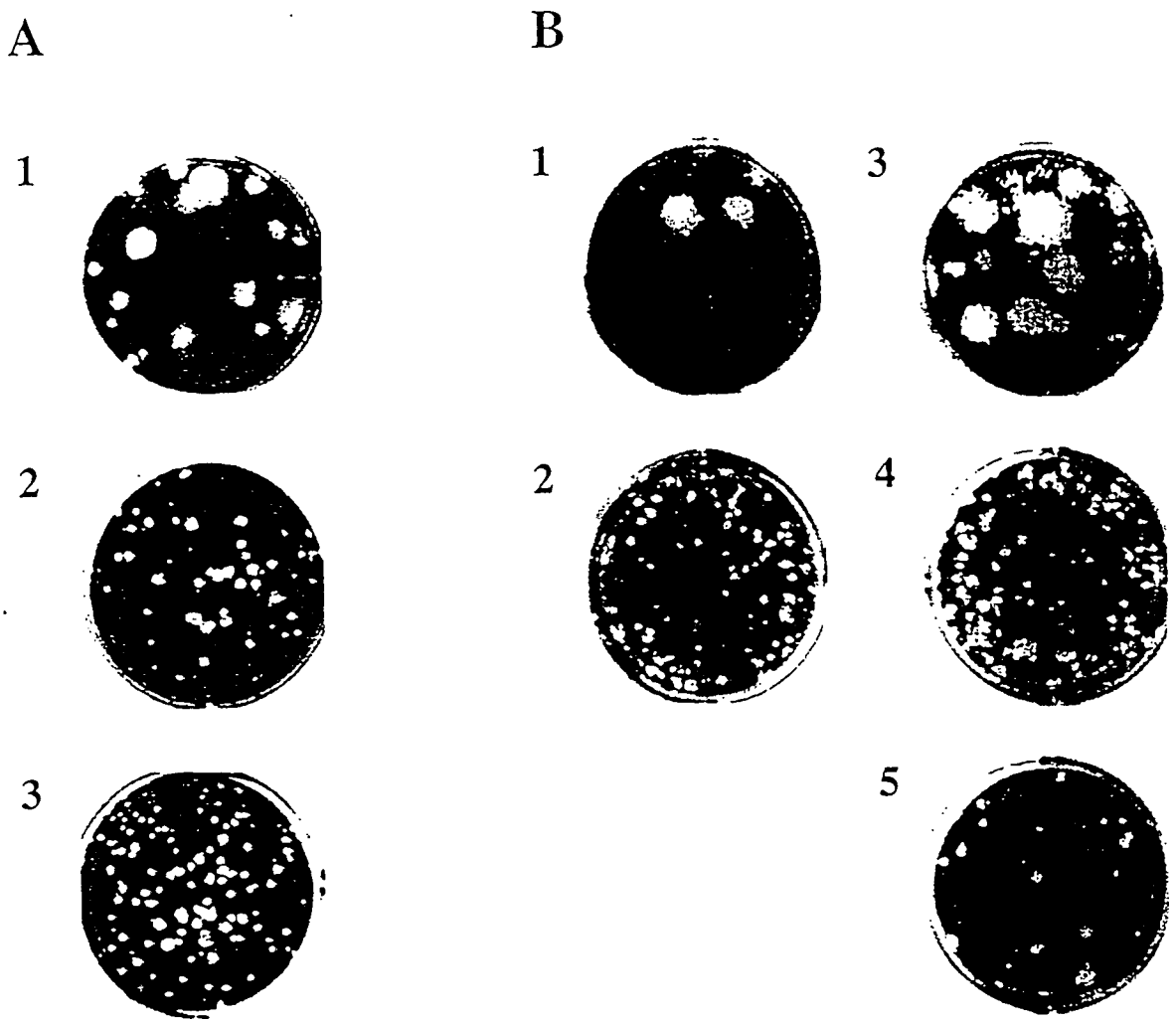

Fig. 2. Plaque size of the recombinant viruses.

HeLa cell monolayers were infected with serial dilutions of the indicated recombinant viruses for 72 h at 37°C under 0.9% agarose and then coloured by crystal violet staining. Each well had a diameter of 35 mm.

Panel A: 1) vM16; 2) vMCS/Nef; 3) vMCS/Nef-2AF.

Panel B: 1) vMC0S; 2) vMC0S-Δgag/nef; 3) vMCS; 4) vMCS-Δpol/gag/nef; 5) vMCS-Δpol/gag/nef-2AF.

protein G sepharose beads (British MRC AIDS Reagent Research Program) (figs. 4A and B, I.P. lanes). A similar *in vitro* translation analysis of RNAs transcribed from the pMCS-Δpol/gag/nef and pMCS-Δpol/gag/nef-2AF cDNAs also confirmed the synthesis of additional non-Mengo viral proteins which migrated with molecular weights compatible with the Δpol/gag/nef product and with the Δpol/gag/nef-L and Δpol/gag/nef-2AF-L fusion proteins (fig. 4C). Once again, the presence of the 2AF sequence allowed efficient separation (more than 70% cleavage) of the Δpol/gag/nef polypeptide from the L protein in the case of the translation of the RNA derived from pMCS-Δpol/gag/nef-2AF (fig. 4C).

Fig. 3. Genetic stability of the recombinant viruses.

Genetic stability of the recombinant viruses as determined by analysing sequential passages of the virus stock for the continued presence of the inserted sequences. HeLa cell monolayers were infected with the indicated viruses at a m.o.i. of 10 PFU/cell and cytoplasmic RNAs were extracted using NP-40 buffer and purified as described (Marc *et al.*, 1989). RT-PCR reactions were programmed with total cytoplasmic mRNA corresponding to 10^5 cells (lanes 1-4, upper panel; lanes 1-5, lower panel) or with 0.1 μg of the parental plasmid DNAs as controls (lanes 5-7, upper panel; lanes 6-9, lower panel). Amplification was performed with the Mengo-virus-specific primers M-609 and M1212.

Upper panel: lane M marker; lane 1=vMCS/nef passage (p) 1; lane 2=vMCS/nef p5; lane 3=vMCS/nef-2AF p1; lane 4=vMCS/nef-2AF p5; lane 5=pMCS; lane 6=pMCS/nef; lane 7=pMCS-nef-2AF; lane 8=negative control.

Lower panel: lane M=marker; lane 1=vMC0S-Δgag/nef p10; lane 2=vMCS-Δpol/gag/nef p1; lane 3=vMCS-Δpol/gag/nef p10; lane 4=vMCS-Δpol/gag/nef-2AF p1; lane 5=vMCS-Δpol/gag/nef-2AF p10; lane 6=pMCS; lane 7=pMC0S-SΔgag/Δnef; lane 8=pMCS-Δpol/gag/nef; lane 9=pMCS-Δpol/gag/nef-2AF; lane 10=negative control.

Immunization of BALB/c mice with the Mengo virus HIV1 *nef* recombinants

To determine whether the two Mengo virus recombinants expressing HIV1 Nef could induce anti-HIV1-Nef immune responses, BALB/c mice were infected once i.p. with 10^6 PFU of either the parental virus vM16 or the recombinant viruses, vMCS/nef or vMCS/nef-2AF. A control group was immunized with a mixture of the two recombinant vaccinia viruses expressing HIV1 Nef aa 73-147 and 148-206. The mice were euthanized three weeks after inoculation. Anti-Mengo-virus neutralizing antibodies developed in mice immunized with vM16 and vMCS/nef-2AF, but not in mice immunized with vMCS/nef, suggesting that vMCS/nef was unable to multiply in the animals (table I). None of the mice developed antibodies to the HIV1 Nef protein as determined by ELISA (table I). The CTL response to HIV1 Nef was analysed using an *in vitro* peptide restimulation test as described by Sauzet *et al.* (1996). The recombinant Mengo virus vMCS/nef-2AF induced a weak CTL response against an HIV1 Nef CTL peptide spanning positions 182-198 (Asakura *et al.*, in press). In contrast, the combined recombinant vaccinia viruses expressing the same region of HIV1 Nef not only induced a stronger CTL response against the 182-198 peptide, but also induced a response directed to a second peptide at position 73-81 (Culmann-Penciolilli *et al.*, 1994). Neither the vaccinia virus, nor the Mengo virus recombinants, induced any CTL targeted to the Nef peptide located at position 132-147 (Culmann-Penciolilli *et al.*, 1994; Asakura *et al.*, in press) (table I). No proliferative responses were induced by either the Mengo virus or the vaccinia virus HIV1 Nef recombinants, as analysed by direct measurement of ^{3}H-thymidine incorporation into splenocytes from immunized mice in reponse to stimulation by HIV1 Nef-specific peptides (data not shown). Indeed, proliferation induced by the recombinants was never more than 10% in excess of that obtained with the negative controls (incubation with medium alone or with an irrelevant peptide; see "Materials and Methods"). This was confirmed by the lack of IL2 production as measured by ^{3}H-thymidine incorporation into CTL.L2 cells cultured in the presence of supernatant obtained from peptide-stimulated splenocytes (data not shown). Conversely, significant proliferation was observed with positive control cultures which had been incubated in the presence of concavalin A "Materials and Methods".

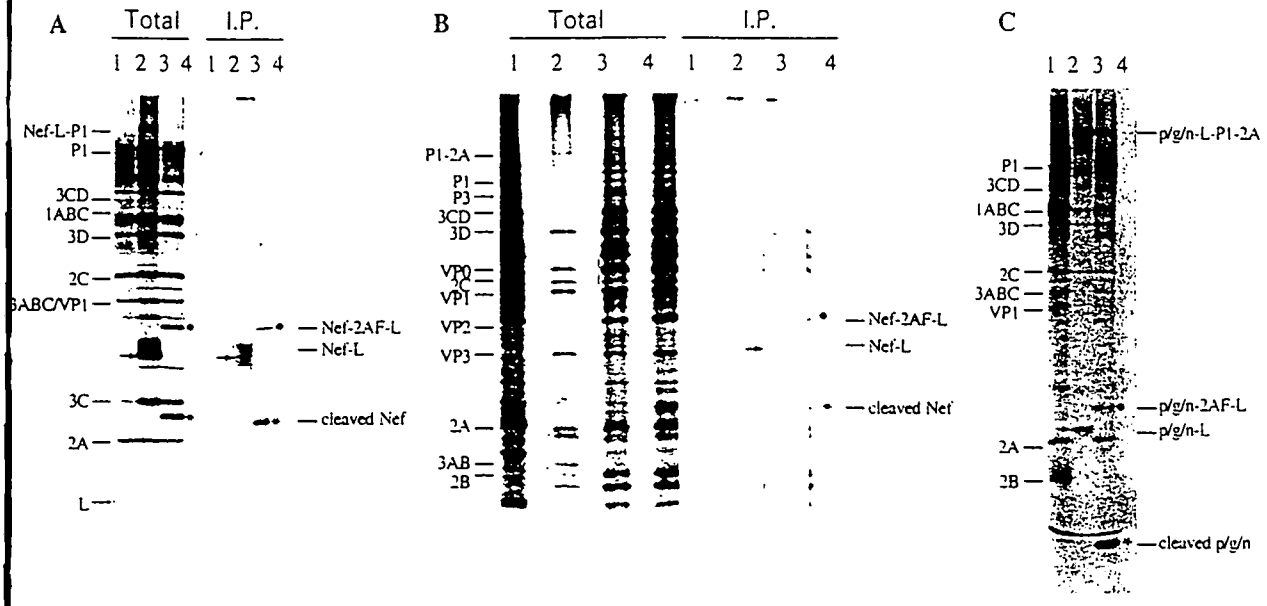

Fig. 4. SDS-PAGE analysis of the proteins expressed by the recombinant Mengo viruses.

Panel A. In vitro translation of mRNAs derived from the Mengo virus/HIV1 Nef recombinants. Purified mRNAs were prepared as described (Borman and Jackson, 1992) and reticulocyte lysate translation reactions (see "Materials and Methods") were programmed with 40 μg/ml (final RNA concentrations) of mRNAs derived from pMCS (lane 1), pMCS/Nef (lane 2), and pMCS/Nef-2AF (lane 3); lane 4=no RNA. After a 3-h incubation at 30°C, reactions were analysed by 20% SDS-PAGE (total lanes), or were immunoprecipitated using anti-Nef serum (see "Materials and Methods") (I.P. lanes).

Panel B. ^{35}S-labelled total cell cytoplasmic protein extracts were prepared as described in "Materials and Methods" from HeLa cells infected with either vM16, vMCS/Nef or vMCS/Nef-2AF at an m.o.i. of 50-60 PFU/cell. Extracts were either directly analysed by 20% SDS-PAGE (total lanes) or were subjected to immunoprecipitation with anti-Nef serum (I.P. lanes). Lane 1=mock-infected cells; lane 2=vM16; lane 3=vMCS/Nef; lane 4=vMCS/Nef-2AF.

Panel C. Expression of the SIV CTL epitopes as demonstrated by *in vitro* translation. Translation reactions programmed with 40 μg/ml of mRNAs (final concentration in extract) derived from pMCS (lane 1), pMCS-Δpol/gag/nef (lane 2), and pMCS-Δpol/gag/nef-2AF (lane 3), (lane 4=no RNA) were incubated for 3h at 30°C prior to analysis of labelled translation products by 20% SDS-PAGE.

In all cases, the positions of the various Mengo virus proteins are indicated. The positions of the heterologous proteins are indicated (p/g/n represents the translation product of the string of SIV pol, gag and nef CTL epitopes). Proteins expressed as fusion proteins with L are marked by an arrow, those separated from L by the FMDV 2A sequence are marked either with an asterisk (when cleavage has occurred at the 2A site) or with a filled circle (for the non-cleaved heterologous protein-2AF-L form). The predicted sizes of the Nef-2AF-L, Nef-L and cleaved forms of Nef are 31,26 and 17 kDa, respectively.

Similar results have been observed by others: a vaccinia virus recombinant expressing the entire HIV1 nef protein induced a CTL response in the absence of a detectable proliferative response (N. Bitton and E. Gomard, personal communication). One explanation for such an observation might be that proliferative responses are normally measured around 10 days postimmunization, whereas CTL responses are typically measured after 21 days.

Given the relatively poor capacity of the Mengo virus HIV1/Nef recombinants to induce

Table I. Immunogenicity of HIV1 Nef recombinant viruses in mice.

Immunogen	Ab titre Mengo virus neutralization	Anti-Nef Ab	CTL 73-81 (*) 100:1	50:1	25:1 (**)	132-147 100:1	50:1	25:1	182-198 100:1	50:1	25:1
vM16	320	<10	0	0	0	0	0	0	0	0	0
Vaccinia/Nef	<20	<10	16	11	9	8	6	4	27	18	12
vMCS/nef	<20	<10	0	0	0	0	0	0	0	0	1
vMCS/nef-2AF	80	<10	11	3	0	0	0	0	11	10	9

Mice were infected via the i.v. route with 10^7 PFU each of the recombinant vaccinia viruses expressing HIV1 Nef aa 73-147 and 148-206, or via the i.p. route with 10^6 PFU of the indicated Mengo virus recombinants. The animals were sacrificed on day 21 and spleen cells were prepared as described (Sauzet *et al.*, 1996). CTL results are given as % specific lysis (adjusted to 2 significant figures) of P815 target cells infected with recombinant vaccinia viruses expressing HIV1 Nef. Background, defined as % lysis of cells infected with wild-type vaccinia virus (Copenhagen strain), was subtracted. Three effector to target cell ratios were used as indicated. Anti-Mengo-virus neutralizing antibody (Ab) titres were determined as described (Reed and Muench, 1938). Anti-Nef antibodies were determined as described in "Materials and Methods".

(*) Nef aa residues in the peptides used for *in vitro* amplification of the CTL population.

(**) Effector to target ratio.

CTL responses in BALB/c mice (as compared to equivalent vaccinia virus recombinants), further studies in macaques were not deemed justifiable.

Immunization of macaques and BALB/c mice with the SIV CTL recombinant viruses

In a first experiment, three rhesus macaques were immunized twice, one month apart, by the i.m. route with 10^6 PFU of vMC0S-Δgag/nef. A control animal was immunized with vM16. All of the animals became infected, as demonstrated by the development of anti-Mengo-virus neutralizing antibodies (table IIa), but no CTL to SIV Gag (182-190) or Nef (155-178) epitopes could be detected using an *in vitro* peptide restimulation assay as described (Bourgault *et al.*, 1994) and autologous peptide-pulsed *Herpesvirus papio*-transformed B lymphocytes as target cells, even though our method could detect significant anti-Nef CTL responses in a positive control animal which had previously been immunized with lipopeptides corresponding to amino acids 155-178 of Nef (Bourgault *et al.*, 1994; data not shown). In a second experiment, four rhesus macaques of Indian origin were immunized via either the i.m. or i.v. routes with 10^6 PFU of the recombinant virus vMCS-Δpol/gag/nef, while two control animals received vM16. Due to the fact that the SIV Gag CTL epitope (182-190) is Mamu-A01-restricted, only animals that had a Mamu-A01-like haplotype were included in the study. As the haplotypes of the animals were determined using a serological assay (Den Haan *et al.*, 1996), discrimination between Mamu-A01 and Mamu-A01-like could not be made. Surprisingly, as judged by the levels of anti-Mengo-virus antibody responses, none of the animals that received the recombinant had become infected, including those inoculated by the i.v. route (table IIb), while both controls were readily infected. A second immunization with 10^7 PFU via alternative (o.n. or i.v.) routes was therefore given. After this second immunization, two of the animals developed very low titres of neutralizing antibodies to Mengo virus, while the other two macaques again resisted infection (table IIb). Three months later, the animals were again immunized, this time with 2×10^7 PFU of vMCS-Δpol/gag/nef-2AF. The rationale for this third immunization was to determine whether the presence of a cleavage site allowing expression of the heterologous protein separate from L protein would increase infectivity of the recombinant *in vivo*. All 4 animals developed high titres of anti-Mengo-virus neutralizing antibodies (table IIb) in response to this high-dose inoculation, but no CTL or antibody

Table IIa. Immunogenicity of recombinant Mengo virus expressing SIV Gag and Nef CTL epitopes in macaques.

Macaque	Immunogen	Neutr. Ab titre after 1st immunization	Neutr. Ab titre after 2nd immunization
92194	vM16	5,500	5,500
92193	vMC0S-Δgag/nef	700	1,800
92195	vMC0S-Δgag/nef	2,500	6,500
92196	vMC0S-Δgag/nef	2,500	6,500

The animals received two immunizations one month apart of 10^6 PFU each, of the indicated viruses via the i.m. route. Anti-Mengo-virus neutralizing antibody (Ab) titres 30 days after each immunization were determined as described (Reed and Muench, 1938).

Table IIb. Immunogenicity in macaques of recombinant Mengo viruses expressing SIV Pol, Gag and Nef CTL epitopes.

Macaque	Immunogen	Route	Dose	Neutr. Ab titre	Immunogen	Route	Neutr. Ab titre
0140	vM16	i.v./o.n.	$10^6/10^7$	2500/2500	vM16	i.v.	8,000
0142	vM16	i.m./i.v.	$10^6/10^7$	11000/11000	vM16	i.m.	12,000
0143	vMCS-Δpol/gag/nef	i.v./o.n.	$10^6/10^7$	<100/400	vMCS-Δpol/gag/nef2AF	i.v.	>16,000
0144	vMCS-Δpol/gag/nef	i.v./o.n.	$10^6/10^7$	<100/200	vMCS-Δpol/gag/nef2AF	i.v.	6,000
0146	vMCS-Δpol/gag/nef	i.m./i.v.	$10^6/10^7$	<100/<100	vMCS-Δpol/gag/nef2AF	i.m.	4,000
0150	vMCS-Δpol/gag/nef	i.m./i.v.	$10^6/10^7$	<100/<100	vMCS-Δpol/gag/nef2AF	i.m.	2,500

Macaques were inoculated with 10^6 PFU of the indicated virus via the i.v. or i.m. routes. When it became clear that the recombinant did not infect the animals, a second dose of 10^7 PFU was given 60 days later via the i.v. or o.n. routes. All animals were subsequently inoculated for a third time, but this time using 2×10^7 PFU of vM16 or the recombinant vMCS-Δpol/gag/nef-2AF via the i.v. or i.m. routes. Anti-Mengo-virus neutralizing antibody (Ab) titres 30 days after each immunization were determined as described (Reed and Muench, 1938).

esponses to the expressed epitopes were detectable (data not shown). Again it was verified that he assay method was capable of detecting anti-Nef CTL responses in the positive control animal which had been immunized with lipopeptides corresponding to aa 155-178 of Nef (Bourgault *et al.*, 1994; data not shown).

The apparent lack of infectivity of some of hese recombinants for macaques, in contrast to previous reports (Altmeyer *et al.*, 1994; Osario *et al.*, 1996a), prompted us to investigate the infectivity of these recombinants for mice. BALB/c nice were immunized i.p. with 10^6 PFU of either he recombinant viruses or the parental poly(C) truncated or deleted Mengo virus vectors vM16 Duke and Palmenberg, 1989) or vMC0 (Duke *et al.*, 1990). All of the animals were infected, but the neutralizing antibody titres in the animals receiving the recombinant viruses were substantially lower than those found in animals immunized with the parental vector virus (table III). Furthermore, no antibodies to any of the expressed epitopes nor any CTL targeted to SIV Nef 155-178 could be demonstrated in the animals. The absence of an anti-Nef CTL response in this experiment is perhaps not surprising, since Winter *et al.* (1995) failed to demonstrate CTL epitopes in this region of SIV Nef in BALB/c mice. Similarly, the lack of antibodies to the expressed epitopes in both mice and macaques might be explained by the fact that these short epitopes do not necessarily include any B epitopes.

Table III. Neutralizing antibody titres of BALB/c mice immunized with the Mengo virus recombinants.

Immunogen	Neutralizing antibody titre
vM16	320
vMC0S	140
vMC0S-SIVΔgag/Δnef	30
vMCS-SIVΔpol/Δgag/Δnef	100
vMCS-SIVΔpol/Δgag/Δnef-2AF	80

Groups of 4 mice were inoculated once via the i.p. route with 10^6 PFU of the indicated virus preparations. Mice were sacrificed 21 days postinfection, and anti-Mengo-virus neutralizing antibody titres were determined on HeLa cells as described (Reed and Muench, 1938) using sera pooled from the 4 mice which had received each immunogen.

DISCUSSION

While the immune mechanisms involved in protection from HIV1 and SIV infection remain unclear, it is likely that a successful vaccine will need to induce both neutralizing antibody and CTL responses. A strong CTL response against the regulatory viral proteins Nef, Tat and Rev, which are expressed early in the viral replication cycle, could lead to elimination of infected cells before release of new particles can occur (Culmann *et al.*, 1989; Winter *et al.*, 1995). In this respect, the Nef protein is an attractive target, since (i) Nef mRNA represents the most abundant viral transcript in the first hours of viral replication (Robert-Guroff *et al.*, 1990), and (ii) Nef is highly immunogenic in HIV-infected individuals (Culmann *et al.*, 1989, 1991; Lahmamedi-Cherradi *et al.*, 1992) and SIV-infected macaques (Bourgault *et al.*, 1992; Venet *et al.*, 1992; Bourgault *et al*, 1994). Although the exact role of Nef in HIV and SIV infection has not been determined, there is good evidence that it is required for maintaining high virus loads in SIV-infected macaques (Kestler *et al.*, 1991). Furthermore, it has been demonstrated that some HIV1-infected long-term non-progressors carry viruses containing truncated *nef* sequences (Deacon *et al.*, 1995; Kirchhoff *et al.*, 1995). Additionally, in the SIV macaque model, it has been shown that infection with a live *nef*-deleted SIV can induce protection from subsequent challenge with virulent SIV (Daniel *et al.*, 1992). Gallimore *et al.* (1995) also demonstrated that a vaccinia virus SIV Nef recombinant could induce protection from SIV challenge in macaques if the CTL precursor frequency is ≥ 1:10,000. Similarly, the *gag* gene products are also present in large amounts in infected cells and thus might prove to be useful targets for induction of anti-HIV1 or anti-SIV CTL responses (Miller *et al.*, 1990; Venet *et al.*, 1992).

With these hypotheses in mind, recombinant Mengo viruses expressing HIV1 Nef (aa 65-206) or SIV Pol (590-598), Gag (182-190) and Nef (155-178) CTL epitopic regions were constructed. The heterologous sequences were expressed either as a fusion protein with the Mengo virus L protein, or in cleaved form through introduction of the FMDV 2A protein, which was shown to undergo autocatalytic cleavage (Ryan and Drew, 1994). Whilst the N-terminal 64 aa of the HIV1 Nef protein were absent from these recombinants, the regions known to contain CTL epitopes in mice have been mapped to the central and C-terminal regions of the protein (Michel *et al.*, 1992; Asakura *et al.*, 1997). The recombinant viruses were viable and genetically stable *in vitro* for several passages, but all had extremely small plaque phenotypes. Although cleavage of the heterologous proteins from L, as mediated by the 2AF sequence, seemed to be efficient, it did not seem to influence the plaque size of the recombinants, as both types of recombinant viruses had a similar plaque phenotype. While the Mengo virus HIV1 Nef recombinants both had a similar small plaque phenotype *in vitro*, the recombinant Mengo virus vMCS/nef, that expressed HIV1 Nef fused to L, failed to infect BALB/c mice, while the recombinant that expressed HIV1 Nef cleaved from L (vMCS/nef-2AF) was infectious *in vivo*. Similar results were obtained for the recombinant Mengo viruses expressing SIV Pol, Gag and Nef CTL epitopes arranged as a string of beads upstream of the Mengo virus polyprotein. In mice, neutralizing antibody titres to these recombinants were significantly lower than those obtained in mice immunized with the

parental vM16 (table III). Moreover, the recombinant virus vMCS-Δpol/gag/nef, that expressed the CTL epitopes fused to L, failed to infect rhesus macaques, while the recombinant that expressed the CTL epitopes cleaved from L (vMCS-Δpol/gag/nef-2AF) was infectious for the animals. Thus it appears that some Mengo virus recombinants that express heterologous proteins fused to L are severely attenuated *in vivo*, leading to loss of infectivity for animals. In addition, for certain of these recombinants, infectivity for animals could not be correlated with *in vitro* viability. Moreover, at least one recombinant (vMCS-Δpol/gag/nef), which failed to infect macaques, retained at least some infectivity for BALB/c mice, suggesting that infectivity *in vivo* for mice and macaques was not always equivalent. However, while we consider it unlikely in the light of the results obtained upon inoculation of macaques with vMCS-Δpol/gag/nef-2AF, we cannot formally exclude the possibility that the vMCS-Δpol/gag/nef recombinant replicated in the macaques without inducing detectable levels of neutralizing antibodies to Mengo virus, or that the different routes of infection of the macaques that were tested dramatically altered their susceptibility to Mengo virus infection.

It has recently been reported that the Mengo virus L protein is involved in shut-off of host cell protein synthesis (Zoll *et al.*, 1996). The same authors also demonstrated that deletion of L resulted in host-cell-restricted virus growth, and it is possible that this phenomenon could explain the results obtained with certain of these Mengo virus recombinants. Indeed, extracts from vMCS/nef-infected HeLa cells show that these recombinants induced very little shut-off of host-cell protein synthesis (fig. 4). It is therefore possible that insertion of the foreign genes into L has inhibited its function sufficiently to prevent infection *in vivo*, although still allowing infection *in vitro*. However, this harmful effect of fusing proteins to L does seem to depend on the nature of the inserted sequence, as Mengo virus recombinants expressing a 450-bp region of HIV1 gp120 or a CTL epitope from LCMV fused to L were not only infectious *in vitro*, but also infectious and immunogenic *in vivo* (Altmeyer *et al.* 1994, 1995). In these respects, the failure of vMCS-Δpol/gag/nef to infect macaques, given that the theoretically more attenuated vMC0S-Δgag/nef apparently retained significant infectivity for these animals, is especially puzzling. At the present time, we have no concrete explanation for this discrepancy. The vMC0S recombinant has a totally deleted poly(C) tract, a deletion which has previously been shown to severely attenuate Mengo virus infectivity for mice (Duke *et al.*, 1990), and which would thus be expected to result in a less infectious virus than the vMCS recombinant which still retains about 50% of its poly(C) tract. In this respect, it is perhaps important to note that there was little observable difference in plaque size between the theoretically more attenuated vMCOSΔgag/nef and the vMCS-Δpol/gag/nef recombinants (fig. 2). Plaque size is, at least in theory, a good indicator of overall viral replicative efficiencies. Whether the additional Pol CTL epitope (9 aa) in vMCS-Δpol/gag/nef is able to so drastically alter virus infectivity for rhesus macaques is under investigation.

The failure of the HIV1 Nef recombinants to induce antibody responses to the Nef protein is disappointing, as a Mengo virus recombinant expressing 450 bp from the V3-C4 region of HIV1 gp120 induced strong humoral immune responses in both mice and macaques (Altmeyer *et al.*, 1994). However, it remains possible that a booster immunization three weeks to one month later might have led to the development of an anamnestic antibody response in the mice. In the case of the SIV CTL recombinants, the lack of antibodies to the expressed CTL epitopes is less unexpected, since these short epitopes have not been shown to contain complete B-cell epitopes.

In the case of the SIV CTL recombinants, the possibility that the "string of beads" approach using the 3G linkers might not have allowed the correct processing of the polypeptides by the proteosome, and subsequent presentation of the optimal peptides in combination with MHC class I molecules on the cell-surface, cannot be dismissed. Indeed, it has recently been demonstrated that the nature of the flanking residues in such polyepitope antigens is of critical importance,

with polyglycine and proline being detrimental to correct recognition of the N-terminally situated epitope in such a "string of beads" (Bergmann *et al.*, 1996). However, the 24-aa region from SIV Nef contains 3 overlapping internal CTL epitopes (Bourgault *et al.*, 1994) and is the last "bead" in the string. Since these 3 C-terminal epitopes were not separated by 3G linkers, one might have expected that they be presented correctly. Alternatively, it could be speculated that the high polymorphism of the outbred macaque population might have resulted in the use of animals which did not possess the correct MHC molecules. The possibility that Nef-induced immunosuppression via downmodulation of MHC class I receptors on the cell surface (Schwartz *et al.*, 1996) could account for the failure of these recombinants to elicit effective CTL responses seems unlikely, since SIV (Winter *et al.*, 1995; Gallimore *et al.*, 1995) and HIV1 (Michel *et al.*, 1992) Nefs have successfully been expressed in several viral and bacterial vectors and have been able to elicit cellular immune responses. Similarly, Bourgault *et al.* (1994) have demonstrated that immunization of macaques with synthetic lipopeptides corresponding to similar regions of Nef could induce specific CTL. Furthermore, in the study presented here, recombinant vaccinia viruses expressing HIV1 Nef and, to a lesser extent, the vMCS/Nef-2AF recombinant were able to induce CTL targeted to HIV1 Nef in BALB/c mice. Whatever the precise reasons for the lack of detectable CTL responses observed in this study, none of the possible factors discussed above would account for the inability of these recombinants to productively infect the susceptible animals used.

In summary, our results indicate that, although attenuated Mengo virus has proven to be a good vector for induction of CTL to a short linear epitope of LCMV (Altmeyer *et al.*, 1995) and also to an epitope in HIV1 gp120 (Altmeyer *et al.*, 1994), it was only weakly immunogenic for HIV1 Nef and also failed to induce CTL directed to selected epitopes of SIV Pol, Gag and Nef. These disappointing results additionally demonstrate that the nature of the insert plays an important role in the immunogenicity of live recombinant Mengo virus vectors, at least when the insertions are made in the Mengo virus L protein. It remains to be determined if the problem of compromised immunogenicity observed here with longer foreign gene insertions is restricted solely to this candidate live viral vector.

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Immunogénicité des recombinants du virus Mengo qui expriment Nef du VIH1 ou des épitopes CTL de Gag, Pol et Nef du SIV

Des virus Mengo recombinants exprimant des gènes hétérologues se sont avérés sans danger et immunogènes à la fois chez les primates et chez la souris, chez lesquels ils induisent une réponse immunitaire à la fois humorale et cellulaire. Des virus Mengo recombinants exprimant soit une grande partie (aa 65-206) du produit du gène *nef* du VIH1, soit des régions épitopiques lymphocyte T cytotoxique (CTL) localisés dans les protéines Gag (aa 182-190), Pol (aa 587-601) et Nef (aa 155-178) du SIV ont éte réalisés. Les antigènes hétérologues ont été exprimés sous forme de protéines de fusion avec la protéine leader (L) du virus Mengo, ou sous forme clivée grace à l'introduction du site de clivage autocatalytique 2A du virus de la fièvre aphtheuse en aval du gène hétérologue. Des macaques rhésus et des souris BALB/c ayant reçu les recombinants virus Mengo/SIV n'ont pas développé de réponses CTL spécifiques du SIV, alors qu'un des recombinants exprimant la protéine Nef du VIH a induit une faible réponse CTL dirigée contre un peptide en position 182-198 de la protéine Nef. En revanche,

les souris BALB/c immunisées avec des recombinants du virus de la vaccine exprimant la protéine Nef du VIH1 ont développé une forte réponse CTL dirigée contre le peptide en position 182-190 et ont également développé une réponse CTL à un second peptide en position 73-81. Les résultats indiquent que les virus Mengo recombinants exprimant la protéine Nef du VIH1 ou des épitopes CTL du SIV sont faiblement immunogènes. Un des virus Mengo recombinants exprimant des épitopes CTL du SIV en fusion avec la protéine L n'a pas infecté les macaques, même après inoculation à de fortes doses; de même, le recombinant exprimant la protéine Nef du VIH1 sous forme de protéine de fusion avec L n'est pas capable d'infecter les souris BALB/c. Ces résultats montrent que l'expression de certaines séquences hétérologues sous forme de protéines de fusion avec la protéine L peut résulter en une perte de la capacité des recombinants d'infecter des animaux qui sont normalement sensibles.

Mots-clés: Virus Mengo, VIH1, SIV, CTL; Nef, Pol, Gag.

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Homologous and heterologous protection from HIV-1 infection in chimpanzees

A MAJOR problem in the development of a vaccine for the human immunodeficiency virus type 1 (HIV-1) is that of virus variability, particularly the hypervariability of the envelope [1]. HIV-1 isolates identified to date form two groups, the M group and the recently identified O group [2]. The M group is divided into at least nine subtypes or clades (designated A through I) on the basis of sequence homologies in the *env* gene [3]. Although some clades are found preferentially in certain regions, there does not appear to be strict localization of clades to precise geographical areas. Virus isolates belonging to clades A, B, C, D and E have been recovered from patients in the Central African Republic [4], as well as in England [5] and Russia [6]. This diversity creates a formidable obstacle for the development of an HIV-1 vaccine.

To study intra- as well as interclade cross-protection, isolates belonging to subtypes other than B should be developed for use in the HIV chimpanzee model. During a recent study focusing on the diversity of the V3 region of HIV-1 strains from the Central African Republic, several clade E viruses were isolated [4]. One of these isolates, CAR/E4002, was adapted to growth on chimpanzee peripheral blood mononuclear cells (PBMC), and a chimpanzee challenge stock was prepared. In this manuscript, we describe the titration of this stock and an interclade challenge of immunized animals using this virus.

Titration of the HIV-1 CAR/E4002 stock

HIV-1 CAR/E4002 is a clade E virus isolated from the blood of a patient from Bangui in the Central African Republic presenting with stage III HIV-associated disease [4]. The virus was adapted to grow on chimpanzee PBMC, and a stock was prepared in chimpanzee PBMC for use in vaccine challenge experiments. The in vitro titer of this stock is 4000 50% tissue culture infective doses ($TCID_{50}$)/mL. To evaluate its infectivity two naive chimpanzees were inoculated intravenously with either 8,000 or 100 $TCID_{50}$; both animals became infected, as shown by the appearance of HIV-1 specific antibodies in their serum and isolation of virus from PBMC. Furthermore, to test whether the virus could be transmitted mucosally, a naive female chimpanzee was inoculated with 500 $TCID_{50}$ by the cervico-vaginal route; this animal also became infected. Due to successful infection by both routes, the virus is now being titrated in chimpanzees. Preliminary results indicate that an animal inoculated with 20 $TCID_{50}$ becomes infected, but that an animal that received 2 $TCID_{50}$ remained uninfected (table I). The 50% chimpanzee infective dose (CID_{50}) of the E4002 stock thus appears to be between 2 and 20 $TCID_{50}$.

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Table I. Titration of the CAR/E4002 chimpanzee challenge stock.

*Dose**	*Chimpanzee*	*Assay*	*Weeks post-infection*					
($TCID_{50}$)			*2*	*4*	*6*	*8*	*10*	*12*
20	C-010	Virus in PBMC	-	+	+	+	+	+
		Antibodies (WB)	-	-	+/-	+	++	++
2	C-038	Virus in PBMC	-	-	-	-	-	-
		Antibodies (WB)	-	-	-	-	-	-

* Titration in vitro of the virus inoculum in parallel with inoculation of chimpanzees gave actual doses of 12 $TCID_{50}$ and < 1 $TCID_{50}$, respectively. PBMC: peripheral blood mononuclear cells; WB: Western blot.

Inter clade cross-challenge with HIV-1 CAR/E4002

We recently showed that two chimpanzees (C-323 and C-483) immunized with gp160-MN/LAI and V3-MN were protected from challenge with HIV-1 SF2, an heterologous clade B strain [7]. These animals remained virus negative after seven months of follow-up. To determine whether they could be protected from an interclade heterologous challenge, they were boosted with gp160-MN/LAI and V3-MN and challenged 2 months later with 100 $TCID_{50}$ of the HIV-1 E4002 chimpanzee challenge stock. Both animals, as well as an unimmunized control animal (C-425), were virus positive at week 2 post-challenge and have remained persistently virus positive by co-culture of PBMC. After challenge, the immunized animals also developed a strong anamnestic antibody response as well as antibodies specific for a clade E V3-loop peptide (table II). Neutralizing antibodies to E4002 (infectivity-reduction assay in human PBMC) appeared only at 7 months post-challenge (table II). Thus, the immunization regimen that protected the two chimpanzees from an intra-clade heterologous challenge (HIV-1 MN vs SF2) failed to protect them from an interclade heterologous challenge (HIV-1 MN vs E4002).

Immunization of chimpanzees with ALVAC-HIV-1 (vCP250)

We previously reported that two chimpanzees immunized with a recombinant canarypox vector (ALVAC-HIV-1 vCP125) expressing the gp160 gene of HIV-1 MN and boosted with soluble rgp160-MN/LAI were not protected from challenge with HIV-1 SF2 [7]. Failure of this vaccination regimen might be explained by the dose of canarypox ($10^{6.1}$ $TCID_{50}$) being too low. To determine whether higher doses of recombinant canarypox virus might confer protection, we immunized two naive male chimpanzees (C-401 and C-451) intramuscularly with $4x10^8$ plaque forming units of ALVAC-HIV-1 vCP250. This is a recombinant canarypox expressing the gp120 (with transmembrane segment), *gag* and protease genes of HIV-1 LAI. The animals received a total of 5 doses of vCP250 which were given at months 0, 1, 5, 9 and 11. No subunit boosts were given. Enzyme immunoassay (EIA) and anti-V3 antibody titers remained at very low levels until

Table II. Anti-V3 and neutralizing antibody titers in chimpanzees after E4002 challenge.

	C-323			C-483		
Months after challenge	*Anti-V3 MN* ELISA*	*Anti-V3 E* ELISA*	*E4002# neutralization*	*Anti-V3 MN* ELISA*	*Anti-V3 E* ELISA*	*E4002# neutralization*
0	19,848	1,091	0	95,242	2,377	0
1	19,341	19,984		134,772	232,010	
2	14,574	14,858		77,500	90,588	
3	11,287	8,191	0	54,740	48,974	0
4	8,611	4,003		42,231	30,999	
5	5,466	2,675		31,476	22,862	
6	5,712	2,651		33,064	39,763	
7	5,148	4,695	0.5	30,411	28,395	0.7

* Anti-V3 antibody titers were determined by ELISA using plates coated with the indicated V3 peptide; # E4002 neutralization titers

Table III. Antibody titers of chimpanzees immunized with vCP250 before and after challenge.

	C-401			C-451		
Time (months)	*EIA*	*Anti-V3 LAI*	*Neutr Ab*	*EIA*	*Anti-V3 LAI*	*Neutr Ab*
6	400	2,114	4	400	712	4
7	400	984	0	200	366	0
8	100	ND	0	100	ND	0
9*	100	758	0	< 100	328	0
10	400	8,856	32	200	848	4
11*	200	3,607	16	200	342	< 4
12*	800	22,237	128	200	7,947	32
13	200	ND	64	6,400	ND	> 512
14	200	ND	32	6,400	ND	> 512

* vCP250 boost; # HIV-1 LAI (IIIB) challenge; EIA: enzyme immuno assay; ND: not done.

after the fourth immunization at 9 months when an increase in anti-V3 antibody titers was observed. This rise was more pronounced after the fifth immunization at 11 months (table III). The EIA titers, however, remained at low levels. Neutralizing antibody titers (determined by the syncytium-inhibition assay) appeared only after the fourth immunization and were markedly increased after the fifth immunization.

One month after the last boost, the immunized animals and a naive control animal (C-353), were challenged intravenously with $6x10^5$ IIIB-infected PBMC (equivalent to 20 infective doses) from an infected chimpanzee [8]. The EIA, anti-V3 and neutralizing antibody titers of C-401 were 3- 4-fold higher than those of C-451 at time of challenge (table III). The anti-canarypox antibody levels of C-401 and C-451 were 159.60 UE/mL and 59.46 UE/mL, respectively. A cut-off of 0.6 UE/mL is considered to be positive in human volunteers, which indicates that the animals developed a strong anti-canarypox antibody response. Western blot (WB) analysis of serum from the animals showed that the anti-*env* responses to vCP250 were weak as compared to the anti-*gag* responses (fig 1). PBMC from the control animal and from one of the immunized animals (C-451) were virus positive at 4 and 2 weeks post-challenge, respectively (not shown). C-451 also showed a strong anamnestic antibody response by EIA and neutralizing antibody assays (table III) and the development of new WB bands with intensification of bands already present (fig 1). Of interest is the fact that the neutralizing antibody titer of the unprotected animal at time of challenge was at the apparent threshold level of 32, whereas that of the protected animal was 128. The second animal (C-401) is still virus negative at this time, and its antibody titers are stable or slowly decreasing (table III).

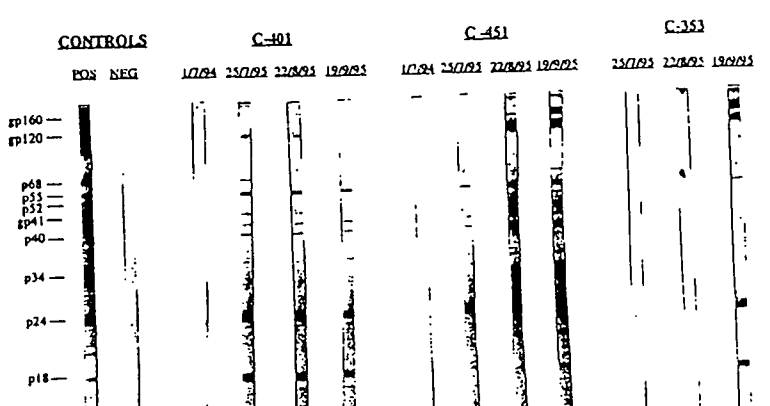

Fig 1. Western blots of chimpanzees immunized with ALVAC vCP250 and challenged with HIV-1 LAI (IIIB).

Conclusion

Previous studies have suggested the existence of a correlation between neutralizing antibody titers and protection from HIV-1 infection in the chimpanzee model [7-9]. In agreement with these findings, we observed that the vCP250-immunized animal with a neutralizing antibody titer of 128 was protected from challenge, whereas the other one, that had a titer of 32, was not protected. This experiment also shows that recombinant HIV-1 canarypox viruses can induce protective neutralizing antibody titers when used for repeated immunizations and at high doses.

In the interclade cross-challenge experiment, however, the results were less encouraging. The animals were protected from an intraclade heterologous challenge despite the complete absence of neutralizing antibodies to the challenge virus (HIV-1 SF2); however, protection did correlate with neutralizing antibodies to the homologous virus used for immunization (HIV-1 MN) [7]. At the time of interclade challenge, the animals had no neutralizing antibodies to HIV-1 CAR/E4002 and were not protected. The question that remains is whether the animals were protected from HIV-1 SF2 challenge by a mechanism other than neutralization, or by low level neutralizing antibodies that might be neutralizing in vivo, but cannot be detected using current neutralization assays. The identification of an HIV-1 strain from a clade other than B that readily infects chimpanzees will facilitate future studies to evaluate potential HIV-1 vaccines. We are currently testing HIV-1 strains from additional clades for possible use in the HIV-1 chimpanzee model.

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Failure of a Human Immunodeficiency Virus Type 1 (HIV-1) Subtype B-Derived Vaccine To Prevent Infection of Chimpanzees by an HIV-1 Subtype E Strain

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Generation of an effective vaccine against human immunodeficiency virus type 1 (HIV-1) must overcome problems associated with extensive genetic diversity. Although we previously reported vaccine-induced protection of chimpanzees against infection with an HIV-1 strain different from the one used to make the immunogens, both the HIV-1 vaccine and challenge strains were classified within subtype B. To determine whether the HIV-1-specific immunity elicited might also prevent infection by a strain of HIV-1 from a different clade, the same chimpanzees were given booster inoculations with the rgp160-MN/LAI (recombinant hybrid gp160 molecule) and V3-MN immunogens and then were challenged by intravenous inoculation of a comparable dose of a subtype E HIV-1 from the Central African Republic. Both animals became infected with the subtype E virus, indicating that intraclade vaccine-mediated protection does not predict interclade protection, at least in the context of intravenous challenge and the HIV-1 strains used. This study has important implications for planned phase III efficacy trials of similar vaccine preparations in Thailand where HIV-1 subtype B and E strains cocirculate.

The extreme genetic diversity of human immunodeficiency virus type 1 (HIV-1) is a major problem to overcome in the development of an effective vaccine (25). HIV-1 strains characterized to date form two major groups, designated M and O, with group M further divided into at least nine equidistant subtypes or clades (A through I) on the basis of sequence homologies in the *env* gene (5, 15–17, 30). Because viruses representing multiple subtypes can be found on more than one continent and within a single country (10, 19, 20, 24, 26, 31), an efficacious vaccine must provide protection from HIV-1 infection or disease after immunization with antigens from a different HIV-1 strain, irrespective of whether the transmitted and vaccine strains are from the same (intraclade) or different (interclade) clades. Phase III vaccine efficacy trials are currently being planned in which existing candidate vaccines generated from HIV-1 subtype B strains will be tested in four countries (Thailand, Brazil, Rwanda, and Uganda) in which multiple subtypes are cocirculating (6, 31). It is expected that one of the first trials will be conducted in an injecting drug user population in Thailand, where HIV-1 subtypes B and E are prevalent (26). Since the candidate vaccine proposed for use in such trials consists of recombinant gp120 (rgp120) derived from the HIV-1 subtype B strain MN, it is important to evaluate the immune responses elicited by such vaccines for their ability to induce cross-reactive immune responses to subtype E strains and to protect against infection in an animal model.

Previous studies demonstrated that chimpanzees could be protected from infection by intravenous challenge with both cell-free and cell-associated HIV-1 when the challenge and vaccine strains were the same: that is, when the challenge was homologous (2, 4, 9, 12). Recently, we and another group also showed that chimpanzees immunized with Env-derived antigens from the subtype B HIV-1 strains MN and LAI could be protected from cell-free virus challenge with another subtype B strain, HIV-1_{SF2} (3, 14). These results demonstrated that, under certain conditions, it was possible to prevent infection in a heterologous intraclade HIV-1 challenge. The study described here was done to determine whether those could be extended to include vaccine-mediated protection from a heterologous interclade HIV-1 subtype E challenge: that is, whether infection by HIV-1 could be prevented when the vaccine was generated from an HIV-1 strain from a different clade.

As previously reported (14), two adult male chimpanzees (*Pan troglodytes*), C-323 and C-483, were immunized with a V3 peptide derived from HIV-1_{MN} (V3-MN) and a purified hybrid gp160 molecule (rgp160-MN/LAI), which lacked a cleavage site and was composed of the gp120 and gp41 moieties from the MN and LAI strains, respectively. During 7 months of follow-up after intravenous challenge with HIV-1_{SF2}, both animals appeared to be protected from infection, as evidenced by failure to isolate or to detect virus in peripheral blood mononuclear cells (PBMCs) or in cells obtained by biopsy from lymph nodes. Attempts to detect HIV-1 included cocultivation of chimpanzee cells with uninfected human PBMCs previously stimulated with phytohemagglutinin (PHA) and nested PCR for proviral DNA. Furthermore, during this period, HIV-1 serum antibody titers to whole virus, gp120, and V3, as well as neutralizing antibody activity, declined progressively, indicating that antigenic stimulation of the immune system as a result of HIV-1_{SF2} replication did not occur (14). Because no evidence of HIV-1 infection was obtained, these two chimpanzees were given booster inoculations with 176 μg of rgp160-MN/LAI and

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TABLE 1. Cell-associated viral burden in peripheral blood lymphocytes from immunized and naive chimpanzees

Chimpanzee	Minimum no. of PBMCs required for HIV isolation at wk[a]:				
	2	4	6	8	12
C-425	3.2×10^3	3.2×10^3	3.2×10^3	6.4×10^4	8×10^4
C-444	ND[b]	8.0×10^4	2.0×10^5	4.0×10^5	6×10^5
C-323	2.0×10^5	8.0×10^3	ND	8.0×10^3	4×10^4
C-483	4.0×10^5	4.0×10^5	$>2.0 \times 10^6$	$>2.0 \times 10^6$	2×10^6

[a] PBMCs were serially diluted 1:5, and duplicate aliquots were cocultured with 2×10^6 PHA-stimulated human PBMCs in 24-well plates. Culture supernatants were monitored for RT activity for 6 weeks. Values are the lowest number of chimpanzee PBMCs required for at least one of the two wells to be RT positive. The symbol > implies no viral RT activity was detected at the highest number of chimpanzee PBMCs cultured.

[b] ND, not determined.

200 μg of V3-MN formulated in incomplete Freund's adjuvant (ISA51; Seppic). Approximately 10 weeks later, the animals were challenged with an intravenous inoculation of 100 50% tissue culture infectious doses ($TCID_{50}$s) of a subtype E strain of HIV-1, 90CR402 (previously designated CAR/E4002), from the Central African Republic (24). This HIV-$1_{90CR402}$ challenge stock, which had been adapted in vitro to infect and replicate in chimpanzee PBMCs, was shown to have a chimpanzee minimal infectious dose between 2 and 5 $TCID_{50}$s (1). The challenge dose, therefore, was equivalent to that of HIV-$1_{LAI(IIIB)}$ which was used in a previous vaccine challenge experiment in chimpanzees (12). A naive control chimpanzee, C-425, was inoculated with the same dose of HIV-$1_{90CR402}$ in parallel with the immunized animals. The chimpanzees were housed at the Laboratory for Experimental Medicine and Surgery in Primates, New York University, in accordance with institutional guidelines and standard practices for the humane care and use of chimpanzees in biomedical research. Before all procedures, the chimpanzees were anesthetized by intramuscular injection of ketamine hydrochloride (10 mg/kg of body weight).

After inoculation of virus, blood samples were obtained from the chimpanzees every 2 weeks for 8 weeks and then at monthly intervals. PBMCs were cocultured with PHA-stimulated normal human PBMCs in RPMI-1640 medium containing 10% fetal bovine serum, interleukin 2 (10 U/ml), and antibiotics. Culture supernatants were tested approximately every 5 days for cell-free reverse transcriptase (RT) activity, at which time an equal volume of medium was replaced; newly stimulated normal human PBMCs were added every 10 days (8). HIV-1 was identified in cultures of PBMCs from all three animals at 2 weeks after inoculation of HIV-$1_{90CR402}$. Subsequent cultures were virus positive at all times tested during 15 months of follow-up, with the exception that all cocultures of C-483's PBMCs obtained at 36 weeks or later have been RT negative, which is consistent with this animal having a lower viral burden. To test this possibility, cryopreserved PBMCs obtained from each animal during the first 12 weeks after challenge were titrated in duplicate by limiting dilution to determine numbers of infectious cells, as described previously (8). All PBMCs from each animal were titrated at the same time on indicator PBMCs from the same uninfected human donor to allow a more accurate comparison of changes over time.

While the minimum number of infectious cells required to detect virus in PBMCs from C-323 was in the same range as those obtained from two unimmunized control animals (C-425, this study; and C-444, originally used to verify the infectivity of this HIV-1 clade E virus stock for chimpanzees [1]), more cells from C-483 were required to detect virus at all times (Table 1). These results indicated that C-483 had approximately 10-fold fewer circulating infectious PBMCs than the other three animals. Since lymph nodes appear to be a reservoir for HIV-1, at 24 weeks after challenge, single-cell suspensions of lymph node tissue from the two immunized animals were titrated as described above; the minimum numbers of lymphocytes required for detection of virus in peripheral lymph nodes from C-323 and C-483 were 8×10^3 and 8×10^4, respectively. These numbers are consistent with chimpanzee C-323 having a higher viral burden than C-483. In addition, the results showed that C-323 had approximately a 10-fold greater number of infectious cells in lymph nodes than most animals infected with HIV-$1_{LAI(IIIB)}$ at similar times after virus inoculation (which generally are comparable to the results obtained with C-483's cells) (7, 14). Because an insufficient number of lymphocytes was obtained from the control animal's lymph node biopsy, it was not possible to titrate its cells.

At the time of and after challenge with HIV-$1_{90CR402}$, serum samples from the chimpanzees were tested for various HIV-1-specific antibody responses. First, with a commercial enzyme immunoassay (EIA) kit (Sanofi Diagnostics Pasteur; Marnes-la-Coquette, France) to evaluate total antibodies, a strong anamnestic response was detectable in both immunized chimpanzees at 4 weeks; the control animal seroconverted at 6 weeks after inoculation of virus (Fig. 1). Second, immunoblot assays revealed that both chimpanzees, which had been immunized only with HIV-1 *env* gene products, developed antibodies to $p55^{gag}$, $p24^{gag}$ and $p18^{gag}$ that were detected at 2 weeks after challenge and persisted thereafter (data not shown). Third, to distinguish between antibodies induced by the HIV-1_{MN} immunogens and those produced in response to HIV-$1_{90CR402}$, serum samples were tested by EIA for reactivity to the V3-MN peptide and to a V3 peptide from the HIV-1 subtype E strain CAR/E4031 (CTRPSNNTRTSVRIGPGQVFYKTGDIIGDI RRAYC), which differs from the V3 consensus sequence of subtype E HIV-1 strains from the Central African Republic by only four amino acids (24, 25). Although serum from both immunized chimpanzees exhibited some cross-reactivity to the HIV-$1_{CAR/E4031}$ V3 peptide at the time they were challenged with HIV-$1_{90CR402}$, the titers were 18- and 40-fold lower than those to the V3-MN peptide (Fig. 2). Consistent with overall antibody titers to HIV-1, the serum anti-V3 titers to the clade

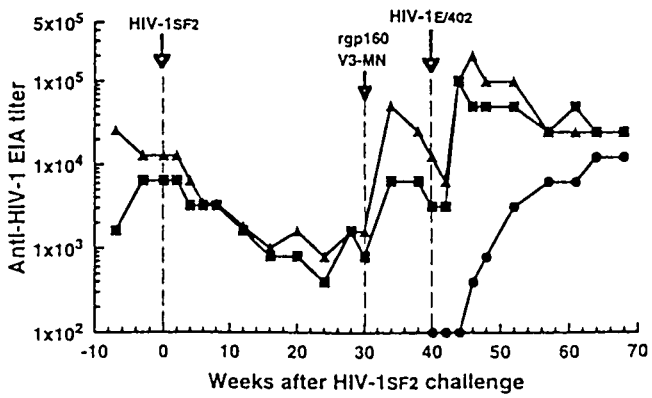

FIG. 1. Serum antibody titers to HIV-1 relative to initial HIV-1_{SF2} challenge. Antibody titers were determined with a commercial HIV-1 EIA kit (Sanofi) and serial twofold dilutions of chimpanzee serum samples; titers are defined as the last dilution of serum that gave an optical density reading above the cutoff value. The times at which chimpanzees were inoculated with the two HIV-1 strains are indicated, as is the timing of the last immunization. ■, C-323; ▲, C-483; ●, naive control C-425.

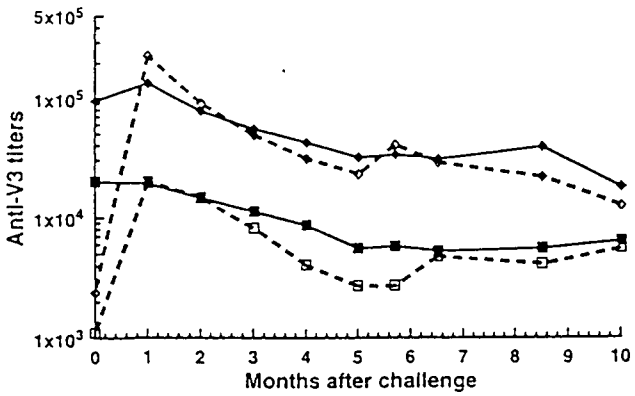

FIG. 2. Serum antibody titers to peptides corresponding to the V3 loops from HIV-1 strains MN and CAR/E4031 after challenge of chimpanzees C-323 (■ and □) and C-483 (◆ and ◇) with HIV-$1_{90CR402}$. Titers were determined with an enzyme-linked immunosorbent assay with microtiter plates coated with V3 peptides derived from HIV-1_{MN} (solid lines) or HIV-$1_{CAR\ E4031}$ (dashed lines).

E virus increased 18- and 97-fold at 4 weeks after challenge and then declined. In contrast, antibody titers to the V3-MN peptide did not increase in either animal after challenge, but rather decreased. In both animals, titers to the two V3 peptides plateaued at comparable levels at 5 months after infection.

Finally, a neutralization assay that measures reduction of infectivity for normal human PBMCs was used to test serum samples for this activity. Briefly, a 1:50 dilution of serum and serial dilutions of a stock of HIV-$1_{90CR402}$ with a titer of 4,000 $TCID_{50}$s/ml were incubated for 1 h before addition of PHA-stimulated human PBMCs to each mixture. After an additional 1-h incubation, the cells were washed twice, resuspended in medium, and distributed into four wells of 24-well tissue culture plates. Supernatant fluids were tested for HIV-1 $p24^{gag}$ antigen on days 6 and 9 with a commercial EIA kit (Sanofi Diagnostics Pasteur), and reduction in virus titers was determined by the Spearman-Karber method. No neutralization activity was detected in serum from either immunized chimpanzee at the time of HIV-$1_{90CR402}$ challenge (data not shown). At approximately 1 year after the challenge inoculation, however, 10-fold reductions in the infectivity titer of HIV-$1_{90CR402}$ were detected in serum samples from both the control and immunized animals.

The serological results and, in particular, the antibody reactivity to the V3 peptides indicated that both chimpanzees were infected with the subtype E HIV-1 strain: however, because they had been exposed previously to HIV-1_{SF2}, it was important to verify the identity of the virus they harbored. Lymph node biopsies were performed on both chimpanzees 24 weeks after inoculation of HIV-$1_{90CR402}$, and genomic DNA was isolated from single-cell suspensions for PCR amplification of proviral DNA. A nested set of primers that flank the V3 loop and recognize isolates from multiple HIV-1 clades was used: outer pair, env1-C (5′-TGTCAGCACAGTACAATGTACAC ATGGAAT-3′) and env1-H (5′-TAGTGCTTCCTGCTGCTC CCAAGAACCC-3′) (840 bp); inner pair, env1-D (5′-CAAC TGCTGTTAAATGGCAGTCTAGCAGAA-3′) and env1-F (5′-ATATAATTCACTTCTCCAATTGTCCCTCAT-3′) (660 bp). PCR amplification, performed as described previously (28), generated products of the expected size (660 bp) that were purified (Geneclean kit; Bio 101, Vista, Calif.), cloned, and transfected into competent cells (TA cloning kit; Invitrogen, San Diego, Calif.). Nucleotide sequences from multiple clones were obtained by the dideoxynucleotide chain termination method (Sequenase version 2 kit; U.S. Biochemicals, Cleveland, Ohio) and were compared with the sequence in this region for HIV-$1_{90CR402}$ (24) and with the consensus sequence for HIV-1 subtype E strains (25). Because no lymph node cells from the control, C-425, were available at 24 weeks, proviral DNA from PBMCs of this animal was used. As an additional comparison, the consensus sequence of 10 independent TA clones generated with the same primers from proviral DNA of PBMCs obtained 4 weeks after intravenous inoculation of chimpanzee C-444 was evaluated.

Of interest, all five clones from lymph node cells of C-483 had fewer mutations in the region sequenced than those from the other two animals challenged at the same time; moreover, none of these mutations resulted in amino acid changes in the V3 loop (Fig. 3). The amino acid sequences of the V3 loop of all nine TA clones from C-444 at 4 weeks were also identical to that of the published sequence of HIV-$1_{90CR402}$, with the exception that one clone had an R→G change at amino acid position 3. In contrast, mutations in proviral DNA in PBMCs from C-425 at 24 weeks resulted in amino acid changes in the V3 loop in five of six clones. One additional clone from C-425 had a one-base insertion that generated a premature stop codon before the V3 loop. All five clones from C-323 differed and had from two to four amino acid changes in the V3 region. Furthermore, amino acid changes in two of the five clones resulted in alteration of the consensus GPGR sequence at the tip of the loop: one clone had GSGR, and the other had GPER. Although variants in the GPG motif have been identified, including an S for the P in multiple HIV-1 strains from different clades, the two G residues rarely vary among the group M HIV-1 strains. Specifically, only 4 of 660 HIV-1 strains in the sequence database have an amino acid other than G in the third position, and none so far identified has an acidic residue such as the glutamic acid (E) found in one clone from C-323 (25). The significance of this particular change, if any, is unknown.

In summary, the gp160/V3-MN immunization regimen that protected two of two chimpanzees from an intraclade challenge with HIV-1_{SF2} failed to protect the same animals from a heterologous interclade challenge with HIV-$1_{90CR402}$. In the previous heterologous intraclade challenge, the animals were protected from infection with HIV-1_{SF2} despite the absence of neutralizing antibodies to the challenge virus; however, protection did correlate with neutralizing antibodies to HIV-1_{MN}, from which the immunogens were derived (14). Likewise, at the time of the present interclade challenge, the animals had

E/90CR402	CTRPFKKVRISARI GPGRVFH TTGNIKGDIRKAYC
C-444 9 cls	-------------- ------- --------------
1 clone	--G----------- ------- --------------
C-425 2 cls	-------------- ------Y --------------
clone-2	----------G--- ------Y ---S----------
clone-3	-------------- ------- --------------
clone-4	-------------- ------- ----------HR
clone-7	----S--------- ------- --------------
C-323 clone-1	--------I----- -----S- --------------
clone-3	-------------V -S---SY --------------
clone-5	----------M- --E--S- --------------
clone-6	--------I----- -----SY --------------
clone-7	-------------- -----SY --------------
C-483 5 cls	-------------- ------- --------------
Cons. CAR/E	----SNNT-T-IT- ---Q--Y R--D-I--------

FIG. 3. Amino acid sequences of the V3 loop of proviral DNA in cells recovered from chimpanzees infected with HIV-$1_{90CR402}$. Proviral DNA was isolated at 24 weeks after challenge from lymph node cells (C-323 and C-483) or PBMCs (C-425) or at 4 weeks from PBMCs (C-444). cls, clones; Cons., consensus.

no detectable neutralizing antibodies to the challenge virus, HIV-$1_{90CR402}$, but neutralizing antibodies to HIV-1_{MN} were detectable (data not shown); however, these animals were not protected from infection. Consistent with this result, in other experiments to be reported elsewhere, an ongoing anti-HIV-1 immune response in chimpanzees infected with the $HIV_{LAI(IIIB)}$ strain for as long as 32 months did not prevent superinfection by the same dose of HIV-$1_{90CR402}$ administered intravenously (7, 11). The identity of the protective mechanism against HIV-1_{SF2} infection in the initial study remains unresolved. It is possible that the chimpanzees were originally protected by a mechanism other than neutralization or, alternatively, that the assays employing human PBMCs are not sensitive enough to detect low levels of cross-reactive neutralizing antibodies.

Under certain conditions of transmission, as demonstrated previously in the HIV-infected chimpanzee model, neutralizing antibodies appear to prevent or contribute to prevention of infection by HIV-1 (2, 9, 12). However, recent extensive studies with large panels of serum samples and viruses representative of most HIV-1 subtypes have shown that, although some serum samples could neutralize across clades, in general, neutralization serotypes did not correlate with genetic subtypes (18). Failure to detect cross-neutralization between subtype B and E viruses was also observed when pooled serum samples selected for high neutralization activity against multiple homologous virus strains were tested reciprocally against viruses representing the other subtype (21). Similar results also were obtained when different sera were tested for the ability to bind various gp120 molecules (22). Because none of the assays described above gave all-or-nothing results with the various combinations of sera and viruses tested, it may yet be possible to identify those epitopes that elicit the most broadly cross-reactive functional antibodies. The feasibility of this goal, which is important for vaccine development, is supported by observations that some monoclonal antibodies to gp120 and gp41 not only bind Env proteins but also neutralize HIV-1 isolates from different clades (23, 29).

Although the two immunized chimpanzees were not protected from infection by the HIV-1 subtype E strain, the quantitative data suggest that the response elicited against subtype B Env gp160 and V3 peptide might have had some impact on HIV-$1_{90CR402}$ replication after challenge. The animal with the higher serum reactivity to HIV-1 antigens, C-483, had fewer infectious cells both in its peripheral circulation at early times and in the lymph node at 24 weeks. In addition, the finding that no amino acid changes in the V3 loop were identified in proviral DNA from this chimpanzee, whereas multiple changes occurred in this region in proviral DNA from both C-323 and C-425, is consistent with less viral replication in C-483 and thus fewer opportunities to accumulate mutations. However, the latter point is speculative, and no definitive conclusions can be made because only two immunized and two naive chimpanzees were studied.

The present study failed to demonstrate interclade protection against HIV-1 subtype E infection after immunization with subtype B Env antigens. A caveat of this experiment, however, is that the challenge inoculum was administered intravenously, which is the most efficient route by which to establish HIV-1 infection, especially compared with mucosal routes (13). Furthermore, it is reasonable to assume that the most efficient route of infection is likely to be the most difficult one to protect against. Thus, the potential efficacy of any HIV-1 vaccine candidate, whether against intra- or interclade infection, should be evaluated for its ability to elicit protective immunity against mucosal infection, not only because success is more likely, but also because the major routes of HIV-1 transmission worldwide are across mucosal surfaces (27). Our results are directly relevant to planned phase III HIV-1 vaccine efficacy trials in Thailand, where both HIV-1 subtypes B and E are present in the target population of injecting drug users (6, 26). Moreover, they suggest that it might be prudent to reevaluate the design of these initial phase III trials, particularly with respect to the immunogens and the study population in which the vaccines will be tested.

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Characterization and Titration of an HIV Type 1 Subtype E Chimpanzee Challenge Stock

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ABSTRACT

A subtype E human immunodeficiency virus type 1 (HIV-1) isolate from the Central African Republic (E/90CR402) was adapted to growth on chimpanzee peripheral blood mononuclear cells (PBMCs) by cocultivation of irradiated, infected human PBMCs with chimpanzee PBMCs. The resulting virus was passaged in chimpanzee PBMCs to generate a stock of chimpanzee-adapted virus. Although its V3 region sequence was identical to that of the parental isolate, the chimpanzee-adapted virus had a syncytium-inducing phenotype as opposed to the non-syncytium-inducing phenotype of the parental virus. After demonstrating in one animal each that the passaged virus could infect chimpanzees following intravenous (i.v.) or cervical inoculation, the i.v. infectious titer of the stock was determined. Exposure of three chimpanzees to different doses of the virus indicated that the titer was between 2 and 5 $TCID_{50}$. Thus, the HIV-1 E/90CR402 chimpanzee challenge stock established persistent infections in chimpanzees by both the i.v. and genital routes and should be valuable for future HIV-1 vaccine studies to evaluate cross-protection between HIV-1 subtypes.

INTRODUCTION

A MAJOR PROBLEM in the development of a vaccine for the human immunodeficiency virus type 1 (HIV-1) is that of virus variation, particularly the hypervariability of the envelope.[1,2] HIV-1 isolates characterized to date form two groups: group M and the recently identified group O.[3–6] Group M viruses are divided into at least 10 subtypes (designated A through J) on the basis of sequence homologies in the *env* or *gag* genes.[2] In North America and Western Europe, virus isolates belonging to subtype B predominate, whereas different combinations of subtypes are found in Africa, Asia, and other parts of the developing world. For example, virus isolates belonging to subtypes A, B, C, D, and E have been recovered from patients in the Central African Republic (CAR),[7] as well as in England[8] and Russia.[9,10] However, the spread of any virus strain is potentially global owing to the increase in international travel. In fact, subtype D and E isolates have been identified in the United States.[11,12] To further complicate the problem, not only dual infections but also recombinant viruses between two distinct HIV-1 subtypes have been described in humans.[13–17] Thus, it is necessary to develop multivalent HIV-1 vaccines that can prevent infection by viruses belonging to all subtypes of HIV-1.

HIV-1 infection of chimpanzees is the only animal model currently available for use in vaccine studies involving experimental challenge. To date the LAI(IIIB) strain of HIV-1 has been used in most vaccine trials in which chimpanzees were challenged; however, this strain is not representative of clinical isolates of HIV-1 because it was adapted to grow in T cell lines.[18–22] More recently, primary isolates of HIV-1 were shown to replicate in chimpanzee peripheral blood mononuclear cells (PBMCs) and to cause persistent infection in the animals,[23–25] but these isolates all belong to clade B. To study intra- as well as interclade cross-protection, challenge stocks of isolates belonging to subtypes other than B must be developed for use in

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the HIV-1–chimpanzee model. Furthermore, because of the limited availability of chimpanzees for biomedical research, it is vital that carefully evaluated stocks of HIV-1 be used for experiments involving challenge of immunized animals. We describe here the adaptation of a CAR subtype E virus isolate to growth in chimpanzee PBMCs and the subsequent generation of a chimpanzee challenge virus stock that can persistently infect chimpanzees by both the intravenous (i.v.) and genital routes. This virus stock is available for use in experiments to evaluate the protective efficacy of candidate vaccines.

MATERIALS AND METHODS

Cells and virus

SupT1 cells were obtained from the American Type Culture Collection; MT2 cells were a kind gift from N. Yamamoto (Yamaguchi University School of Medicine, Japan). Human and chimpanzee PBMCs were obtained from healthy HIV-seronegative donors and were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS), recombinant interleukin 2 (rIL-2, 500 U/ml) (Proleukin; Chiron Corporation, Emeryville, CA), 1% glutamine, 1% PSN (penicilline, streptomycin, and neomycin), anti-interferon serum (1 U/ml; Valbiotech, Paris, France), and Polybrene (0.5 μg/ml; Sigma, St. Louis, MO). MT2 and SupT1 cells were cultured in similar medium, but without addition of rIL-2, Polybrene, and anti-interferon serum. Before infection human PBMCs were stimulated by incubation with phytohemagglutinin (PHA, 200 μg/ml; Murex Diagnostics, England), whereas chimpanzee PBMCs were stimulated with concanavalin A (ConA) (Boehringer Mannheim, Indianapolis, IN) at 10 μg/ml.

HIV-1 strain E/90CR402 was isolated in 1990 by coculture of PHA-stimulated donor human PBMCs with PBMCs from an African male patient from Bangui, CAR, who had stage IV HIV-associated disease (Fatigue, weight loss, herpes zoster, recurrent respiratory infections, diarrhea and lymphadenopathy), as defined by the Centers for Disease Control (Atlanta, GA). This isolate, which was identified originally as HIV-1 E4002 in a study by Murphy *et al.*,[7] has been cloned, and the entire nucleotide sequence determined.[26]

Preparation of chimpanzee-adapted virus stock

An aliquot of the original HIV-1 E/90CR402 isolate frozen in 1990 and kept at −70°C was thawed and used at a dilution of 1:100 to infect a culture of 4×10^6 PHA-stimulated human PBMCs. Cultures were stimulated with fresh donor PBMCs every 7 days, and the reverse transcriptase (RT) activity was determined twice weekly. On day 9 of culture (RT activity, 279,000 cpm/ml), the infected cells were irradiated with 5000 rads using a ^{137}Cs source. An aliquot of 2×10^6 irradiated cells was mixed with 6×10^6 fresh ConA-stimulated PBMCs from a naive chimpanzee (C-380). Supernatant fluids collected at days 16 (RT activity 85,700 cpm/ml) and 20 (RT activity 37,100 cpm/ml) were pooled, and an aliquot of this pool were used to infect 2×10^7 ConA-stimulated PBMCs from a second naive chimpanzee (C-444) at dilutions of 1:10 and 1:100. Supernatant fluids collected at days 23 (RT activity, 148,000 cpm/ml) and 26 (RT activity, 200,000 cpm/ml) were pooled in a total volume of 30 ml, which was reserved for stock. An aliquot of the pooled virus was then used to infect 8×10^7 fresh ConA-stimulated PBMCs from C-444 at dilutions of 1:10 and 1:100. Supernatant fluid (70 ml) from the two cultures was collected on day 13 (RT activity, 44,700 and 138,300 cpm/ml) and added to the 30 ml previously harvested to obtain a final volume of 100 ml.

In vitro *infections and titration of virus infectivity*

To test the ability of the virus to replicate in human and chimpanzee PBMCs, human primary macrophages, and in SupT1 and MT2 cell lines, 10^6 cells were infected with 0.25 ml of a 1:10 dilution of virus. Culture supernatants were tested every third day for 30 days for RT activity and p24 antigen using a commercial enzyme immunoassay (EIA) (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France). Mitogen-stimulated human or chimpanzee PBMCs (4×10^6 cells) were incubated for 60 min at 37°C with 10-fold serial dilutions of virus (from 1:100 to 1:10,000) in 500 μl of RPMI 1640 with 5% FCS. The cells were then washed twice, resuspended at 1×10^6 cells/ml in culture medium, and 1-ml aliquots were distributed into 4 wells of a 24-well tissue culture plate. Supernatant fluids were tested for p24 antigen on days 6 and 9. The 50% tissue culture infectious dose ($TCID_{50}$) was determined from the quadruplicate cultures using the Spearman–Kärber formula.[27]

Virus neutralization

Neutralization activity was evaluated using an infectivity reduction assay, as described by Pellegrin *et al.*[28] Briefly, serial dilutions of the chimpanzee-adapted virus stock were incubated for 60 min at 37°C with a 1:50 dilution of serum from the infected chimpanzees or with monoclonal antibodies at concentrations of 10, 20, or 50 μg/ml. Monoclonal antibodies 2F5, 2G12, and IgG1b12, which can neutralize some subtype E primary isolates when used at high concentrations,[29–31] were obtained from the British Medical Research Council AIDS Reagent Project (Potters Bar, UK). Following virus adsorption to 4×10^6 PHA-stimulated human PBMCs for 60 min at 37°C, the cells were extensively washed and 1-ml aliquots of the cell suspensions (10^6 cells) were distributed into 4 wells of a 24-well tissue culture plate. Neutralization activity was expressed as decreased virus infectivity titers, determined as described above for *in vitro* titrations, with a reduction of ≥ 0.5 $\log_{10}$ being considered significant.

Polymerase chain reaction and sequence analysis

To verify the identity of the passaged virus, DNA was extracted from infected PBMCs of chimpanzee (C-444) (passage 1), and the V3 region of HIV-1 was sequenced after amplification by polymerase chain reaction (PCR), as previously described, using the ES7 and ES8 primers.[32] The PCR fragments were directly sequenced using the PRISM Ready Reaction AmpliTaq FS dideoxy terminator (Perkin-Elmer, Norwalk, CT). The sequence was aligned with reference sequences from the HIV-1 database using the multiple aligned sequence editor (CLUSTAL). Phylogenetic analysis was performed using the PHYLIP package.

To detect HIV-1 proviral DNA in PBMCs from infected

chimpanzees, a nested PCR was performed using 1 μg of DNA with primers corresponding to the 5′ long terminal repeat (5′ LTR) of the HIV-1 E/90CR402 genome. Primer sequences were as follows: outer set, 5′ GCA GGG GAA CCC ACT GCT 3′ and 5′ GGT CTG AGG GAT CTC TA 3′; and inner set, 5′ TTG TTA GAC CAG GTC GAG C 3′ and 5′ CAG ACC ACT CTA GAC TGA G 3′. The PCR products were transferred to a nylon membrane and hybridized using a radiolabeled probe with the following sequence: 5′ GTG GTG TGT GCC CGT TGT TAG G 3′.

Susceptibility of chimpanzees to infection

Adult chimpanzees (*Pan troglodytes*) from the Laboratory for Experimental Medicine and Surgery in Primates (LEMSIP), New York University (Tuxedo, NY), and the Centre International de Recherche Medicale (CIRMF) in Franceville, Gabon, were housed in biosafety level 2 facilities in accordance with institutional guidelines and standard practices for the containment of infectious diseases and the humane care and use of chimpanzees in biomedical research.[33] Before all procedures the chimpanzees were anesthetized by intramuscular injection of ketamine hydrochloride (10 mg/kg). All chimpanzees were seronegative for antibodies to HIV-1 core and envelope antigens by Western blot assay (Diagnostic Biotechnology, Singapore). The animals were inoculated by i.v. injection in the antecubital vein, using 1 ml of virus at appropriate dilutions. Blood samples were collected from all the chimpanzees on the day of virus inoculation, every 2 weeks for 8 weeks, and at monthly intervals thereafter.

The presence of virus in chimpanzee PBMCs was monitored by PCR (described above) and cocultivation of the PBMCs of each animal with PHA-stimulated human PBMCs and periodic assays of culture supernatants for RT activity and/or p24 antigen.[34,35] Freshly stimulated human PBMCs were added to the cultures every 10 days for 30 days. Single-cell suspensions of lymph node biopsy tissue and cells from bone marrow aspirates were also evaluated for the presence of virus by cocultivation with PHA-stimulated human PBMCs. Numbers of infectious PBMCs were determined by culturing successive fivefold dilutions of chimpanzee PBMCs with human PBMCs, as described above.

Titers of total serum HIV-1-specific antibodies were determined by serial twofold dilutions, using a commercially available EIA kit according to manufacturer instructions (Elavia; Sanofi Diagnostics Pasteur). The titer was defined as the reciprocal of the last dilution to give an optical density reading above the cut-off value. Western blot assays were done using a serum dilution of 1:100 and commercially available kits (Sanofi Diagnostics Pasteur and Diagnostic Biotechnology).

RESULTS

Characterization of the chimpanzee-adapted HIV-1 E/90CR402 stock

During a study on the diversity of V3 region sequences of HIV-1 isolates from the CAR, nine subtype E viruses were isolated.[7] Three of these isolates, as well as two isolates from Thailand, were tested for their ability to replicate in chimpanzee PBMCs. Since none of those tested replicated to detectable levels using direct culture methods, one of the isolates, HIV-1 E/90CR402, was selected for adaptation to growth on chimpanzee PBMCs. As described in Materials and Methods, 100 ml of a chimpanzee-adapted E/90CR402 stock was generated; this virus stock has an RT activity of 42,000 cpm/ml, a p24 antigen concentration of 115 ng/ml, and titers of 3000 and 4000 $TCID_{50}$/ml on human and chimpanzee PBMCs, respectively.

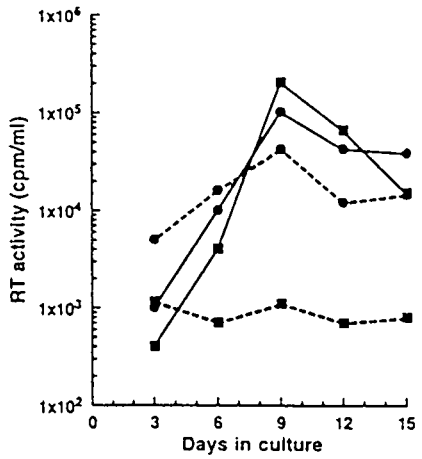

FIG. 1. Growth of the parental HIV-1 E/90CR402 isolate (■) and the chimpanzee-adapted virus (●) in human (solid lines) and chimpanzee (dashed lines) PBMCs.

Comparison of biologic properties of the parental and chimpanzee-adapted strains revealed that both viruses replicated efficiently in human PBMCs and macrophages, but only the chimpanzee-adapted HIV-1 E/90CR402 was able to replicate in chimpanzee PBMCs (Fig. 1). In addition, the chimpanzee-adapted virus had a syncytium-inducing (SI) phenotype, as shown by growth on MT2 cells with formation of syncytia,[36,37] whereas the phenotype of the parental isolate was nonsyncytium inducing (NSI).

Comparisons of the parental and chimpanzee-adapted HIV-1 E/90CR402 strains for their ability to be neutralized also revealed differences. First, polyclonal serum from three chimpanzees infected with the chimpanzee-adapted HIV-1 E/90CR402 stock (see below) was tested at a 1:50 dilution for neutralization of both strains. Sera obtained from all three animals at 4 or 8 months after infection, and at later times, neutralized the chimpanzee-adapted strain (Fig. 2), but not the parental isolate. Second, monoclonal antibodies 2F5, 2G12, and IgG1b12, which had some neutralizing activity against other, but not all, primary HIV-1 subtype E strains,[31] were tested; none of these monoclonal antibodies neutralized either the chimpanzee-adapted virus or the parental isolate. Furthermore, serum from patient 4002 was unable to neutralize either the parental, or the chimpanzee-adapted E/90CR402 viruses.

To confirm that the chimpanzee-adapted virus was HIV-1 strain E/90CR402, and also to determine whether the phenotypic changes were accompanied by genotypic changes, the nucleotide sequence of the V3 region of the chimpanzee-adapted virus was determined. The resulting DNA sequence was aligned with two previously reported sequences (4002-5 and 4002-6) derived from infection of SupT1 cells with virus from patient

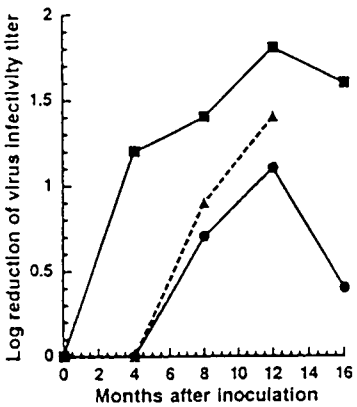

FIG. 2. Serum-neutralizing antibody responses to HIV-1 E/90CR402 of chimpanzees infected by the intravenous (solid lines) or cervical (dashed line) route. Chimpanzees: C-380 (▲); C-425 (●); C-444 (■).

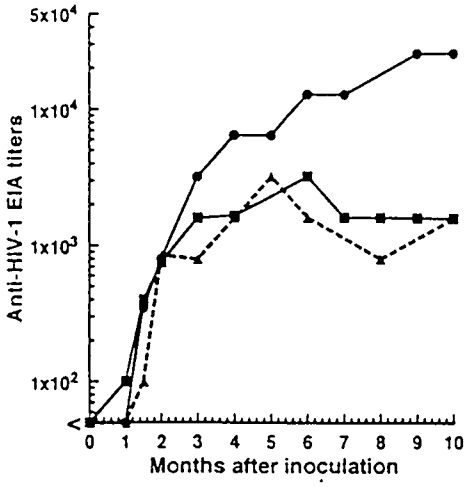

FIG. 3. Serum antibody responses of chimpanzees after intravenous (solid lines) or cervical (dashed line) infection with HIV-1 E/90CR402. Chimpanzees: C-380 (▲); C-425 (●); C-444 (■).

4002.[7] These two sequences differ at the tip of the V3 loop: clone 4002-5 encodes GPGRV while clone 4002-6 encodes GPGAI. The V3 sequence of the chimpanzee-adapted HIV-1 E/90CR402 is identical to that of the original clone 4002-5, except for a single amino acid change from threonine to arginine at position 368. This change corresponded to the last amino acid sequenced and may reflect technical difficulties. As expected, phylogenetic analysis using neighbor joining with the Kimura two-parameter distance matrix and 100 replicate bootstraps confirmed that the chimpanzee-adapted virus was derived from the original subtype E strain.

In vivo *infectivity of the chimpanzee-adapted E/90CR402 virus stock*

To determine whether the chimpanzee-adapted E/90CR402 virus stock was infectious *in vivo*, the naive chimpanzee (C-444), whose PBMCs were used in the adaptation process, was inoculated i.v. with both cell-free virus (8000 $TCID_{50}$) and autologous PBMCs infected with the virus. Because the animal became readily infected, as shown by isolation of virus from PBMCs, initially at 2 weeks postinoculation, and seroconversion to HIV-1 antigens (Fig. 3), a second naive chimpanzee (C-425) was inoculated by the i.v. route with 100 $TCID_{50}$ of the cell-free virus stock. In addition, a naive female chimpanzee (C-380) was inoculated atraumatically by depositing 500 $TCID_{50}$ of the cell-free virus stock in 0.25 ml into the cervical canal.[37a] Both animals became infected and seroconverted, as detected by EIA (Fig. 3), Western blot analysis, and development of neutralizing antibodies (Fig. 2). Virus was isolated initially from their PBMCs at weeks 2 and 4 postinoculation, respectively, and persistent infections were established by both routes, as evidenced by maintenance of antibody titers and continued isolation of virus. During the first year after infection, the frequency of isolation of HIV-1 from the PBMCs of the three chimpanzees infected with HIV-1 E/90CR402 was comparable to that of chimpanzees infected with the LAI(IIIB) strain. Virus was isolated on approximately 75 or 55% of attempts from those animals infected with either strain by the i.v. or cervical route, respectively. Plasma RNA levels in the E/90CR402-infected chimpanzees at 6 weeks after inoculation were 741, 2439, and 331 RNA copies/ml (Amplicor RT-PCR kit; Roche Molecular Systems, Nutley, NJ), with the lowest level in the animal infected by the cervical route. Although these data suggest that lower viral burdens are established after mucosal infection, this may be an artifact of the small number of animals. Virus was also isolated from lymph node biopsies performed on C-380 at 12 and 24 weeks, and from a bone marrow aspirate from C-444 at 12 weeks.

In vivo *titration of the HIV-1 E/90CR402 chimpanzee challenge stock*

To estimate the chimpanzee infectious dose (CID) *in vivo*, the chimpanzee-adapted E/90CR402 stock was titrated in two phases in a total of three chimpanzees. Initially two chimpanzees (C-010 and C-038) were inoculated with 20 and 2 $TCID_{50}$, respectively. C-010 but not C-038 became infected, as shown by the detection of HIV-1-specific antibodies by immunoblot (not shown) and EIA (Fig. 4), and isolation of virus (Table 1). These results indicated that the CID of the HIV-1

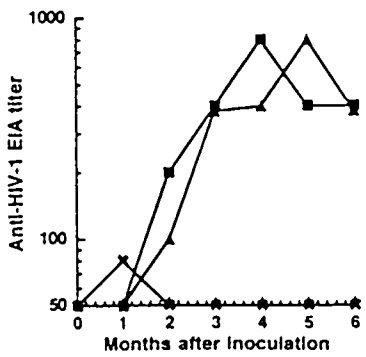

FIG. 4. Serum antibody responses of chimpanzees inoculated intravenously with different doses of HIV-1 E/90CR402. Chimpanzee C-022 (■), 5 $TCID_{50}$; C-038 (×), 10 $TCID_{50}$; C-010 (▲), 20 $TCID_{50}$.

TABLE 1. VIRUS RECOVERY FROM CHIMPANZEE PBMCs AFTER INOCULATION OF HIV-1 E/90CR402

Chimpanzee	Inoculum ($TCID_{50}$)	Weeks after inoculation[a]									
		0	2	4	6	8	10	12	16	20	24
C-010	20	−	−	+	+	+	+	+	+	+	+
C-038[b]	10	−	−	+	−	−	−	−	−	−	−
C-022	5	−	−	+	+	+	+	+	+	+	+
C-038[b]	2	−	−	−	−	−	−	−	−	−	−

[a]Isolation of HIV-1 was attempted by coculture of chimpanzee PBMCs with human PBMCs. +, Cultures were positive for RT activity or $p24^{gag}$ antigen; −, all assays for HIV-1 were negative.

[b]C-038 was initially inoculated with 2 $TCID_{50}$, but did not appear to become infected; therefore, C-038 was reinoculated with 10 $TCID_{50}$ of virus after a 6-month interval.

E/90CR402 challenge stock was between 2 and 20 $TCID_{50}$. To obtain a more accurate estimate of the CID, chimpanzee C-038, which had remained virus negative during 6 months of follow-up, was reinoculated with 10 $TCID_{50}$, and a third chimpanzee (C-022) was given 5 $TCID_{50}$. Both animals became infected; virus was isolated from their PBMCs (Table 1), and HIV-1-specific antibodies were detected (Fig. 4). Thus, the CID of the HIV-1 E/90CR402 chimpanzee challenge stock is between 2 and 5 $TCID_{50}$.

Infectious virus was isolated from the PBMCs of chimpanzee C-038, however, on only one occasion, 4 weeks after inoculation (Table 1), at which time the number of infectious PBMCs per 10^6 cells was twenty-five 50% infectious doses (ID_{50}). This quantity was comparable to that obtained in the other two chimpanzees during the first 4 months after infection: for C-010 (recipient of 20 $TCID_{50}$) numbers of infectious cells per 10^6 PBMCs ranged from <4 to 56 ID_{50} and for C-022 (recipient of 5 $TCID_{50}$) from 17 to 934 ID_{50}. Thus, there was no association between virus dose and viral burden during acute infection. C-038 was, furthermore, only positive for HIV-1 proviral DNA in PBMCs, using nested PCR with primers specific for the 5′ LTR of the HIV-1 E/90CR402 genome, on two occasions (4 and 6 weeks postinoculation).

Consistent with failure to isolate virus except at one time, serum antibodies to HIV-1 by EIA in C-038 were transient (Fig. 4). This animal developed an antibody response that initially gave immunoblot bands of low intensity to both *env* and *gag* gene products. The antibody response to gp160 detected by immunoblot was transient, completely disappearing by 6 months after inoculation, and anti-Gag (p24 and p18) responses, while still present 6 months after infection, were decreasing in intensity.

DISCUSSION

By irradiation of infected human PBMCs and subsequent cocultivation with mitogen-stimulated chimpanzee PBMCs, a subtype E HIV-1 isolate from the CAR, E/90CR402, was adapted to grow, and a virus stock was then prepared on chimpanzee PBMCs. Titration of this virus showed that it had a CID of less than 5 $TCID_{50}$, which is comparable to that of the HIV-1 LAI(IIIB) strain[18] previously used in most chimpanzee challenge studies.[19,20,22] This chimpanzee challenge stock is the first one from an HIV-1 subtype other than B that has been characterized. In general, most primary isolates of HIV-1 do not replicate in chimpanzee PBMCs.[24,38] Of several subtype A, B, C, and D primary isolates that we tested for replication in chimpanzee PBMCs, only one subtype A and several type D strains replicated efficiently in chimpanzee PBMCs using direct culture methods (F. Barré-Sinoussi and P. N. Fultz, unpublished data); a second subtype A isolate was adapted to grow in chimpanzee PBMCs using the methods described here (F. Barré-Sinoussi, unpublished data).

Previous studies in the HIV-1 chimpanzee model that evaluated vaccine-mediated protection have used the T cell line-adapted HIV-1 LAI(IIIB) strain, but considerable evidence suggests that the tropism and neutralization sensitivity of primary isolates of HIV-1 differ from those of T cell line-adapted viruses.[39–41] It has been suggested that (1) V3-specific antibodies in serum of HIV-1-seropositive patients play fundamentally different roles in neutralization of laboratory-adapted strains versus primary isolates, and (2) antibodies directed to epitopes outside of the V3 loop are more important in the neutralization of HIV-1 primary isolates.[42] Primary isolates of HIV-1, however, can be neutralized by antisera from some infected patients, especially sera from long-term nonprogressors, and by some monoclonal antibodies such as those directed to gp41 or the CD4-binding site in gp120.[43,44] HIV-1 neutralization depends not only on the association of antibodies with linear epitopes whose structure might vary in a way predictable from knowledge of genotypes, such as those found in the V3 loop, but also with discontinuous epitopes within the monomeric and oligomeric forms of the HIV-1 envelope glycoproteins.[45–50] It has been demonstrated that although putative neutralization serotypes do not directly correspond to genetic subtypes, some correlation between neutralization serotypes and genetic subtypes exists under certain conditions.[51,52] Furthermore, Mascola *et al.*[53] demonstrated that the phylogenetic classification of HIV-1 subtypes B and E corresponds to two distinct neutralization serotypes. Neutralization serotypes are more likely to be influenced by antibodies to complex epitopes, such as those dependent on the native conformation of the envelope glycoproteins.[51] Therefore, to mimic natural infection with the virus as closely as possible, it is important to prepare challenge stocks of primary isolates of HIV-1 that have been passaged only in PBMCs. This criterium was met with the HIV-1 E/90CR402 isolate used for preparation of the chimpanzee-adapted stock (available as 1-ml cryopreserved aliquots), which should be valuable for studies on cross-clade immunity to HIV-1 in the chimpanzee model.

Despite limited passage in PBMC cultures, the chimpanzee-adapted virus had an SI phenotype, as shown by replication in MT2 cells with formation of syncytia, which contrasted with the NSI phenotype of the parental virus.[36,37,54] Interestingly, a similar phenotypic change was observed for the subtype A virus, which was adapted to grow in chimpanzee PBMCs using similar methods (F. Barré-Sinoussi, unpublished data). Thus, it appears that adaptation of the parental virus to growth on chimpanzee cells occurred either through selection of a minor SI variant in the parental quasispecies or through mutation. Kusumi *et al.*[55] showed that *in vitro* propagation of HIV-1 by coculture resulted in selective amplification of a single clone found in the parental quasispecies *in vivo*. Although previous studies have indicated that changes in HIV cell tropism result from mutations in the *env* V3 region,[56–61] the parental and chimpanzee-adapted viruses had identical C3–V3 loop sequences in spite of their phenotypic differences. However, other regions of the HIV-1 genome can also influence NSI/SI phenotypes. De Mareuil *et al.*[62] showed that fusogenic determinants of an HIV-1 subtype D isolate, NDK, from Zaire are located not only in the envelope glycoprotein, the matrix protein, and the C-terminal portion of Vpr, but also that the $p18^{gag}$ protein may be involved in these properties.[63] It was also demonstrated that the maintenance of an SI phenotype is associated with positively charged residues in the V2 region of gp120.[64] More recently, Sabri *et al.* also demonstrated a lack of correlation between V3 amino acid sequence and SI phenotype of some HIV-1 isolates.[65]

The chimpanzee-adapted HIV-1 E/90CR402 strain persistently infected chimpanzees by both the i.v. and cervicovaginal routes. During the acute phase of infection with the subtype E strain, viral burdens, as measured by RNA copies per milliliter, were comparable to those observed in chimpanzees infected with the subtype B strains SF2 and DH12, but were up to 1000-fold lower than those routinely observed in chimpanzees infected with the IIIB(LAI) strain (P. N. Fultz, unpublished data). Infection of chimpanzees with HIV-1 via the genital route was first demonstrated by Fultz *et al.*,[66] who used a high dose of the HIV-1 LAI(LAV-1b) strain, which was generated in human PBMCs after *in vivo* passage in chimpanzees. In the present study, persistent infection of a female chimpanzee was established by inoculation of only 500 $TCID_{50}$ of HIV-1 E/90CR402 via the genital route. This is important since the majority of HIV-1 infections worldwide, particularly in the developing world, are transmitted through vaginal intercourse.[67–69] Therefore, it is critical that evaluation of the protective efficacy of vaccines against mucosal challenge be done with HIV-1 strains representing several subtypes. Results of a study in Thailand by Kunanusont *et al.*[70] indicated that HIV-1 transmission in concordant heterosexual couples was less efficient when the index case harbored a subtype B virus, than in cases where a subtype E virus was present. This could imply that the subtypes primarily circulating in the developing world (subtypes A, C, D, and E) are more easily transmitted via the genital route than the subtype B strains circulating in the United States and Western Europe. In support of this hypothesis, it has been shown that subtype E strains, when compared with subtype B viruses, replicated more efficiently and to higher levels in dendritic Langerhans cells isolated from skin.[71] Similar cell types are found in the mucosal epithelium.

In the *in vivo* titration of the E/90CR402 stock, chimpanzee C-038 received two inoculation of virus, first 2 $TCID_{50}$ and then 10 $TCID_{50}$. Only at one time, 4 weeks after the second exposure, was virus isolated from its PBMCs; this was followed by development of antibodies that gave faint bands to both *env* and *gag* gene products on immunoblot and an extremely low antibody titer by EIA. HIV-1 proviral DNA was detected in PBMCs on only two occasions, 4 and 6 weeks after the second exposure. That the animal had been inoculated initially with a subinfectious dose of virus raises the question of whether the first exposure induced partial protection from subsequent challenge. It was demonstrated that inoculation of macaques with a subinfectious dose of SIV induced T cell-proliferative responses that correlated with protection from subsequent challenge with a higher dose of virus.[72] Miller *et al.*[73] demonstrated that some macaques inoculated with cell-free SIV by the vaginal route developed a transient viremia in the absence of an antibody response. Pauza *et al.*[74] also showed that rectal inoculation of a subinfectious dose of SIVmac251 protected against subsequent challenge with a higher dose of virus in the absence of seroconversion. A role for $CD8^+$ T lymphocytes was suggested when depletion of these cells from PBMCs led to a significant increase in SIV replication *in vitro*. The mechanism of protection, however, did not appear to be mediated by cytotoxic T lymphocytes.[75] This is reminiscent of the results obtained by Walker *et al.*,[76] who demonstrated that $CD8^+$ lymphocytes of HIV-1-seropositive patients could suppress replication of HIV-1 in PBMCs in a dose-dependent manner. A similar phenomenon was also demonstrated in HIV-1-infected chimpanzees.[77] Whether soluble factors, such as RANTES, MIP-1α, and MIP-1β, mediated this activity is not known.[78] The rapid clearance of HIV-1 E/90CR402 from the PBMCs of C-038 after the second inoculation of virus might be due to similar mechanisms or factors; these possibilities are being investigated.

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Challenge of Chimpanzees Immunized with a Recombinant Canarypox-HIV-1 Virus

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To evaluate the potential protective efficacy of a live recombinant human immunodeficiency virus type 1 (HIV-1) canarypox vaccine candidate, two chimpanzees were immunized five times with ALVAC-HIV-1 vCP250, a recombinant canarypox virus that expresses the HIV-$1_{IIIB(LAI)}$ gp120/TM, *gag*, and protease gene products. One month after the last booster inoculation, the animals were challenged by intravenous injection of cell-associated virus in the form of peripheral blood mononuclear cells from an HIV-$1_{IIIB(LAI)}$-infected chimpanzee. One chimpanzee with a neutralizing antibody titer to HIV-$1_{IIIB(LAI)}$ of 128 at the time of challenge was protected, whereas both the second animal, with a neutralizing antibody titer of 32, and a naive control animal became infected. At 5 months after challenge, the protected chimpanzee and a third animal, previously immunized with various HIV-1_{MN} antigens, were given a booster inoculation. The two animals were challenged intravenously 5 weeks later with twenty 50% tissue culture infectious doses of cell-free HIV-1_{OH12}, a heterologous subtype B isolate. Neither chimpanzee had neutralizing antibodies to HIV-1_{OH12}, and neither one was protected from infection with this isolate. The immune responses elicited by vaccination against HIV-$1_{IIIB(LAI)}$ or HIV-1_{MN} did not, therefore, protect the animals from challenge with the heterologous cell-free HIV-1_{OH12}.

INTRODUCTION

Recombinant canarypox virus vectors induce both humoral and T-cell-mediated immunity against diverse pathogens in animals (Baxby *et al.*, 1992; Taylor *et al.*, 1992). Furthermore, a recombinant canarypox–rabies G protein virus proved to be safe and immunogenic in humans (Cadoz *et al.*, 1992). In a recent study ALVAC–HIV-1 vCP125, a recombinant canarypox virus expressing the gp160 *env* gene product of HIV-1_{MN}, induced HIV-1-specific neutralizing antibodies and cytotoxic T-lymphocyte responses in 90 and 40% of human volunteers, respectively, when combined with a subunit rgp160 vaccine in a prime-boost regimen (Pialoux *et al.*, 1995). A similar regimen, however, failed to protect chimpanzees from a heterologous challenge with cell-free HIV-1_{SF2} (Girard *et al.*, 1995). A possible explanation for this failure was that the dose [$10^{6.1}$ 50% tissue culture infectious doses (TCID50)] of canarypox virus used to immunize the chimpanzees was too low. We report here on the immunization of chimpanzees with a higher dose of a recombinant canarypox–HIV-1 virus, followed by challenge with homologous and heterologous HIV-1 strains.

MATERIALS AND METHODS

Animals

Adult male chimpanzees (*Pan troglodytes*) were housed at the Laboratory for Experimental Surgery in Primates (LEMSIP), New York University, in biosafety level 2 facilities in accordance with institutional guidelines and standard practices for the containment of infectious diseases and the humane care and use of chimpanzees in biomedical research (Moor-Jankowski and Mahoney, 1989). Before all procedures the chimpanzees were anesthetized by intramuscular (im) injection of ketamine hydrochloride (10 mg/kg).

Study design

Two naive chimpanzees (C-401 and C-451) were immunized via the im route with 4×10^8 plaque-forming units (PFU) of ALVAC–HIV-1 vCP250, a recombinant canarypox virus that expresses not only the HIV-$1_{IIIB(LAI)}$ gp120 and the transmembrane segment from gp41 (gp120/TM), but also the *gag* and protease gene products. The animals received a total of five doses of vCP250 at Months 0, 1, 5, 9, and 11. No subunit booster inoculations were given.

Challenge viruses

For preparation of the cell-associated HIV-$1_{IIIB(LAI)}$ challenge stock, a chimpanzee (C-087) was inoculated via

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the intravenous (iv) route with 100 TCID50 of the NIH HIV-$1_{IIIB(LAI)}$ stock (Arthur *et al.*, 1989). The animal became readily infected and a peripheral blood mononuclear cell (PBMC) stock obtained 3 months after infection was used to infect a second naive chimpanzee (C-435). This animal also became infected as demonstrated by persistent isolation of virus from its PBMCs (Fultz *et al.*, 1992). At 4 months after inoculation, a stock of its PBMCs was prepared, aliquoted, and cryopreserved. The titer of this stock is 30 infectious cells per 10^6 PBMCs.

DH12 is a clinical isolate that readily infects chimpanzees and multiplies in their cells *in vitro* (Shibata *et al.*, 1995). A chimpanzee challenge stock of HIV-1_{DH12} was obtained from Malcolm Martin and Alan Schultz at the NIH. The chimpanzee infectious dose of this stock is between 6 and 16 TCID50 (Shibata *et al.*, 1996).

Virus isolation and serology

The presence of virus in PBMCs was monitored by cocultivation of each animal's PBMCs with phytohemagglutinin-stimulated human indicator PBMCs and periodic assays of culture supernatants for reverse transcriptase activity (Fultz *et al.*, 1986) or p24*gag* antigen (Hollinger *et al.*, 1992). Freshly stimulated human PBMCs were added every 10 days.

Serial twofold dilutions of serum samples were tested for total anti-HIV-1 antibodies with a commercially available enzyme immunoassay (EIA) kit (Diagnostics Pasteur, Marnes-la-Coquette, France). Titers were defined as the reciprocal of the last serum dilution to give an optical density reading above the cut-off recommended by the manufacturer. Antibodies to HIV-1 strains IIIB(LAI) and DH12 V3-loop peptides were also determined by EIA, as previously described (Girard *et al.*, 1995). Western blot assays were done using a commercially available kit (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) and a serum dilution of 1:100. Neutralizing antibodies to HIV-$1_{IIIB(LAI)}$ were measured using a quantitative syncytium inhibition assay on CEM-SS cells (Nara *et al.*, 1987).

Anti-gp120 antibody titers and avidity were determined using an EIA. Briefly, duplicate microtiter plates (Maxisorp, Nunc) were coated with concanavalin A at 2.5 μg/well for 1 hr at room temperature, after which they were washed with PBS containing 1% Tween 20 and coated with 50 ng/well of rgp120 (British MRC AIDS Reagent Research Project), which was derived from a clinical isolate of HIV-1 (HIV-1_{GB8}) (Farrar *et al.*, 1991; Jones *et al.*, 1995). Sequential twofold dilutions of sera in PBS containing 2% bovine serum albumin were added and incubated for 2 hr at 37°. Binding antibodies were detected by standard methods with alkaline phosphatase-conjugated anti-human IgG and *p*-nitrophenyl phosphate substrate using one of the duplicate plates, whereas the other plate was first soaked with PBS containing 8 *M* urea and then processed. The anti-gp120 antibody avidity index (AI) was then calculated as described (Hedman *et al.*, 1989; Clements *et al.*, 1995) from the difference in optical density (OD) values at 405 nm in corresponding wells. The AI was determined over a range of serum dilutions in order to control for variations in antibody concentration.

CTL assays

$CD8^+$-specific CTL responses in the animals were evaluated using a peptide-restimulation assay and autologous Epstein–Barr virus-transformed B-lymphoblastoid cell lines as target cells (Walker, 1993). PBMCs were stimulated *in vitro* with pools of overlapping 20-mer peptides corresponding to amino acids 30 to 510 in Env and 139 to 369 in p24*gag* of HIV-1_{SF2}; culture medium was RPMI 1640 containing 10% fetal bovine serum, 5% human interleukin-2 (IL-2) (Schiaparelli), 100 IU/ml rIL-2 (Cetus Corp.), antibiotics, and 10 μg/ml of each peptide. After 8 days in culture, $CD8^-$ cells were purified using magnetic beads coated with anti-CD8 antibodies (Dynal, Lake Success, New York) and tested for cytolytic activity in a standard ^{51}Cr release assay (Erickson *et al.*, 1993) against autologous target cells pulsed with the homologous peptide pool (10 μg/ml of each peptide). Control targets were pulsed with a heterologous peptide pool.

RESULTS AND DISCUSSION

Two chimpanzees were immunized with ALVAC–HIV-1 vCP250, a recombinant canarypox-HIV-1 virus expressing the HIV-$1_{IIIB(LAI)}$ gp120/TM, *gag*, and protease gene products. The animals were bled regularly and their immune responses to HIV-1 monitored. Anti-V3 IIIB(LAI) antibody titers remained at low levels until after the fourth immunization (9 months) when an increase in titers was observed in serum from C-401 (Table 1); C-451 had an increase in anti-V3 antibody titers only after the fifth immunization at 11 months. Total anti-HIV-1 antibody titers, however, remained at low levels (Fig. 1). WB assays done at the time of challenge (12 months) demonstrated that although a good anti-p24*gag* antibody response was seen in both immunized animals, p17*gag* antibodies were observed only in C-401, and that the anti-Env response was weak in both chimpanzees (Fig. 2; lanes BC, first challenge). Neutralizing antibodies to HIV-$1_{IIIB(LAI)}$ were detected only after the fourth and fifth immunizations for C-401and C-451, respectively (Table 2). No neutralizing antibodies to a clinical isolate of HIV-1 (BZ167) were detected using a resting cell assay, as described by Zolla-Pazner *et al.* (1995).

One month after the last booster inoculation, the immunized animals and a naive control (C-353) were challenged via the iv route with 6×10^5 viable PBMCs (equivalent to 20 infectious doses) from an HIV-$1_{IIIB(LAI)}$ infected chimpanzee (C-435). In three previous experiments, this dose of cell-associated virus from the same stock of

TABLE 1

Anti-V3 Antibody Titers of Chimpanzees Immunized with ALVAC-HIV-1 vCP250

	Chimpanzees		
		C-401	
Months[a]	C-451 V3-IIIB(LAI)	V3-IIIB(LAI)	V3-DH12
0	589	330	ND
6	710	2,100	ND[b]
9[c]	330	760	ND
10	850	8,900	ND
11[c]	340	3,600	ND
12[d]	7900	22,000	ND
17[c]	ND	8,800	<6
18[e]	ND	32,000	<6
19	ND	38,000	22
20	ND	20,000	140
21	ND	15,000	170

[a] Months after first immunization.
[b] ND, not done.
[c] Booster inoculation with vCP250.
[d] Challenge with cell-associated HIV-$1_{IIIB(LAI)}$.
[e] Challenge with cell-free HIV-1_{SF2}.

cryopreserved PBMCs, readily infected three of three naive chimpanzees (Fultz *et al.*, 1992; Saksena *et al.*, 1993, and unpublished data). The total anti-HIV-1, anti-V3 IIIB(LAI), and neutralizing antibody titers of C-401 were two- to fourfold higher than those of C-451 at time of challenge (Fig. 1, Tables 1 and 2). The anti-canarypox antibody levels of C-401 and C-451 as determined by EIA were 160 and 60 EIA units (EU)/ml, respectively, indicating a strong immune response to the virus vector (a cut-off value of 0.6 EU/ml is considered positive in human volunteers). C-401 was, therefore, in all aspects, a better responder than C-451 to the canarypox virus-HIV-1 vaccine candidate. No CTL activity directed to either Env or p24*gag* epitopes were, however, detected in either of the chimpanzees at any of the time points tested, including the day of challenge. The lack of a CTL response following five immunizations with a live recombinant canarypox virus is surprizing, but it should be noted that a significant CTL reponse, using a different recombinant canarypox–HIV-1 virus (vCP125), was seen in only 40% of human volunteers (Pialoux *et al.*, 1995), while chimpanzees immunized with this recombinant failed to develop a CTL response (Girard *et al.*, 1995). Another explanation for this lack of CTL activity might be that the assay was not sensitive enough. However, CTL activity could be demonstrated in HIV-1_{SF2}-infected chimpanzees, starting at 6–8 weeks postinoculation, using this assay method (C Walker, personal communication).

After challenge, the chimpanzees were bled every 2 weeks for 8 weeks and then at monthly intervals. PBMCs from the control animal and C-451 were initially virus positive at 4 and 2 weeks after challenge, respectively. Chimpanzee C-451 also showed a strong anamnestic

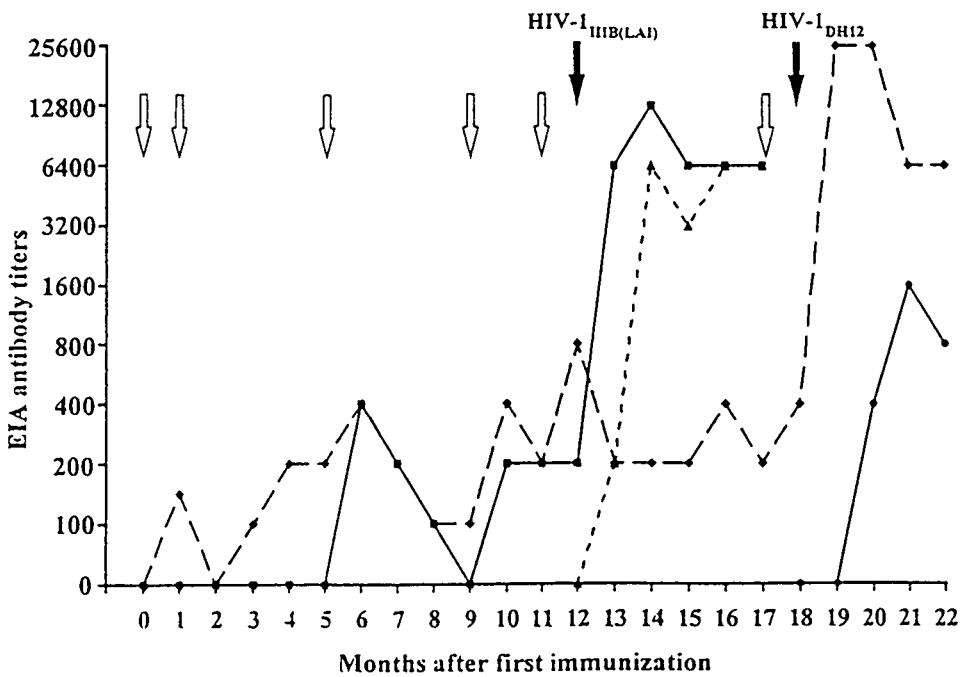

FIG. 1. Anti-HIV-1 antibody responses in chimpanzees. Animals C-451 and C-401 were immunized with ALVAC–HIV-1 vCP250 (open arrows) and challenged with HIV-$1_{IIIB(LAI)}$ and/or HIV-1_{DH12} (closed arrows). Animals C-353 and C-573 were included as naive unimmunized controls. Antibody titers were determined by EIA using serial twofold dilutions of chimpanzee serum samples; titers are defined as the last dilution of serum that gave an OD reading above the cut-off value recommended by the manufacturer. C-451 (■, solid line); C-401 (♦, dashed line); C-353 (▲, dotted line); and C-573 (●, solid line).

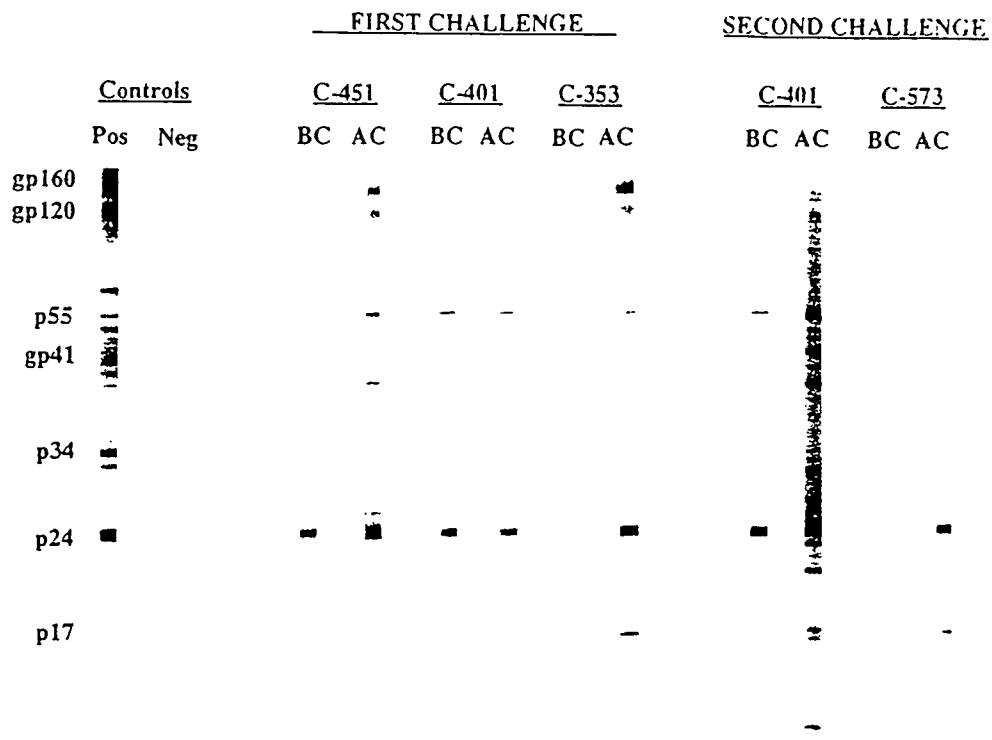

FIG. 2. HIV-1 antigen-specific responses of chimpanzees at time of challenge (BC) and 8 weeks after challenge (AC). WB assays were performed ing a serum dilution of 1:100. First challenge: HIV-$1_{IIIB(LAI)}$; second challenge: HIV-1_{DH12}.

tibody response by both EIA (Fig. 1) and neutralizing tibody assays (Table 2). Consistent with these results, B analysis showed not only that bands representing v and Gag antibody reactivity had intensified, but also t novel serum reactivities had appeared (Fig. 2, lane C, first challenge). In contrast, C-401 remained virus negative during 5 months of follow-up, and its antibody titers decreased slowly by all assays (Figs. 1 and 2, Table 2). Of interest was the finding that the neutralizing antibody titer of the unprotected animal (C-451) at time of challenge was 32, whereas that of the protected animal (C-401) was 128. Although no firm conclusions can be drawn from a study involving only two animals, this observation agrees with previous results that suggested an association between neutralizing antibody titers and protection from HIV-1 infection in the chimpanzee model (Berman *et al.*, 1990; Girard *et al.*, 1991, 1995; Fultz *et al.*, 1992; Bruck *et al.*, 1994; Zolla-Pazner *et al.*, 1996). Similar results were obtained for HIV-2 (Putkonen *et al.*, 1991a,b), while Van Rompay *et al.* (1996) demonstrated that protection from oral SIV infection of neonatal macaques born from immunized mothers correlated with the titers of neutralizing antibodies of the immunized females. Furthermore, it was recently demonstrated that the protection from SIV infection conferred by live attenuated SIVΔ*nef* vaccines is associated with the levels of high-avidity neutralizing antibodies in the immunized animals (Wyand *et al.*, 1996). This result also suggests that recombinant canarypox–HIV-1 viruses can induce protective neutralizing antibody titers in some chimpanzees when used at a high dose in repeated immunizations.

After 5 months of follow-up, C-401 was again inoculated im with 4×10^8 PFU of vCP250, and 1 month later it was challenged with 20 TCID50 of cell-free HIV-1_{DH12}

TABLE 2

HIV-$1_{IIIB(LAI)}$ Neutralizing Antibody Titers

Months[a]	Chimpanzees C-451	Chimpanzees C-401
6	4	4
9[c]	0	0
10	4	32
11[c]	0	16
12[d]	32	128
13	>512	64
14	>512	32
18[e]	ND[f]	64

Neutralizing antibody titers were expressed as the reciprocal of the tion of serum that resulted in 90% reduction of the number of syncv- ormed on CEM-SS cells by HIV-$1_{IIIB(LAI)}$ (Nara *et al.*, 1993).
Booster inoculation with vCP250.
Challenge with cell-associated HIV-$1_{IIIB(LAI)}$.
Challenge with cell-free HIV-1_{DH12}.
ND, not done.

TABLE 3

Lack of Correlation among Anti-gp120 Antibody Titers, Avidity of Antibodies to gp120, and Protection from Challenge

Experiment	Chimpanzee	Immunogens	Anti-gp120 titer[a]	Avidity index[b]	Challenge virus	Protection
1	C-451	vCP250	50	19	IIIB (cells)	No
	C-401	vCP250	100	23	IIIB (cells)	Yes
2	C-401	vCP250	100	25	DH12	No
	C-489	vCP205 + gp140 MN/LAI + V3-MN peptide + MN-T/B peptide	400	80	DH12	No
3[c]	C-477	vCP125 + gp140 MN/LAI	800	91	SF2	No
	C-641	vCP125 + gp140 MN/LAI	200	90	SF2	No
	C-483	gp140 MN/LAI + V3 MN peptide	3200	96	SF2	Yes
	C-323	gp140 MN/LAI + V3 MN peptide	1600	89	SF2	Yes

[a] Titers expressed as the reciprocal of the last serum dilution to give an OD reading of ≥0.1 at 405 nm.

[b] Avidity index = (OD urea-treated well/OD untreated well) × 100; All values <30% are considered to indicate low avidity antibody (Hedman *et al.*, 1989).

[c] Described in Girard *et al.* (1995).

(1 ml of a 1:400 dilution of the virus stock provided by Malcolm Martin) together with a naive control (C-573). The gp120 gene of HIV-1_{DH12} has only 80% homology with that of HIV-$1_{IIIB(LAI)}$ (Shibata *et al.*, 1996), making this a heterologous intraclade challenge. Both C-401 and the control animal readily became infected, as shown by virus isolation from PBMCs at 2 weeks after challenge and thereafter. C-401 developed a strong anamnestic response to HIV-1 (Figs. 1 and 2), and infection of both animals elicited antibodies to a V3 peptide from HIV-1_{DH12} (Table 1). At the times of the last vCP250 inoculation and subsequent HIV-1_{DH12} challenge, a third animal (C-489) was also included. This chimpanzee had been immunized previously with a variety of HIV-1 immunogens, including vCP205 (a recombinant canarypox virus expressing the *env* gp120 with transmembrane region, *gag*, and protease genes of HIV-1_{MN}), a HIV-1_{MN} T- and B-cell chimeric peptide, recombinant gp140 MN/LAI, and a V3 MN peptide. Like C-401, chimpanzee C-489 was not protected from HIV-1_{DH12} challenge; virus was isolated from its PBMCs and its antibody titer to HIV-1 increased (not shown).

At time of challenge, C-489 had an anti-HIV-1_{MN} neutralizing antibody titer of 8, but no neutralizing antibodies to HIV-1_{DH12} (not shown) as determined using a quantitative immunohistochemically based infectivity reduction assay on PBMCs (Nara *et al.*, in press). Similarly, C-401 had an anti-HIV-$1_{IIIB(LAI)}$ neutralizing antibody titer of 64, but no neutralizing antibodies to HIV-1_{DH12}. The lack of protection from HIV-1_{DH12} challenge, therefore, was associated with the absence of neutralizing antibodies to the challenge virus. However, we previously showed that two chimpanzees immunized with a recombinant gp140 MN/LAI molecule and V3 MN peptide were protected from a heterologous intraclade challenge with HIV-1_{SF2}, despite the absence of demonstrable neutralizing antibodies to HIV-1_{SF2} (Girard *et al.*, 1995). Berman *et al.* (1996) also demonstrated that three chimpanzees immunized with recombinant gp120 MN were protected from heterologous challenge with HIV-1_{SF2}. Sera from the protected animals were unable to neutralize HIV-1_{SF2} infectivity for PBMCs, but they did neutralize infectivity for AA5 cells of a T-cell-line-adapted HIV-1_{SF2} variant. Since HIV-1_{SF2} replicates poorly in chimpanzees (Girard *et al.*, 1995), it is possible that protection of chimpanzees from infection with this virus is easier to achieve than it is from other subtype B strains. This possibility is supported by the fact that chimpanzees infected with HIV-1_{SF2} could readily be superinfected with HIV-$1_{IIIB(LAI)}$ or HIV-1_{DH12} (Fultz *et al.*, 1987; Fultz, 1995; Shibata *et al.*, 1996). However, at least one chimpanzee infected with HIV-$1_{IIIB(LAI)}$ was superinfected with HIV-1_{DH12} (Fultz, 1995). In contrast, Shibata *et al.* (1996) could not demonstrate HIV-1_{DH12} superinfection of HIV-$1_{IIIB(LAI)}$-infected chimpanzees. Considerable evidence suggests that the neutralization sensitivity of primary isolates of HIV-1 differs from that of T-cell-line-adapted viruses (Daar *et al.*, 1990, Bou-Habib *et al.*, 1994, Hanson 1994). Antibodies directed to epitopes outside of the V3 loop are more important in the neutralization of primary isolates, while V3-targeted neutralizing antibodies are important for neutralization of T-cell-line-adapted strains (Vancott *et al.*, 1995). The lack of neutralizing antibodies could, therefore, be explained by the fact that HIV-1_{DH12} is a primary isolate, whereas the IIIB(LAI) strain is T-cell adapted.

The presence of high-avidity anti-gp120 antibodies has also been implicated as an important correlate for protective antibody responses against HIV-1 and SIV infection (Devash *et al.*, 1990; Clements *et al.*, 1995). The sera from the immunized chimpanzees were, therefore, tested for such antibodies by EIA. Sera from chimpanzees immunized with rgp140 MN/LAI combined with either a recombinant canarypox virus expressing the gp120/TM of HIV-1_{MN} (ALVAC-HIV-1 vCP125) or a V3 MN peptide

and then challenged with HIV-1_{SF2} (Girard *et al.*, 1995) were also included in this experiment. The results demonstrated that the two chimpanzees immunized with vCP250 (C-401 and C-451) had low anti-gp120 antibody titers with low avidity, while the chimpanzees that had immunization regimens that included envelope subunits had higher antibody titers with high avidity (Table 3). However, no correlation between anti-gp120 antibody avidity and protection from challenge was noted in any of these chimpanzees. Although the HIV-1_{GB8} gp160 *env* gene shows only approximately 84% overall homology with the gp160 *env* genes from IIIB(LAI), MN, and SF2, Vella *et al.* (1995) demonstrated that the rgp120 GB8 was recognized by all sera from HIV-1-infected patients tested. Furthermore, hyperimmune sera raised against rgp120 derived from HIV-1 strains IIIB(LAI), MN, SF2, or GB8 showed comparable recognition of the HIV-1_{GB8} gp120 by EIA.

Altogether, these results leave the question of immune correlates of protection from HIV-1 in the chimpanzee model unanswered and suggest that intraclade cross-protection among HIV-1 isolates will not be easily achieved and might differ from isolate to isolate. The induction of antibodies with broad spectrum neutralizing activity against multiple primary isolates remains at this time one of the major goals in the development of an efficacious HIV-1 vaccine.

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Genital Infection of Female Chimpanzees with Human Immunodeficiency Virus Type 1

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ABSTRACT

To develop an animal model for mucosal HIV-1 infection, adult chimpanzees were inoculated without trauma by depositing the virus inoculum at the entrance to the cervical canal with a rigid catheter to which flexible tubing was attached. By this procedure, persistent infections were established in some chimpanzees with various infectious doses of either cell-associated HIV-$1_{LAI(IIIB)}$ (peripheral blood mononuclear cells from an infected chimpanzee) or with cell-free HIV-1 strains representing subtypes B and E, but not with a subtype A strain. Although some animals did not become infected until after the second or third cervicovaginal exposure, one chimpanzee was clearly infected after one exposure by several criteria, including virus isolation, but this animal did not seroconvert. A second chimpanzee appeared to be resistant to infection despite repeated mucosal exposures at irregular intervals. However, lymphocytes from both of these animals exhibited low-level proliferative responses to HIV-1 but not SIV antigens. Despite these apparently abortive or latent infections, after exposure to HIV-1 by the intravenous route, both animals developed systemic infections and seroconverted. Overall, 8 of 10 chimpanzees were infected systemically after one to three cervicovaginal exposures to HIV-$1_{LAI(IIIB)}$. The results indicate that (1) HIV-1 productive infection of female chimpanzees by the cervicovaginal route generally requires more than one exposure, just as with humans; (2) low level infections without seroconversion can be established after mucosal exposure to HIV; and (3) vaccine efficacy studies involving a single virus challenge of immunized chimpanzees by the cervicovaginal route probably will not be possible.

INTRODUCTION

EPIDEMIOLOGICAL DATA show that worldwide approximately 80% of human immunodeficiency virus (HIV) infections are transmitted by heterosexual contact.[1–3] That heterosexual transmission can occur by both male-to-female and female-to-male routes is supported by detection of both cell-free virions and HIV-infected cells in genital secretions of males and females.[4–10] The majority of cases, however, are the result of transmission from an infected male to his female partner of virus present in either preejaculatory fluid or semen; both of these secretions are known to contain white blood cells.[4,6–8] Using various techniques, such as virus isolation by culture with susceptible indicator cells, immunohistochemistry, and polymerase chain reaction (PCR), investigators have detected less than 10 to more than 10^4 proviral DNA copies of HIV per milliliter in semen samples from men at all stages of infection, including those taking antiretroviral drugs.[6,7,11] Furthermore, Gupta *et al.*[12] used a nucleic acid sequence-based amplification (NASBA) technique to detect up to 2.8×10^5 and 1.3×10^7 HIV-1 RNA copies/ml of seminal plasma and whole semen, respectively.

Using normal reproductive tract tissues obtained from hysterectomy patients, Howell *et al.*[13] demonstrated that viable tissue fragments or isolated cells could be infected with both T cell line-adapted and primary strains and that infection was not related to stage of the menstrual cycle at which the tissues were obtained. Tissues in which productive infections were established included uterus, fallopian tube, cervix, and ectocervix, but not vaginal mucosa. The actual mechanisms involved in

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transmitting HIV or HIV-infected cells across the vaginal and cervical mucosae are unknown, but it has been postulated that HIV is transported by epithelial cells from the lumen to the basolateral side of the epithelium where lymphocytes, macrophages, or Langerhans cells in the submucosa can be infected.[14–18] This hypothesis is supported by evaluation of tissues from macaques infected vaginally with simian immunodeficiency virus (SIV) or a pathogenic chimeric simian–human immunodeficiency virus (SHIV)[19,20] and by *in vitro* experiments using human epithelial cell lines.[21–24] These latter studies showed that HIV-infected lymphocytes can attach and transmit virus directly to cervical epithelial cell lines, a process that was enhanced by the presence of human seminal plasma.[21,22]

Because mechanisms of transmission of HIV across mucosal surfaces are not understood, it is not possible to define specific immune responses that might be involved, and, therefore, that should be elicited by a vaccine in order to prevent infection via the female genital tract. Although models of mucosal infection of macaques with various SHIVs that encode an HIV-1 glycoprotein are in development,[20,25] these models are not as ideal as HIV-1 infection of chimpanzees. To date, successful transmission of HIV-1 to a chimpanzee by a mucosal route has been reported only once, and in that instance, a high dose of cell-free HIV-$1_{LAI(LAV\text{-}1b)}$ was deposited in the vaginal vault without obvious trauma.[26] Thus, the current study was undertaken to develop a model for HIV-1 genital infection of chimpanzees for use in the evaluation of vaccine strategies for eliciting protective mucosal immunity.

MATERIALS AND METHODS

Animals

Adult female chimpanzees *(Pan troglodytes)* were housed at the Laboratory for Experimental Medicine and Surgery in Primates, New York University (Tuxedo, NY), in biosafety level 2 facilities in accordance with institutional guidelines and standard practices for the containment of infectious diseases and the humane care and use of chimpanzees in biomedical research. Before all procedures, chimpanzees were anesthetized by intramuscular inoculation of ketamine hydrochloride (10 mg/kg). As well as could be determined, the animals were inoculated at the peak of ovulation, as evidenced by edema of the genital area. When necessary, menstrual cycles were synchronized by administration of depoprevera; this treatment did not appear to influence whether a productive infection was established. Before inoculation of virus, a speculum was used to facilitate visual inspection of the vagina for absence of blood.

Virus stocks

Several strains of HIV-1 were used in attempts to infect chimpanzees via the genital route. Cell-free virus stocks included the following strains: (1) LAI(IIIB), a challenge stock generated from infection of the H9 cell line (titer, 10^4 infectious units/ml on chimpanzee peripheral blood mononuclear cells [PBMCs]) and titrated in chimpanzees by intravenous inoculation (1 chimpanzee infectious dose [CID] is ~4 tissue culture 50% infectious doses [$TCID_{50}$]), obtained from L. Arthur[27]; (2) C90/LAI(IIIB), a stock (titer, 10^4 $TCID_{50}$/ml) generated by infection of chimpanzee PBMCs with virus recovered from chimpanzee C-090 at 4 weeks after vaginal inoculation of HIV-$1_{LAI(IIIB)}$-infected PBMCs from chimpanzee C-435 (see below); (3) DH12, a subtype B primary isolate for which a challenge stock (titer, 8×10^3 $TCID_{50}$/ml) was generated by infection of chimpanzee PBMCs and titrated in chimpanzees by intravenous inoculation (1 CID is 6 to 16 $TCID_{50}$), obtained from M. Martin and A. Schultz[28,29]; (4) 90CR402, a subtype E primary isolate adapted to replicate in chimpanzee PBMCs and with which a challenge stock (titer, 4×10^3 $TCID_{50}$/ml) was generated in chimpanzee PBMCs and titrated in chimpanzees by intravenous inoculation (1 CID is 2 to 5 $TCID_{50}$)[30]; and (5) 92UG029, a subtype A primary isolate (obtained from S. Osmanov),[31] for which a stock (titer, 1200 $TCID_{50}$/ml in chimpanzee PBMCs) was generated in chimpanzee PBMCs. The cell-associated HIV-1 stock was generated by cryopreserving multiple aliquots of PBMCs obtained from chimpanzee C-435 at 4 months after intravenous inoculation of PBMCs from chimpanzee C-087, which was infected with HIV-$1_{LAI(IIIB)}$.[32] Multiple limiting dilution titrations in human and chimpanzee PBMCs indicated that the C-435-derived stock, designated C435P/LAI(IIIB), contained approximately 30 infectious cells (ICs)/10^6 PBMCs.

Cervical/vaginal inoculation

Virus stocks were diluted to the desired concentration in a final volume of 0.25 ml in RPMI 1640 medium supplemented with 25% human seminal plasma (hsp), where indicated, and were drawn into a 1-ml syringe, leaving an air pocket to ensure that all of the inoculum was expelled. A rigid metal catheter to which a short piece of flexible tubing was attached was mounted on the syringe containing the inoculum. After placing a sterile speculum lubricated with water-based KY jelly (Johnson and Johnson, New Brunswick, NJ) in the vagina, a colposcope was used for direct observation as the tip of the tubing was gently inserted 1–2 mm into the endocervical canal, where the contents of the syringe were slowly injected. Occasionally some of the inoculum leaked into the vagina; in these instances, it was not possible to determine the actual amount that remained in the endocervix. Care was taken to prevent trauma to the cervical os, and colposcopic examinations were done after the inoculations to confirm that there was no visible bleeding. The animals were positioned in the ventral decubitus position for the procedure and were left with their hindquarters elevated for approximately 30 min after inoculation. Vaginal washes were performed with the aid of a Kelly proctoscope, which was inserted into the vagina until the dorsal and ventral fornix and the external opening of the cervix were visible. Using a syringe to which was attached a Klatskin needle and 5 to 6 cm of an infant feeding tube, 3 ml of phosphate-buffered saline was used to flush the 1 to 2 cm of exposed vaginal wall and cervix. The fluid was drawn into the syringe, and the procedure was repeated two or three times.

Serology

Serial twofold dilutions of serum samples were tested for total anti-HIV-1 antibodies with a commercially available enzyme immunoassay (EIA) kit, according to the manufacturer instructions (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France). Titers were defined as the reciprocal of the highest di-

lution of serum to give an optical density reading above the cut-off value recommended by the manufacturer. Using serum dilutions of 1:100, immunoblot assays were done with a commercially available kit (Sanofi Diagnostics Pasteur).

Virus isolation and infectivity assays

Blood samples were obtained from chimpanzees on the day of inoculation, every 2 weeks for 8 weeks, and at monthly intervals thereafter. The presence of infectious virus in the peripheral circulation was ascertained by coculture of each animal's PBMCs with phytohemagglutinin (PHA)-stimulated normal human PBMCs in RPMI 1640 medium containing 10% fetal bovine serum (FBS), interleukin 2 (10 units/ml), and antibiotics (complete medium). Culture supernatants were tested approximately every 5 days for cell-free reverse transcriptase (RT) activity, at which time an equal volume of medium was replaced; freshly stimulated normal human PBMCs were added every 10 days.[33] In some instances, duplicate cultures of chimpanzee PBMCs, which were maintained in a second laboratory, were monitored for HIV-1 $p24^{gag}$ antigen.[34] To facilitate recovery of virus, some cocultures were established with chimpanzee PBMCs that had been enriched for $CD4^+$ lymphocytes by removal of $CD8^+$ cells with magnetic beads coated with anti-CD8 monoclonal antibodies (Dynabeads; Dynal, Chantilly, VA). Single-cell suspensions of lymph node biopsy tissues and cells from bone marrow aspirates and vaginal washes were also evaluated for infectious virus by cocultivation with human PBMCs, as described above. Cells were removed from vaginal washes by low-speed centrifugation and resuspended in culture medium; some of these cell-free supernatants were used to inoculate cultures of human PBMCs in attempts to isolate HIV-1.

To evaluate virus replication, cryopreserved PBMCs from uninfected or HIV-1-exposed chimpanzees were rapidly thawed, washed, and resuspended in medium, and viable cells were counted in a hemacytometer by trypan blue dye exclusion. Chimpanzee PBMCs were stimulated with PHA for 3 days, then washed and counted. Depending on the experiment, 3 to 5×10^6 PBMCs were aliquoted into either 12-well plates or T25 culture flasks, where they were inoculated with 10^3 $TCID_{50}$ of the HIV-1 strain C90/LAI(IIIB). After letting the virus adsorb for 2 hr, complete medium was added to give a cell concentration of 10^6 PBMCs/ml. Cell-free supernatants were tested periodically for RT activity, as described above.

Proliferative assays

Cryopreserved PBMCs were thawed, washed, and counted, and resuspended at 10^6 cells/ml in RPMI 1640 containing 14% human AB serum (AB–RPMI) and antibiotics. Aliquots of 0.1 ml were placed in wells of 96-well flat-bottom microtiter plates, and 0.1 ml of the following reagents in AB–RPMI medium was added to quadruplicate wells: medium only; a 1:500 dilution of PHA; 2.5 μg of concentrated, heat-inactivated HIV-1 virions (Electronucleonics, Silver Spring, MD); and in some experiments, 2.5 μg of concentrated SIV. After 3 days in a CO_2 incubator at 37°C, 1 μCi of [^{3}H]thymidine in 20 μl was added to each well, and the plates were reincubated for approximately 18 hr before harvesting the cells and determining the amount of radionucleotide incorporated. A comparison of incubation for 3 or 6 days prior to adding the [^{3}H]thymidine showed there was essentially no difference in stimulation indices (SIs), and those that were positive ($SI > 2$) at 3 days were also positive at 6 days; therefore, only the results of the 3-day incubation are reported. The SI is defined as counts per minute (cpm) incorporated in the presence of antigen or mitogen divided by counts per minute incorporated in medium only. When SIs were determined on different days, PBMCs from an animal previously tested were included as an internal standard; the results in comparison with these control cells were always consistent.

PCR and DNA sequence analyses

Oligonucleotide primers that flank the V3 loop (from C2 to V5) and recognize HIV-1 isolates from multiple clades were used to detect proviral HIV-1 DNA by nested PCR, using primers and conditions described previously.[35,36] Two different sets of external and internal primers were used: set 1, 587S/588 (external) and 589/590 (internal)[35]; and set 2, C/H (external) and D/F (internal).[36] Multiple PCR analyses were done using 0.5 to 1.5 μg of genomic DNA from PBMCs and from bone marrow and lymph node biopsies obtained on different dates. For some samples, duplicate vials of cryopreserved cells were tested up to 2 years later by a different individual using the second set of primers identified above. All PCR assays included genomic DNA from a known HIV-infected chimpanzee or a chimpanzee that was never exposed to HIV-1 as positive and negative controls. To minimize the possibility of contamination, reactions were performed in a sterile biosafety hood using positive-displacement pipettors and filtered tips.

RESULTS

Cervical inoculation of chimpanzees with cell-associated HIV-1

Experiment 1. Initial attempts to establish a model for genital infection of chimpanzees employed the cell-associated virus stock, C435P/LAI(IIIB), described above. Two female chimpanzees, C-382 and C-120, were inoculated with 1.2×10^6 and 10^7 viable PBMCs, which contained the equivalent of 35 and 300 ICs, respectively. At 2 weeks after inoculation and multiple times thereafter, virus was isolated from C-120 PBMCs and lymph node cells. HIV-1-specific IgA antibodies were detected in vaginal wash fluids at 3 weeks and at later times[37]; seroconversion occurred 4 weeks after inoculation, as determined by both EIA and immunoblot (not shown). In contrast, virus was isolated from C-382 PBMCs only on three occasions (3, 4, and 6 weeks after exposure), with virus-positive cultures obtained in two separate laboratories. Vaginal washes were performed at multiple times after the cervical inoculation and cultured with human PBMCs, but HIV-1 was never recovered from these washes from either animal. Two independent PCR amplifications of proviral DNA in cells from a lymph node biopsy from C-382 at 33 weeks were positive for HIV-1, but virus was not isolated by cocultivation of lymph node cells or C-382 PBMCs obtained at the same time. Although serum antibodies to HIV-1 were not detected during an 8-month follow-up period, HIV-1-specific IgA antibodies (titer, 1:20) were detected in a vaginal wash obtained 6 weeks after cervical exposure of

C-382.[37] Together these results indicate that C-382 was infected with HIV-1, but that there was minimal replication, insufficient to induce detectable serum antibodies, and a presumptive latent infection was established.

Because of the failure of C-382 to seroconvert, at 35 weeks after the first inoculation, C-382 was exposed cervically to 300 ICs of the same cell-associated virus stock; this dose was the same as that used initially to infect C-120. After this second exposure, virus was not isolated from PBMCs or vaginal washes, using standard cocultivation procedures; C-382 did not seroconvert, and no HIV-1-specific antibodies were detected in a vaginal wash performed at 4 weeks. Therefore, 3 months after the second cervical exposure, we inoculated C-382 by the intravenous route with 30 ICs (10^6 PBMCs) of the cell-associated virus (Fig. 1). HIV-1 was isolated consistently from C-382 PBMCs beginning at 2 weeks, with seroconversion occurring at 4 weeks, demonstrating that protective immune responses against intravenous infection were not present.

Although serum antibodies were not detected before the intravenous inoculation of C-382, it was possible that a cellular immune response had been elicited during the transient viremia. There was no evidence of the presence of cytotoxic T lymphocytes (CTLs), but proliferative responses to HIV-1 antigens or inactivated whole virus were present at low levels during the first 6 months after the initial cervical inoculation (Fig. 2). After C-382 received the higher-dose inoculum at 35 weeks, proliferative responses that had deteriorated to undetectable levels again increased above background levels (SI > 2). Proliferative responses to HIV-1 antigens also were detected in C-120 PBMCs beginning at 12 weeks after mucosal exposure. When equivalent amounts of SIV antigens were used as stimulus in the proliferative assays, the results were negative, with none of the SIs greater than 1 (data not shown).

Note: The remaining experiments described in the following sections were performed over several years and were not necessarily done in the order discussed.

Experiment 2. Because either a persistent or transient viremia was established after the first inoculation of C-120 with 300 ICs and C-382 with 35 ICs, we tested whether an intermediate dose of infectious cells would establish persistent infection via exposure to the female genital tract. The rationale was that this inoculum would help to identify a dose of cell-associated virus for use in future vaccine challenge experiments. Therefore, chimpanzee C-562 was inoculated via the cervical os with 100 ICs from C-435, and at the same time, C-454 was exposed to the same number of infectious cells on the vaginal mucosa (Fig. 1). Neither animal seroconverted, and virus was not isolated from PBMCs, bone marrow, lymph node, or vaginal washes during 34 weeks of follow-up. Although multiple PCR analyses of PBMCs obtained from C-454 between 6 and 12 weeks after inoculation were positive for HIV-1 proviruses, cells from a lymph node biopsy done at 19 weeks were negative. All PCR assays with DNA from C-562 were negative; however, HIV-1-specific IgA antibodies were consistently detected in vaginal washes from C-562.[37] Vaginal washes from C-454 were not evaluated. Both animals developed low-level proliferative responses to HIV-1 antigens by about 30 weeks after inoculation (Fig. 2). However, because neither animal was overtly infected, at 34 weeks, both C-454 and C-562 as well as a naive chimpanzee, C-460, were inoculated via the cervical os with a higher dose, 420 ICs, of the C-435 cell-associated HIV-1 stock. The only evidence of infection in the three animals was a positive PCR assay using genomic DNA from a coculture of C-460 PBMCs, which was initiated 4 weeks after cervical exposure; this culture was also positive for $p24^{gag}$ antigen.

Last, chimpanzee C-304 was exposed to 300 ICs of the C-435 cell-associated stock with similar results; that is, this animal did not seroconvert, nor was virus detected by culture or PCR analysis in PBMCs, bone marrow, or lymph node cells during 29 weeks of follow-up. Thus, of six naive chimpanzees exposed to HIV-1-infected PBMCs by cervicovaginal inoculation, only two chimpanzees were infected unequivocally: C-120, from which virus was persistently isolated, and C-382, from which virus was transiently isolated from PBMCs. However, IgA antibodies to HIV-1 antigens were detected on more than one occasion in vaginal washes from C-120, C-382, and C-562,[37] the only chimpanzees for which this analysis was done. It is possible, therefore, that the other three animals exposed to HIV-1 via the genital mucosa also developed IgA responses. Both the presence of IgA antibodies detectable by EIA and the proliferative responses of PBMCs to HIV antigens indicated that sufficient viral replication had occurred in mucosal tissues to induce these immune responses.

Cervical inoculation in the presence of seminal plasma

Because genital infection of female chimpanzees with HIV-1-infected PBMCs failed to provide a reliable model for future vaccine studies, a second series of experiments was performed. Using the SIV–macaque model, Miller *et al.*[38] showed that adding hsp to the inoculum enhanced infectivity of cell-free SIV by the vaginal route, presumably because it helped to buffer the low vaginal pH. Therefore, the effect of hsp on the viability of PBMCs from the C435P/LAI(IIIB) stock was investigated. When measured after a 3-hr incubation *in vitro*, the addition of 25% hsp to chimpanzee PBMCs resulted in a higher percentage of viable cells (Table 1). To determine whether hsp influenced mucosal infectivity of HIV-1-infected PBMCs, chimpanzee C-460, which previously appeared to experience transient viremia after exposure to 400 ICs (without seroconversion), and a naive animal, C-090, were inoculated with 300 ICs of C-435P/LAI(IIIB) (1×10^7 PBMCs) resuspended in medium containing 25% hsp. Both animals readily became infected as shown by repeated virus isolations from PBMC beginning at 2 weeks after inoculation, followed by seroconversion to HIV-1 antigens. Two sequential attempts to infect C-454 (which had already resisted two cell-associated and two cell-free genital challenges; see below) were then made using this procedure, but neither attempt resulted in detectable infection. Inoculation of C-304 (which apparently was not infected after one previous cell-associated inoculation) with 300 ICs (1×10^7 PBMCs) of these same HIV-1-infected PBMCs in medium with 25% hsp also failed to establish detectable infection. Therefore, cell-associated cervicovaginal challenge using 25% hsp as a buffer resulted in persistent infection after only two of five attempts in four animals (Fig. 1). However, the three failed attempts involved two chimpanzees previously exposed cervically to HIV-infected cells: C-304 and C-454; the latter had been exposed to some form of HIV-1 four times previously. It is possible, therefore, that local mucosal immunity was present

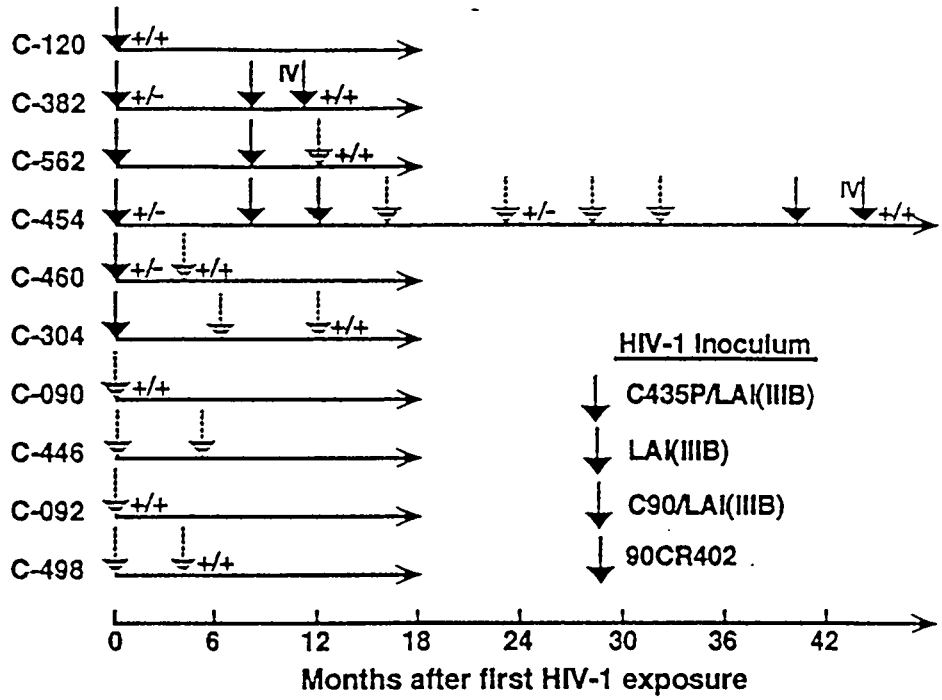

FIG. 1. Time line showing the various HIV-1 inocula given to individual chimpanzees. Each HIV-1 inoculum is identified by colored arrows, as shown in the legend. Solid arrows indicate HIV-1 inocula diluted in RPMI 1640 only, whereas the dashed arrows indicate that the virus was administered in the presence of 25% human seminal plasma. The first "+" sign to the right of some arrows means that virus was isolated from PBMCs and/or one or more PCRs were positive for HIV-1-proviral DNA. The second "+" or "–" sign indicates whether the animal did (+) or did not (–) seroconvert. If no positive or negative signs are adjacent to an arrow, then no evidence of virus or seroconversion was obtained after that inoculation. All inoculations were by the cervicovaginal route except for those indicated by an IV, which were intravenous.

and that these responses influenced the outcome of the second and third attempts.

Cervical inoculation of female chimpanzees with cell-free HIV-1

In a first attempt at genital infection of chimpanzees using cell-free virus, two animals, C-454 and C-562, both of which had resisted two previous attempts at cell-associated virus infection, were inoculated, respectively, with 2000 and 200 $TCID_{50}$ (equivalent to 500 and 50 CID by the intravenous route) of the HIV-$1_{LAI(IIIB)}$ chimpanzee challenge stock diluted in medium only. Virus was isolated from C-562 PBMCs on two occasions (weeks 6 and 8) and from lymph node cells at 8 weeks, but not thereafter; however, the animal did seroconvert. C-454, on the other hand, showed no evidence of infection. Using 1250 $TCID_{50}$ of the same stock diluted into medium containing 25% hsp, a second attempt was made to establish a productive infection in C-454. The animal, however, once again did not develop a systemic infection.

Cervical infection with chimpanzee-passaged cell-free HIV-1

Because it is possible that virus recovered from a chimpanzee infected via the cervical os would be more infectious by a mucosal route, a cell-free stock [designated HIV-$1_{C90/LAI(IIIB)}$] was made from virus recovered from C-090, an animal infected with the C435P/LAI(IIIB) cell-associated HIV-1 by the cervical route. C-304, for which no evidence of infection was obtained after two exposures to cell-associated virus (Fig. 1), was inoculated cervically with 500 $TCID_{50}$ of the C90/LAI(IIIB) stock in the presence of 25% hsp. Virus was isolated initially from PBMCs at 6 weeks, and the animal seroconverted. Two other animals, C-454 and C-446, were inoculated with 125 $TCID_{50}$ of the HIV-$1_{C90/LAI(IIIB)}$ stock in the presence of 25% hsp, but they apparently were not infected. A second unsuccessful attempt was made to infect C-446 using a higher dose, 500 $TCID_{50}$, of the chimpanzee-passaged virus. As part of a vaccine challenge study (data not shown), two naive female chimpanzees, C-092 and C-498, were inoculated with 1250 $TCID_{50}$ in 25% hsp via the cervicovaginal route. C-092 became infected, as indicated by initial virus isolation from PBMCs at 6 weeks after infection and seroconversion. When no evidence was obtained that C-498 was infected, this animal was inoculated again with the same dose of virus, which led to productive infection and seroconversion. The results of cervicovaginal inoculation with cell-free HIV-$1_{C90/LAI(IIIB)}$, therefore, were variable: a dose of 125 $TCID_{50}$ appeared to be insufficient, that of 500 $TCID_{50}$ was effective in one of two attempts, and that of 1250 $TCID_{50}$ was effective in two of three attempts (Table 2).

Inoculation of female chimpanzees with HIV-1_{DH12}

There is considerable evidence that the neutralization sensitivity of T cell line-adapted HIV-1 strains, such as LAI(IIIB), and those of primary isolates differ[39–43]; therefore, it was im-

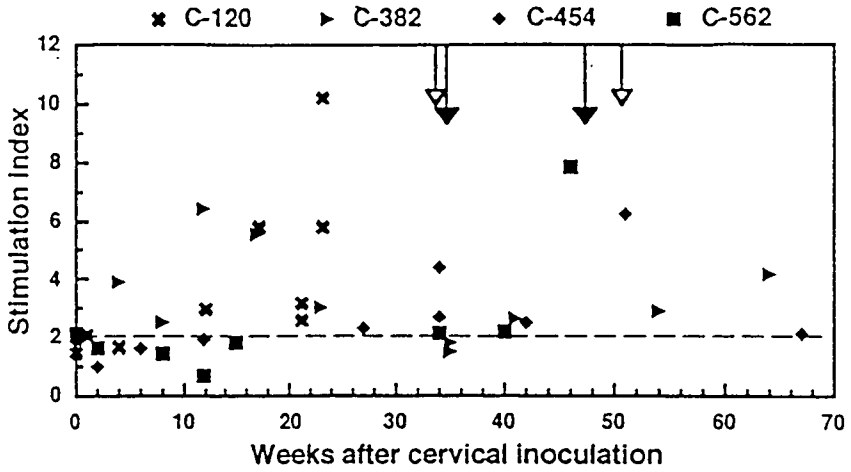

FIG. 2. Proliferative responses of chimpanzee PBMCs to heat-inactivated HIV-1 virions at various times after cervicovaginal exposure to infectious HIV-1. The long arrows with the filled arrowheads indicate times at which C-382 received additional inoculations, and the shorter arrows with open arrowheads indicate times at which C-454 received additional inoculations. Stimulation indices above 2 (dashed line) are considered positive.

portant to establish whether genital infection could occur using a primary isolate. A titrated stock of HIV-1_{DH12}, a dual-tropic primary HIV-1 isolate that had been shown to be infectious for chimpanzees, was selected.[28,29] In a first attempt at genital infection with HIV-1_{DH12}, a female chimpanzee (C-534) was inoculated with 500 $TCID_{50}$ of the cell-free virus stock in the presence of 12.5% hsp via the cervicovaginal route. The animal did not become infected by standard criteria. However, a second female, C-110, exposed previously via the cervical os to a subtype A strain with no evidence of infection (see below), was inoculated with 1000 $TCID_{50}$ in 25% hsp and became infected. This latter result demonstrated that genital infection with a primary HIV-1 isolate was possible.

Inoculation of female chimpanzees with HIV-$1_{92UG029}$

There is some evidence that interclade cross-protection among HIV-1 subtypes may be difficult to achieve by vaccination.[44–46] To evaluate the infectivity by the cervicovaginal route of another primary HIV-1 isolate from a different subtype, a female chimpanzee, C-110, was inoculated with 500 $TCID_{50}$ of the subtype A isolate HIV-$1_{92UG029}$. The animal, however, failed to become infected. Female C-534, which previously was exposed to HIV-1_{DH12} without success, was inoculated via the genital route on two occasions with 1000 $TCID_{50}$ of the HIV-$1_{92UG029}$ stock. Neither of the two attempts led to infection, as evidenced by failure to isolate virus from PBMCs and lack of seroconversion. However, the infectivity of the HIV-$1_{92UG029}$ stock for chimpanzees was demonstrated by inoculating chimpanzee (C-631) with 100 $TCID_{50}$ by the intravenous route. This animal developed a systemic infection within 2 weeks and subsequently seroconverted (data not shown).

Inoculation of female chimpanzees with HIV-$1_{90CR402}$

Since genital infection of female chimpanzees with the subtype A strain was unsuccessful, we tested a primary subtype E isolate from the Central African Republic, HIV-$1_{90CR402}$, that had been adapted to grow in chimpanzee PBMCs.[30,47] Two fe-

TABLE 1. EFFECT OF HUMAN SEMINAL PLASMA ON SHORT-TERM VIABILITY OF CHIMPANZEE PBMCs[a]

Human seminal plasma(%)	*Incubation time (hr)*			
	0.5	*1*	*2*	*3*
0	98	95	69	59
25	97	97	96	94
50	97	91	90	92

[a]PBMCs from an uninfected chimpanzee were incubated at room temperature in RPMI 1640 medium supplemented with antibiotics and the indicated percentage of human seminal plasma. Cell viability was determined at the indicated times by trypan blue dye staining.

TABLE 2. CERVICOVAGINAL INOCULATION OF FEMALE CHIMPANZEES WITH CHIMPANZEE-PASSAGED CELL-FREE HIV-$1_{C90/LAI(IIIB)}$

Chimpanzee	*Previous exposures*[a]	*Dose*[b] *($TCID_{50}$)*	*Infection*[c]
C-446	0	125	No
C-454	6	125	No
C-304	2	500	Yes
C-446	1	500	No
C-092	0	1250	Yes
C-498	0	1250	No
C-498	1	1250	Yes

[a]Number of times the animal had been exposed to either cell-associated or cell-free HIV-1 by inoculation at the cervical *os* before the indicated dose of the C90/LAI(IIIB) strain.

[b]The virus inoculum was diluted in RPMI 1640 containing 25% human seminal plasma.

[c]Disseminated systemic infection accompanied by seroconversion.

male chimpanzees (C-380 and C-366) were inoculated via the cervicovaginal route with 500 $TCID_{50}$ of cell-free HIV-$1_{90CR402}$ in the presence of 25% hsp; both animals became viremic and seroconverted. Although C-454 had not seroconverted after seven genital challenges with various HIV-$1_{LAI(IIIB)}$ virus preparations, it was possible that the subtype E strain might be infectious by the cervical route, especially if subtype B-specific immune responses had been elicited by the previous multiple exposures; therefore, C-454 was exposed to 500 $TCID_{50}$ of the HIV-$1_{90CR402}$ stock. However, no evidence of infection was obtained. Therefore, cervicovaginal inoculation of 500 $TCID_{50}$ of HIV-$1_{90CR402}$ resulted in a systemic infection and seroconversion in both previously unexposed animals, but not in C-454.

Susceptibility of C-454 to HIV-1 infection

The evidence that C-454 might have had an abortive infection with HIV-1 after the various mucosal exposures consisted of (1) sporadic positive PCRs within 12 weeks after the first inoculation and one positive PCR at 12 weeks after the fifth inoculation, and (2) proliferative responses of PBMCs to HIV-1 antigens that developed about 6 months after the first cervical inoculation (Fig. 2). Since it was possible that the PBMCs of C-454 were inherently unable to support efficient replication of HIV-1, we compared the replication efficiency of HIV-1 in lymphocytes from C-454 with replication in cells from other chimpanzees. PBMCs obtained on the day C-454 was exposed cervically for the seventh time to HIV-1 were stimulated with PHA and then infected with HIV-$1_{C90/LAI(IIIB)}$, the virus stock used for that inoculation. In parallel, PBMCs from two uninfected chimpanzees, one of which (C-446) was exposed for the first time by the cervical route at the same time C-454 received inoculum number 7, were also tested. In two separate experiments, the maximum amount of virus released into the culture supernatants from infected cells of C-454 was about 10- to 70-fold lower than that produced by PBMCs from the uninfected animals and C-446 (Fig. 3).

To determine whether the eight prior exposures to HIV-1 in conjunction with the apparent reduced ability to support replication of HIV-1 might influence infection by a parenteral route, C-454 was inoculated intravenously with 10 $TCID_{50}$ of HIV-$1_{C90/LAI(IIIB)}$. Within 2 weeks, virus was isolated from the PBMCs of the animal and at 6 weeks, antibodies to HIV-1 antigens were measurable by EIA and immunoblot assays. This result demonstrated that the apparent resistance of C-454 to disseminated HIV-1 infection after mucosal exposure did not extend to inoculation by the intravenous route.

DISCUSSION

Atraumatic inoculation of chimpanzees via the cervical os with either HIV-1-infected PBMCs or cell-free virus resulted in productive infections and seroconversion, but the efficiency was low. After one exposure of 10 chimpanzees to virus stocks derived from the LAI(IIIB) strain, only 4 animals (C-120, C-382, C-090, and C-092) developed a systemic infection, although C-382 did not develop serum antibodies (Fig. 1). Two of the apparently uninfected chimpanzees (C-460 and C-498) seroconverted and virus was consistently isolated from their PBMCs after a second exposure 4 months later. Another two animals (C-304 and C-562) became unequivocally infected after a third cervicovaginal exposure. Of the remaining two animals, one (C-446) was not reexposed after the second inoculation, and the last animal (C-454) did not seroconvert, nor was virus isolated from its tissues despite eight separate cervicovaginal inoculations with four different virus preparations. However, at least one of several nested PCRs of PBMC proviral DNA obtained 6 and 12 weeks after the first exposure was positive, as was one of six nested PCRs of DNA from a bone marrow aspirate performed 12 weeks after the fifth inoculation. These results suggest that this animal was infected with HIV-1, probably throughout the entire observation period, but that the virus was essentially latent with insufficient antigen production to elicit a detectable antibody response. Thus, cervicovaginal inoculation of adult chimpanzees with HIV-$1_{LAI(IIIB)}$-derived stocks resulted in three different outcomes (Fig. 1): (1)

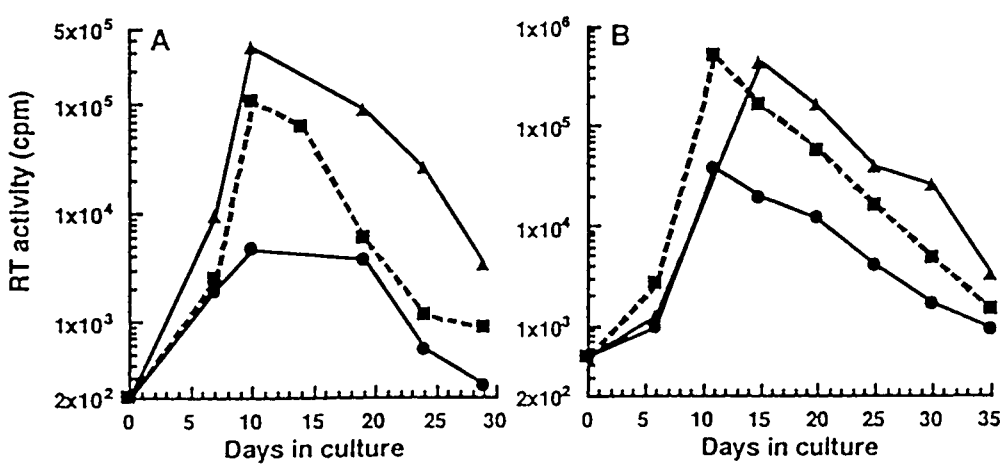

FIG. 3. Replication of HIV-$1_{C90/LAI(IIIB)}$ in PBMCs from C-446 (▲), C-454 (●), and an uninfected chimpanzee (■). PBMCs were obtained either (A) on the day C-446 was initially exposed to HIV-1 mucosally, which was the same day that C-454 was exposed for the seventh time, or (B) 16 weeks after the exposure described in (A). PBMCs from different uninfected animals were used in the two experiments.

no apparent infection, as evidenced by repeated failure to detect virus by standard cocultivation techniques and multiple nested PCRs as well as lack of seroconversion; (2) transient viremia and/or latent infection, as evidenced by detection of virus in PBMCs, lymph nodes, or bone marrow on only a few occasions, but failure to seroconvert; and (3) overt infection with consistent isolation of virus from PBMCs and seroconversion. After one to three exposures, 8 of 10 animals were infected systemically.

Chimpanzee C-382 unequivocally developed a disseminated infection but failed to seroconvert after the first exposure to HIV-1. That this animal had transient anti-HIV IgA responses in vaginal washes and maintained a proliferative response to HIV-1 antigens suggests that the animal may have been persistently infected but that the virus was sequestered in an inaccessible compartment, such as mesenteric lymph nodes. Although it is possible HIV-1 was cleared from its body, this is unlikely owing to the life cycle of retroviruses, in general, and the persistence of HIV-1, in particular. That few of the PCRs were positive merely indicates that the viral burden was low. We used a maximum of 1.5 μg of cellular DNA per PCR; six independent PCRs with this amount of DNA would be equivalent to testing DNA from about 1.3×10^6 cells. If only one cell in 5 to 10 million cells carried a provirus, it is unlikely that it would be detected in peripheral blood cells unless one were to perform at least 50 or more independent PCRs.

Our results are consistent with some reports using the SIV–macaque model, in which animals appeared not to become infected after multiple vaginal or rectal exposures to SIV.[38,48–53] Findings in the SIV–macaque model that resembled our results, especially in the cases of C-382 and C-454, included (1) transient viremia without seroconversion after vaginal inoculation,[38,53] and (2) resistance to a high-dose rectal challenge after exposure to a low dose, but establishment of persistent infections after subsequent intravenous challenge.[52] In addition, mucosal inoculation of macaques with a low dose of SIV, which appeared not to be infectious, induced T cell-proliferative responses that correlated with protection from a subsequent mucosal challenge with a higher dose of virus.[54] Results in HIV–chimpanzee and SIV–macaque models are consistent with one another, and with those of HIV-seronegative individuals at risk for infection and who exhibited T cell-proliferative responses to HIV antigens.[55,56] Furthermore, Mazzoli *et al.*[57] demonstrated not only T cell-proliferative responses, but also mucosal HIV-specific IgA responses without seroconversion, in seronegative partners of HIV-1-seropositive humans. Taken together, these results, in association with the observed discordance between viral burdens in blood and male and female genital secretions,[58–60] provide evidence for compartmentalization of immune responses and sequestering of virus in all three virus–host systems.

In the SIV–macaque model, a role for $CD8^+$ T cells in preventing mucosal infection or sequestering virus in mucosal tissues was suggested after it was shown that removal of these cells from PBMCs led to a significant increase in SIV replication *in vitro*, but the inhibition appeared to be mediated by soluble factors rather than by cytotoxic T lymphocytes.[50] $CD8^+$ T cell-produced soluble suppressor factors, first described in the context of HIV by Walker *et al.*,[61] may or may not include β and CXC chemokines.[62–64] In one study, protection from rectal challenge of immunized macaques appeared to correlate with the production of β-chemokines by lymphocytes depleted of $CD4^+$ cells.[65] In contrast, HIV-1-specific cytotoxic $CD8^+$ T lymphocytes have been detected in exposed but seronegative women and children.[66,67] Furthermore, the relative resistance of $CD4^+$ lymphocytes from exposed, uninfected individuals to infection by various HIV-1 strains was shown to correlate with production of chemokines by the purified $CD4^+$ T cells.[68] Thus, there are substantial data to indicate that seronegative humans and animals exposed mucosally to HIV (or SIV) develop various virus-specific immune responses, some of which can be detected in PBMCs for extended periods. For such responses and memory cells to be induced, the virus must transit the epithelium and replicate, at least to a limited extent. Whether these responses are protective against subsequent mucosal infection by the same or different HIV-1 strains is unknown.

In the studies reported here, multiple cervicovaginal inoculations of various HIV-1 stocks were required to establish systemic infections and seroconversion in adult female chimpanzees. This result is consistent with those obtained following vaginal inoculation of macaques with various SIV strains.[38,53] We generally used lower doses of HIV-1 than were used in most SIV studies which, in part, was dictated by the small volume that could be deposited in the cervical os and the titers of the virus stocks. However, it has been reported that, in the SIV–macaque model, successful vaginal infection was not dependent on the virus dose, but rather on the inherent ability of a particular SIV strain to replicate in macaques.[53] The HIV-1 strains LAI/LAV-1b and JC499, which establish the highest viral burdens in chimpanzees (our unpublished data), were not used in the present studies. However, in another study one female was infected with HIV-1_{JC499} after one exposure to the cervical os.[69]

In one chimpanzee, eight separate inoculations of four different HIV-1 strains [three strains related to HIV-$1_{LAI(IIIB)}$ and one subtype E strain] failed to result in productive infection. However, the animal did exhibit evidence of the presence of memory cells, and occasionally proviral DNA was detected in PBMCs. It is possible that mucosal IgA and memory cell responses elicited by the first, or a subsequent, cervical exposure either prevented infection or facilitated maintenance of a low-level infection localized in mucosal tissues. Irrespective of the mechanisms involved, our results indicate that a strain or dose of either cell-free or cell-associated HIV-1 that will result in systemic infections 100% of the time after cervicovaginal inoculation of adult chimpanzees is unlikely to be identified. Because of the limited numbers of chimpanzees that can be used in one experiment and the erratic success in infecting chimpanzees by the cervicovaginal route, obtaining statistically significant results will be difficult. Thus, vaccine protection studies in which the challenge inoculum is a single genital exposure of chimpanzees to HIV-1 do not seem feasible.

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Genital HIV-1 Challenge of Female Chimpanzees Immunized with A Recombinant Canarypox-HIV-1 Virus

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ABSTRACT

Heterosexual sex accounts for more than 80% of human immunodeficiency virus type 1 (HIV-1) infections worldwide; therefore, an effective vaccine must be able to induce immunity to infections acquired via mucosal routes. To evaluate whether vaccination would prevent genital transmission of HIV-1, five female chimpanzees were immunized five times by a combination of routes, including mucosal, with ALVAC-HIV-1 vCP250, a recombinant canarypox virus expressing the HIV-$1_{LAI(IIIB)}$ gp120/TM *env, gag,* and protease genes. Four of the immunized animals and a naive chimpanzee were challenged 5 weeks after the last boost with 1250 50% tissue culture infectious doses of a chimpanzee-passaged cell-free LAI(IIIB)-derived stock deposited at the cervical *os.* Virus isolation and changes in antibody titers indicated that the control, but none of the vaccinated animals, was infected. After 5-months follow-up, the animals were boosted with vCP250 and challenged cervically 3 weeks later with the same virus stock. Two of the immunized animals developed a disseminated infection, but no evidence of infection of the control and the other three immunized animals was obtained. All four of these animals were challenged a third time; the control animal and one of the three immunized animals were infected systemically. While it appears that two of the five immunized animals were protected after three cervicovaginal HIV-1 challenges, that the naive control required two exposures to HIV-1 before infection was documented suggests that the two chimpanzees might have been naturally resistant or occult infections were established. Only one of the putative protected chimpanzees had HIV-1-specific antibodies in mucosal secretions , and these were low level. Thus, protection, if it occurred, probably was not antibody mediated. These results indicate that, when evaluating protective efficacy in phase III HIV-1 vaccine trials, it may be difficult to distinguish between those individuals actually protected and those with either natural resistance or "silent" infections.

INTRODUCTION

Although more than 80% of human immunodeficiency virus type 1 (HIV-1) infections worldwide are transmitted as a result of heterosexual sex,[1] little is known about the mechanisms of, and immune responses involved in, protection from this route of infection. There is considerable evidence that the immune system consists of two functionally independent compartments and that immune responses induced in one of these are not necessarily reflected in the other. These two compartments are identified as systemic, represented by the bone marrow, spleen and peripheral lymph nodes, and mucosal, represented by mucosa-associated lymphoid tissues and external secretory glands.[2-5] Because pathogens initially encounter different cell types and environments in these two tissues, it is likely that correlates of immune-mediated protection against mucosal infection by HIV-1 will be different from those needed to prevent intravenous infection as well as subsequent progression to disease after infection. In fact, several studies have demonstrated that protection from diseases transmitted via mucosal membranes of the respiratory and gastrointestinal tracts correlates with the level of antibodies in the corresponding secretions rather than with that in serum.[2]

HIV can be transmitted by and can be found in semen as cell-free virus or virus-infected cells.[6,7] Thus, both humoral and cell-mediated immune responses, which are most effective at removing cell-free virions and infected cells, respectively, will be required to provide optimal protection against HIV infection and/or disease progression. Furthermore, it is known that soluble proteins or peptides formulated with adjuvants primarily induce antibodies and helper T-cell responses whereas recombinant live virus vectors are more efficient at inducing cytotoxic T-lymphocyte (CTL) responses. In fact, studies in humans and nonhuman primates have demonstrated that immunization with a live virus expressing HIV antigens in

combination with purified proteins or peptides elicited better humoral and cellular immune responses to HIV or SIV than either form of immunogen alone.[8-10]

To date, in all of the studies using the HIV-1-chimpanzee model to assess protection by vaccine-elicited immune responses, the animals were challenged intravenously. In those chimpanzees in which no evidence of infection was obtained, the only apparent correlate of protection was the titer of neutralizing antibodies to the envelope glycoprotein of the homologous virus.[11-17] In some of these, the V3 region had been targetted specifically in the immunization regimen.[12-15] However, whether these antibodies would have prevented infection across a mucosal surface is not known.

Only two studies demonstrating HIV-1 infection of chimpanzees following exposure of the virus to a mucosal surface have been published. In an early study, one chimpanzee exposed vaginally to a high dose of cell-free HIV-$1_{LAI(LAV-1b)}$ became infected and seroconverted.[18] More recently, we described a model in which chimpanzees were exposed either to cell-free or cell-associated HIV-1 by depositing the inoculum atraumatically at the opening of the cervical canal.[19] Although infections were established with both forms of virus, some chimpanzees required two or more exposures before disseminated infections accompanied by seroconversion were documented. In addition to cell-free and cell-associated HIV-$1_{LAI(IIIB)}$, infections with a cell-free inoculum of another subtype B strain, DH12, and a subtype E strain, 90CR402, were established by the cervicovaginal route.[19] We describe here mucosal immunization and subsequent genital challenge of female chimpanzees with cell-free HIV-1 and demonstrate that protection against mucosal HIV-1 infection can be achieved through vaccination. The role of vaccine-induced immune responses, however, is unclear. In contrast to studies involving intravenous challenge of vaccinated chimpanzees, the results are more difficult to interpret because of inherent

differences in the mucosal environment of individual animals and the possibility of occult infections being established.

MATERIALS AND METHODS

Animals and virus inoculations

Adult female chimpanzees (*Pan troglodytes*) were housed at the Laboratory for Experimental Medicine and Surgery in Primates, New York University, in biosafety level 2 facilities in accordance with institutional guidelines and the Animal Welfare Act. Before all procedures, chimpanzees were anesthetized by intramuscular inoculation of ketamine hydrochloride (10 mg/kg). All virus inoculations were done when the animals were at or near the peak of estrous, employing administration of depoprovera to synchronize menstrual cycles when necessary. A speculum was used to inspect the vagina visually, thus ensuring that no blood was present before virus inoculations, which were done as described previously.[19] The inoculum was deposited at the opening of the cervical canal. Care was taken not to induce trauma, and colposcopic examinations were done to confirm that no bleeding occurred.

Immunogen and study design

Five HIV-naive adult chimpanzees were immunized with ALVAC-HIV-1 vCP250, a recombinant canarypox virus that expresses the HIV-$1_{LAI(IIIB)}$ gp120 fused to the transmembrane (TM) anchor segment from gp41 (gp120/TM) as well as the *gag* and protease genes from this same strain. Each animal received five inoculations containing a total of 4×10^8 plaque-forming units of vCP250 at time 0, 7, 24, 57, and 70 weeks. Of these five chimpanzees, two animals (C-420 and C-440) were immunized by a combination of the intramuscular, vaginal, and rectal routes with 1/3 of the total inoculum administered by each route; two chimpanees (C-422 and C-424), by a combination of the intramuscular, oral, and nasal routes; and one chimpanzee (C-452)

received the entire dose intramuscularly. No booster inoculations with protein subunits were done. Chimpanzees were challenged with approximately 1250 $TCID_{50}$ of cell-free HIV-1 in a volume of approximately 0.3 ml by atraumatic inoculation via the cervicovaginal route. The virus stock, designated C90/LAI(IIIB), was generated from peripheral blood mononuclear cells obtained from a chimpanzee 4 weeks after infection by the cervicovaginal route with HIV-$1_{LAI(IIIB)}$ and has been described.[19]

Virus isolation and quantitation

After HIV-1 challenge, whole blood samples in heparin were obtained from all chimpanzees every 2 weeks for 8 weeks and then at monthly intervals. Isolated PBMC were co-cultured with mitogen-stimulated normal human PBMC, and aliquots of cell-free supernatants were monitored periodically for reverse transcriptase assay to detect virus production. At various times after challenge, vaginal wash fluids, cervical tissue obtained by punch biopsy then minced with scissors, and single-cell suspensions of peripheral lymph node biopsy tissue were also cultured as above.[19]

To quantify viral burdens, 0.5- or 1-ml plasma samples were thawed and tested for cell-free virion RNA by nucleic acid sequence-based amplification (NASBA) using a NucliSens HIV-1 QT kit (Organon Teknika). The sensitivity or lower limit of detection is about 200 RNA copies per ml.

Serologic assays

Serial twofold dilutions of serum samples were tested for total anti-HIV-1 antibodies with a commercially available enzyme immunoassay (EIA) kit, according to the manufacturer's instructions (Sanofi, Seattle, WA). Titers were defined as the reciprocal of the last serum dilution to give an optical density reading above the cut-off value recommended by the manufacturer. Immunoblot assays were done at a serum dilution of 1:100 with a commercially available kit (Diagnostics Pasteur). Antibody

titers to a HIV-$1_{LAI(IIIB)}$ V3-loop peptide were determined by EIA, as previously described.[15] Neutralizing antibodies to HIV-$1_{LAI(IIIB)}$ were measured using a quantitative syncytium-inhibition assay with CEM-SS cells.[20]

Several times, including at the time of the first virus challenge, vaginal, rectal and nasal wash specimens from the immunized animals were collected in PBS with 10 mM LiCl. The dilution factors of the wash specimens were calculated by measuring lithium concentrations in the supernatants, as described by Bélec *et al.*[21] Mucosal secretions, which included whole saliva, and serum samples were tested for anti-gp160 IgG and IgA using a sandwich EIA, previously described.[22] Serum samples were tested for the presence of anti-gp120 antibodies; the avidity of binding of these anti-gp120 antibodies was also determined as described.[16]

RESULTS

Serologic response to immunization

Five adult female chimpanzees were immunized five times each with ALVAC-vCP250. Blood samples were obtained regularly, and humoral immune responses to HIV-1 proteins were evaluated. Serum anti-HIV-1 antibodies were first detected by EIA in one animal after the second (week 7) and in three other animals after the third (week 24) immunization, but these responses were transient in the latter three animals (Fig. 1). The exception was C-440, for which antibody titers increased after the second and third immunizations and then remained relatively stable. After the fourth immunization (week 57), increases in antibody titers to whole virus were observed in all five animals (Fig. 1). With the exception of C-440 and C-452, which showed increases of at least threefold after the fourth immunization, HIV-$1_{LAI(IIIB)}$ anti-V3 antibody titers remained relatively low until after later boosts. C-420's anti-V3 titers, however, only increased substantially after the animal became infected (Table 1).

While the antibody titers of the animals to gp120 Env were low, ranging from < 25 to 100, serum titers to gp160 Env were moderate, ranging from 250 (C-424) to 8,000 (C-440) at time of the first challenge (week 75) (not shown). Immunoblot assays done at the time of the first challenge (week 75) demonstrated that, although anti-p24*gag* antibody responses were easily detected in all of the animals, only three of the four chimpanzees (C-420, C440, and C-452) had detectable anti-p17*gag* antibodies. For all animals, no anti-Env responses were detected by immunoblot (not shown), and antibodies were of low avidity (avidity index < 30%). Anti-HIV-$1_{LAI(IIIB)}$ neutralizing antibody responses, with titers $\geq$ 32 developed in only two animals (C-440 and C-452) (Table 2).

Of the samples tested, no anti-gp160 IgA antibodies were detected in mucosal secretions from any animal (not shown). Chimpanzee C-440, however, did have anti-gp160 IgG antibodies in mucosal secretions tested at a dilution of 1:2. The highest amounts were in rectal washes, with levels approximately twofold higher than those in vaginal washes, fourfold higher than in nasal washes and tenfold higher than in saliva. These relative concentrations of antibodies were consistent with C-440's immunization regimen, which was a combination of rectal, vaginal, and intramuscular. None of the mucosal secretions from other animals had any evidence of anti-gp160 IgG antibodies.

The anti-canarypox antibody levels, as determined by EIA, ranged from 16 (C-420) to 116 (C-440) EIA units (EU)/ml, indicating that all of the animals developed an immune response to the virus vector. (A cut-off value of 0.6 EU/ml is considered positive in human voluteers.)

Cervicovaginal challenge

Five weeks after the last booster inoculation, four of the immunized animals (C-452, C-440, C-420, and C-424) and a naive control (C-092) were challenged via the

cervicovaginal route with 1250 $TCID_{50}$ of cell-free $HIV\text{-}1_{C90/LAI(IIIB)}$. Chimpanzee C-422 was not inoculated with virus because, unexpectedly, blood was in its vagina on the day of challenge. At 6 weeks after challenge, virus was isolated from PBMC from the control animal (C-092), which seroconverted between 8 and 12 weeks after challenge (Fig. 1). In contrast, virus was not isolated from PBMC or lymph node cells from the four immunized animals at any time during 5 months of follow-up (weeks 75 to 96). This finding and the declines in their antibody titers suggested that systemic infections were not established (Fig. 1; Tables 1 and 2).

After 5 months of follow-up, all five of the immunized chimpanzees were again boosted with $4x10^8$ pfu of vCP250, and 3 weeks later (week 99), all of these animals and a second naive control (C-498) were challenged via the cervical *os* with a dose of cell-free $HIV\text{-}1_{C90/LAI(IIIB)}$ equivalent to that used in the first challenge. PBMC from two of the immunized animals (C-452 and C-420) were positive for infectious HIV-1 at 6 weeks after inoculation and thereafter. Virus isolation was accompanied by fourfold increases in antibody titers over those present at time of challenge (Fig. 2). The other three immunized animals appeared not to be infected, as demonstrated by the inability to isolate virus from their PBMC and by decreases in antibody titers (Fig. 2). However, the control animal (C-498) also did not become infected systemically, as indicated by its lack of seroconversion (Fig. 2) and our failure to isolate virus from its PBMC and from lymph node cells obtained by biopsy 6 weeks after challenge. This was not surprising because we never achieved 100% infection with this dose of the C90/LAI(IIIB) challenge virus when naive animals were inoculated via the cervicovaginal route.[19]

After 4 months of follow-up (week 116), the three immunized uninfected animals, as well as the second uninfected control animal (C-498), were again challenged via the cervicovaginal route with 1250 $TCID_{50}$ of the $HIV\text{-}1_{C90/LAI(IIIB)}$ stock. HIV-1 was isolated initially from PBMC from one immunized female, C-424, and

the control animal, C-498, at weeks 4 and 8 after challenge, respectively. Chimpanzee C-424 also exhibited a substantial increase in antibody titers, as assessed by both EIA (Fig. 2) and immunoblot assay (not shown). In contrast, PBMC from the other two immunized females, C-422 and C-440, remained virus negative by coculture assay, and these two animals did not develop an anamnestic antibody response after the third virus challenge (Fig. 2).

Viral Burdens

To determine whether immunization had an effect on viral burdens after systemic infections were established, virion RNA levels in plasma samples obtained early after cervical challenge were quantified by NASBA. Comparison of RNA copy numbers detected in plasma from the two control and three immunized animals that were infected revealed no obvious differences in the two groups (Table 3). In fact, the highest RNA copy number was obtained in plasma samples from C-424, an immunized animal that was infected systemically after the third HIV-1 challenge.

DISCUSSION

In this study we demonstrated that intramuscular immunization alone or in combination with mucosal immunization of female chimpanzees with a recombinant canarypox-HIV-1 virus (vCP250) might have provided protection from infection after cervicovaginal challenge with cell-free HIV-1. However, in contrast to what was demonstrated in previous chimpanzee studies where the intravenous route of challenge was used,[12,14-16] no correlation between infection (or lack thereof) and serum neutralizing antibody titers was noted (Table 4). The four chimpanzees challenged initially at 75 weeks had neutralizing antibody titers ranging from 4 to 64, and all animals appeared to be protected from systemic infections. Likewise, at the

time of the second challenge, the three animals for which no evidence of infection was obtained and the two animals that subsequently became infected had comparable titers of > 8 to 64. Of the three immunized chimpanzees exposed to HIV-1 a third time, both C-422 and C-424 had no detectable serum neutralizing activity (titer, < 4); however, C-422, but not C-424, was protected. It should be reiterated that these animals were immunized only with a nonreplicating recombinant immunogen. In general, a boost with a purified protein is required to elicit high titers of antibodies, including neutralizing antibodies, as shown in studies with nonhuman primates and in phase I/II studies in humans with related recombinant canarypox-HIV-1 vectors.[8-10,23-25] Thus, our present results suggest that systemic neutralizing antibodies are not important in protection from mucosal challenge.

Even though four of the five chimpanzees were immunized by both parenteral and mucosal routes, only one of these four, C-440, developed detectable antibodies in any of the mucosal secretions tested. These antibodies, however, were only of the IgG isotype. That no IgA antibodies to HIV were detected is consistent with a previous study by Lubeck *et al.*[26] in which only IgG responses to HIV-1 were detected in saliva and nasal, vaginal, and rectal secretions from chimpanzees immunized intranasally with a recombinant adenovirus-HIV-1 vaccine. That result, as well as those in the study reported here, contrast with the results of other studies showing that intranasal immunization is an efficient way to induce both humoral and cell-mediated immune responses to a variety of antigens in multiple mucosal sites.[27-31] Although C-440 was not immunized by the nasal route, this animal did generate the highest sustained antibody response of any chimpanzee and, of note, appeared to be protected from infection despite three virus challenges. Whether the low levels of HIV-1-specific antibodies that were found in mucosal secretions from C-440 played a role in preventing systemic infection in this animal cannot be determined.

Although it is possible that cell-mediated immunity might have played a role in the apparent protection from infection of these animals, we did not evaluate CTL in this study. Previous attempts to demonstrate CTL activity in PBMC from two chimpanzees immunized intramuscularly with vCP250 were not successful.[16] Furthermore, in humans and nonhuman primates immunized with various ALVAC constructs, CTL activity has been limited.[9,25,32,33] Using two sequential 14-day in vitro stimulations and a novel method of generating target cells, Ferrari *et al.*[34] were able to detect CTL activity against primary HIV-1 isolates from different clades in PBMC from vaccinated volunteers. Also relevant are the findings of Belyakov *et al.*[35] and Nguyen *et al.*[36], who demonstrated, at least in mice, that $CD8^+$ CTL must be present at the site of mucosal exposure in order to protect against mucosal infection. Furthermore, macaques transiently viremic or aviremic and seronegative after colonic exposure to SIV developed CTL responses, which were localized to the jejunal lamina propria and appeared to protect against subsequent colonic challenge with a heterologous SIV.[37] Thus, the ability to identify CTL activity in peripheral blood may be irrelevant for assessing whether immune responses elicited by candidate vaccines might prevent mucosal transmission. Since we did not attempt to evaluate CTL in cervicovaginal washes or tissue sections, primarily because invasive procedures would be required, we can make no conclusions as to whether CTL might have contributed to the apparent protection observed in this study.

There is one important caveat to the results described above. In our previous experiments to establish a mucosal model of HIV-1 infection in chimpanzees for use in vaccine studies, after one cervical inoculation, only two of eight female chimpanzees became unequivocally infected as shown by multiple reisolations of virus from PBMC and seroconversion.[19] Three of these eight animals never seroconverted after the first exposure despite isolation of virus from PBMC and/or demonstration of the presence of proviral DNA by PCR. Systemic infections with seroconversion were subsequently

established in one of these latter three animals after a second cervical exposure. Of the three uninfected of the original eight animals, two became infected after the third exposure; the last one was not reexposed when it failed to become infected after two cervicovaginal inoculations. Finally, one chimpanzee that was positive for proviral DNA after the first exposure never seroconverted during a 3-year period when it was subjected to eight separate cevicovaginal exposures. These results, and the finding in the present study that one naive control animal became infected and seroconverted only after the second mucosal inoculation, make it difficult to determine whether the immunized chimpanzees actually were protected from infection by vaccination. It is possible the challenge results are merely a reflection of an inoculum containing less than a 100% infectious dose. (The challenge dose was mandated by the titer of the HIV-1 stock and limitations in volume for inoculation at the cervical *os*.) Alternatively, like humans, chimpanzees probably vary naturally in susceptibility to HIV-1 infection across a mucosal surface. Multiple factors related to the cervicovaginal milieu can influence whether HIV-1 is transmitted as a result of any one episode of vaginal sex; these factors include variation in normal vaginal flora and infections with a variety of pathogens, both of which can effect changes in vaginal pH and the presence of inflammatory cytokines.[38-41]

Results in the SIV-macaque model indicate that it is easier to protect against vaginal rather than parenteral transmission of SIV after vaccination.[42] However, the macaque model has also been used to demonstrate unequivocally that transient disseminated infections without seroconversion can be established after rectal or vaginal inoculation, even when high doses of virus are used.[43-45] Similar results were obtained after mucosal transmission of feline immunodeficiency virus (FIV) to cats, where failure to detect FIV and FIV-specific antibodies was associated with suppression of replication by $CD8^+$ lymphocytes.[46] The presence of occult infections might explain the observations that HIV-specific cytotoxic T cells have been detected

in multiply exposed, (apparently) uninfected women and in uninfected children born to HIV-infected women.[47,48] Likewise, it is possible that the chimpanzees in the present study either were protected by non-specific factors or that occult infections were established.

Irrespective of whether vaccine-mediated protection was achieved in the two chimpanzees in which disseminated HIV-1 infections were not established after three cervicovaginal inoculations, the immune responses induced by vaccination with only ALVAC vCP250 were low, but comparable to those seen in human volunteers immunized with related ALVAC constructs. However, as in humans, it appears that boosting with other immunogens, such as purified proteins, will be required to optimize anti-HIV-1 humoral and cell-mediated immune responses, both systemically and in mucosal tissues. The HIV-1-chimpanzee mucosal model might be improved somewhat if a higher-titered stock of a strain of HIV-1 that is more infectious than the C90/LAI(IIIB) strain and is pathogenic for chimpanzees were used. While this study demonstrates that chimpanzees can be used to evaluate vaccine efficacy against cervicovaginal HIV-1 infection, it also illustrates problems that will be associated with phase III human trials. Decisions will have to be made as to whether vaccines are protective, nonspecific factors in the vaginal milieu prevented infection, or occult infections were established.

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FIGURE LEGENDS

Figure 1. HIV-1-specific antibodies in serum samples from chimpanzees immunized with recombinant ALVAC vCP250. Short arrows at the top of the graph indicate times of immunization after the first immunization at week 0. The long dotted arrow indicates the time at which all chimpanzees, except C-422, were inoculated cervically with HIV-$1_{C90/LAI(IIIB)}$.

Figure 2. HIV-1-specific antibodies in serum samples before and after cervical challenges of immunized chimpanzees. Week 70 is the same as week 70 in figure 1 and is the time of the fourth booster immunization. Arrows depict times of immunization with vCP250 or cervical challenge with HIV-$1_{C90/LAI(IIIB)}$, as described in the legend to figure 1.

Table 1. Anti-V3 Antibody Titers in Serum from Chimpanzees Immunized with ALVAC-HIV-1 vCP250 by Systemic and Mucosal Routes

	i.m./vaginal/rectal[a]		i.m./oral/nasal		i.m.	Controls	
Week[b]	C420	C-440	C-422	C-424	C-452	C-092	C-498
27	530	1250	440	710	650	ND[c]	ND
57[d]	500	780	430	390	640	ND	ND
70[d]	500	4000	560	670	2040	ND	ND
75[e]	770	38000	1550	4460	8600	112	ND
96[d]	214	18900	1430	1300	1400	12200	ND
99[e]	843	45400	7040	16600	27000	ND	246
107	15700	18000	7250	4820	110000	ND	177
116[e]	ND	12200	4830	2570	ND	598	<50
124	ND	13600	3170	70300	ND	ND	254
132	ND	9950	2520	ND	ND	ND	ND

[a]Route of immunization of indicated chimpanzees; i.m., intramuscular.

[b]Week after first immunization with ALVAC vCP250.

[c]ND, not done.

[d]Fourth, fifth, and sixth inoculations of ALVAC vCP250 at weeks 57, 70, and 96, respectively.

[e]First, second and third cervical challenges with HIV-$1_{C90/LAI(IIIB)}$ at weeks 75, 99, and 116, respectively.

Table 2. Serum HIV-$1_{LAI(IIIB)}$ Neutralizing Antibody Titers

	i.m./vaginal/rectal[a]		i.m./oral/nasal		i.m.
Week[b]	C-420	C-440	C-422	C-424	C-452
75[c]	8	64	4	16	32
79	8	64	4	8	16
96[d]	<4	<4	<4	<4	<4
99[c]	>8	64	16	32	32
116[c]	32	16	<4	<4	64

[a]Route of immunization of indicated chimpanzees; i.m., intramuscular.

[b]Weeks after first immunization with ALVAC vCP250.

[c]First, second and third cervical challenges with HIV-$1_{C90/LAI(IIIB)}$.

[d]Sixth inoculation of ALVAC vCP250.

Table 3. HIV-1 Virion RNA Levels in Plasma after Cervical Challenge of Chimpanzees

Chimpanzee	vCP250[a]	Challenge number[b]	Weeks after virus inoculation			
			6	8	12	16
C-420	yes	2	16,000[c]	5,600	2,100	73,000
C-422	yes	-	ND	ND	ND	ND
C-424	yes	3	ND	34,000	240,000	ND
C-440	yes	-	< LDL	ND	ND	ND
C-452	yes	2	23,500	3,800	< LDL	< LDL
C-092	no	1	1,500	25,000	2,300	11,000
C-498	no	2	ND	2,000	1,400	ND

[a]Chimpanzee was or was not immunized with ALVAC vCP250.

[b]Number of times an animal was challenged before a systemic infection was established. –, no evidence of infection.

[c]RNA copies/ml plasma, determined by NASBA. < LDL, below the lower detection limit; ND, not done.

Table 4. Neutralizing Antibody Titers and Outcomes of Cervical Challenges of Chimpanzees

Chimpanzee	Route[a]	Challenge no. 1 NAb[b]	1 Inf'd[c]	2 NAb	2 Inf'd	3 NAb	3 Inf'd
C-452	i.m.	32	-	32	+		
C-420	i.m./vaginal/rectal	8	-	> 8	+		
C-440	i.m./vaginal/rectal	64	-	64	-	16	-
C-422	i.m./oral/nasal	4	NC[d]	16	-	< 4	-
C-424	i.m./oral/nasal	16	-	32	-	< 4	+
C-092	-		+				
C-498	-		NC		-		+

[a]Route of immunization.

[b]NAb, neutralizing antibody titers at the time of challenge.

[c]Infection was (+) or was not (-) documented by isolation of virus and an increase in HIV-1-specific antibody titers.

[d]NC, not challenged.

Figure 1. Girard et al.

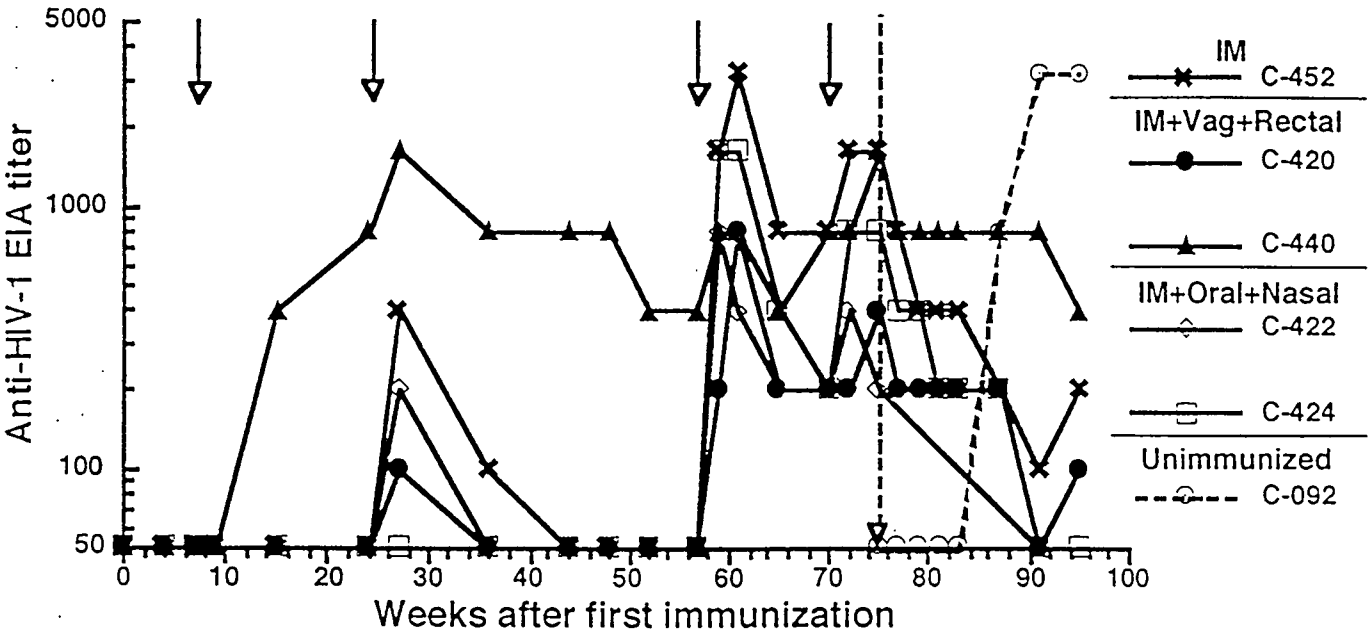
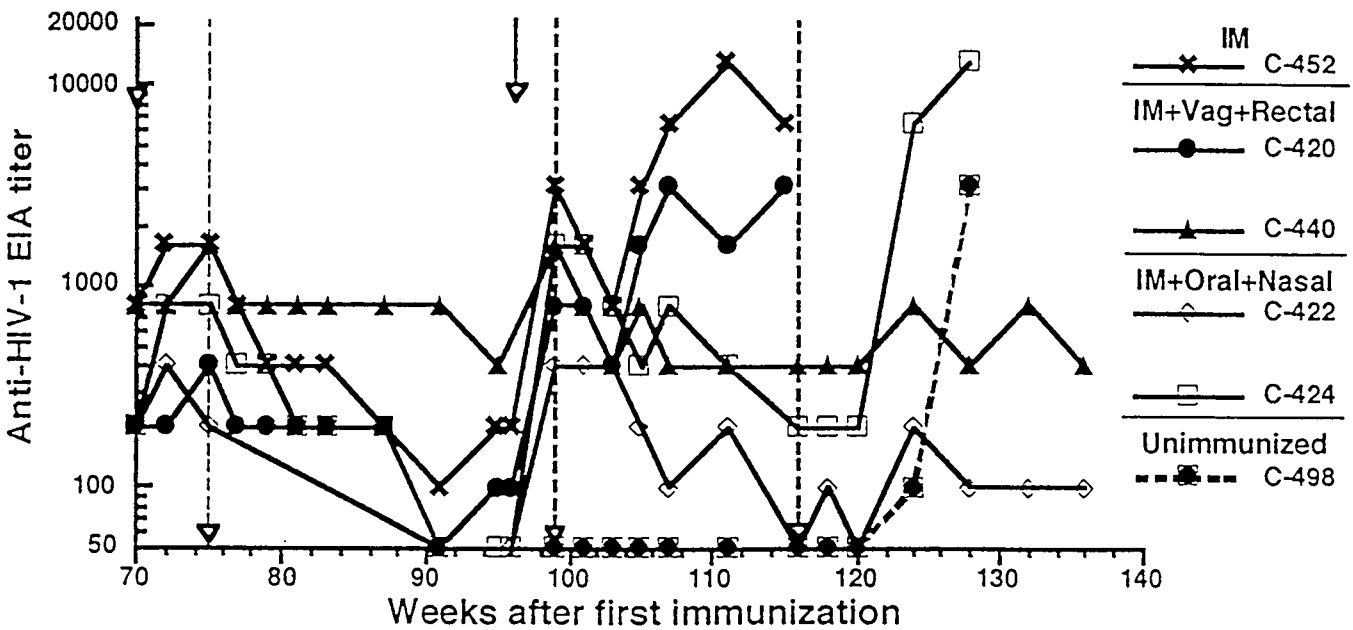


Figure 2. Girard et al.



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L'immunisation du macaque avec les virions purifiés d'un isolat primaire du virus de l'immunodéficience humaine de type 1 induit des anticorps facilitants

Immunisation with whole purified human immunodeficiency virus type 1 particles elicits enhancing antibodies in rhesus macaques

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Lecture par Marc GIRARD

RÉSUMÉ

Nous avons hyperimmunisé six macaques avec des préparations purifiées d'un isolat primaire du virus de l'immunodéficience humaine de type 1 (VIH-1), le VIH BX08. Trois animaux ont reçu du virus pleinement infectieux et trois autres du virus inactivé à la bêta-propiolactone ou au formol. Au lieu d'induire ainsi des anticorps neutralisants, nous avons paradoxalement induit chez les animaux l'apparition d'anticorps capables d'augmenter de 10 à 90 fois le pouvoir infectieux du VIH-1 BX08 pour les cellules mononucléées du sang périphérique en culture. Ces mêmes anticorps augmentent le nombre de syncytia formés en cultures de cellules HeLa CD4⁺ CCR5⁺ exprimant l'enveloppe du VIH-1 BX08. Les vaccins anti-VIH à base de particules virales entières pourraient donc induire des anticorps qui faciliteraient la transmission du VIH et/ou l'évolution de la maladie.

MOTS-CLÉS : HIV-1. IMMUNISATION. LENTIVIRINAE. FACILITATION DÉPENDANTE ANTICORPS.

SUMMARY

Six Rhesus macaques were hyperimmunized with either live infectious human immunodeficiency virus type 1 (HIV-1) or with beta-propiolactone — or formalin — inactivated

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HIV-1. The virus used was HIV-1 BX08, a primary virus isolate grown in human PBMC. Instead of eliciting virus-neutralizing antibodies, this regimen induced antibodies that enhanced HIV-1 infectivity for PBMC by 10 to 90 fold. Enhancement was also seen in a cell-to-cell fusion assay using a Semliki Forest virus replicon to express BX08 gp160 in CD4⁺, CCR5⁺ HeLa cell cultures. These observations raise the concern that whole virus particles-based HIV-1 vaccines might elicit enhancing antibodies that could play a facilitating role in the transmission and/or evolution of the disease.

KEY-WORDS (Index Medicus) : HIV-1. IMMUNIZATION. LENTIVIRUS. ANTIBODY-DEPENDENT ENHANCEMENT.

INTRODUCTION

La glycoprotéine d'enveloppe du virus de l'immunodéficience humaine (VIH) est synthétisée dans la cellule infectée sous forme d'un précurseur polyprotéique, la gp160, qui est glycosylée dans le réticulum endoplasmique, acquiert la structure tertiaire qui la rend capable de se fixer sur le récepteur CD4, s'oligomérise, puis est transportée dans l'appareil de Golgi. La gp160 est ensuite clivée en une sous-unité externe (gp120) et une sous-unité transmembranaire (gp41) qui restent associées l'une à l'autre de manière non-covalente à la surface de la cellule infectée puis du virion, où on les retrouve sous forme de trimères de gp120/gp41 [1, 2].

La fixation du virus à la surface de sa cellule cible nécessite l'interaction de la gp120 avec le domaine amino-terminal du CD4 [3-6]. Le changement de conformation de la glycoprotéine qui s'ensuit [7] aboutit au démasquage du site d'attachement à un deuxième récepteur. Celui-ci est soit le CCR5, récepteur des chimiokines bêta RANTES, MIP-1alpha et MIP-1bêta, soit le CXCR4, récepteur de la chimiokine alpha SDF-1, selon qu'il s'agit de souches de VIH à tropisme macrophagique ou à tropisme lymphocytaire [8-12]. La boucle hypervariable V3 de la gp120 paraît directement impliquée dans le choix du 2e récepteur [13-15].

L'interaction du virus avec son 2e récepteur conduit à la fusion de l'enveloppe virale avec la membrane de la cellule cible. On admet, par analogie avec ce que l'on sait de la pénétration du virus grippal [16, 17], que la gp41, au repos, serait repliée sur elle-même au cœur de l'hétérodimère gp120/gp41. Le déploiement de la molécule entraîne la projection de son extrémité N-terminale très hydrophobe au contact de la membrane plasmique de la cellule cible, dans laquelle elle s'insère (Fig. 1). La gp41 se replierait ensuite sur elle-même, pour amener l'enveloppe du virus au contact intime de la membrane cellulaire [18, 19], prélude à la fusion des deux bi-couches lipidiques.

Le même mécanisme paraît responsable de la formation des syncytia que l'on observe au cours de l'infection à VIH-1 : les cellules infectées expriment en effet à leur surface des molécules de gp120/gp41 qui leur permettent de fusionner avec des cellules non infectées porteuses des récepteurs CD4 et CCR5 ou CD4 et CXCR4, selon le tropisme du virus considéré (Fig. 1).

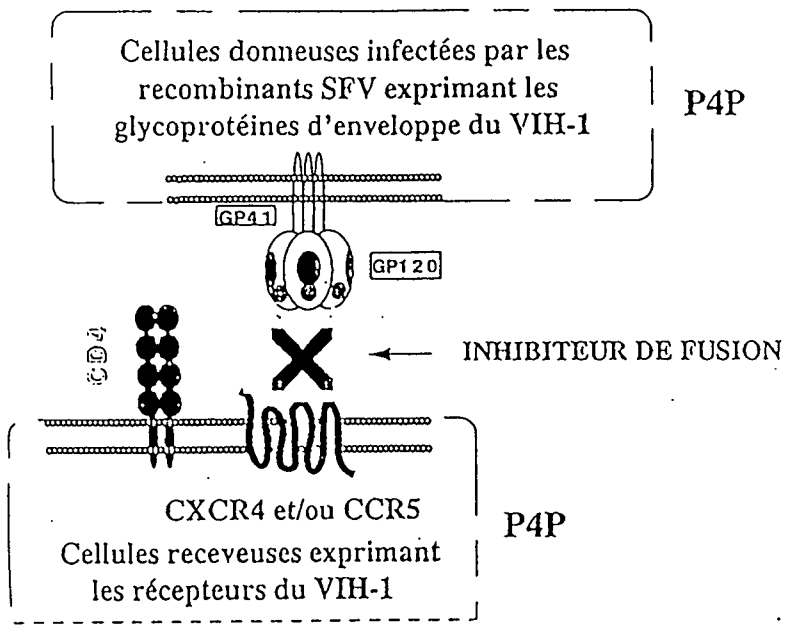

FIG. 1 — Schéma du test de fusion.
L'expression des glycoprotéines gp120/gp41 du VIH-1 (ici représentées sous forme de trimère) à la surface de cellules HeLa P4P infectées par un recombinant SFV-*env*, entraîne la formation de syncytia dans la culture, par interaction des glycoprotéines d'enveloppe du VIH avec les récepteurs CD4 et CCR5 (ou CXCR4). Ce test permet de doser l'activité anti-fusion du sérum ou d'inhibiteurs de la pénétration du virus (voir texte).

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Cependant, il arrive que la pénétration du virus soit aussi facilitée par son interaction avec des anticorps antiviraux non neutralisants [20, 21]. On a ainsi décrit chez les individus infectés par le VIH l'existence d'au moins deux types de ces anticorps que l'on dénommera ici « anticorps facilitants ». Certains agiraient en se fixant sur les particules virales, puis en interagissant avec les récepteurs cellulaires de la partie Fc des immunoglobulines [22, 23] qu'on trouve à la surface des lymphocytes T, des cellules NK, et des macrophages. D'autres agiraient *via* le complément par fixation des complexes anticorps-virions-complément sur les récepteurs du complément CR3 [24-26].

Nous avons fait l'observation paradoxale que les sérums des macaques que nous avions immunisés avec les virions d'un isolat primaire du VIH-1, la souche BX08, étaient non seulement incapables de neutraliser l'infectivité du virus *in vitro*, mais en fait augmentaient le pouvoir infectieux du virus pour les lymphocytes humains du sang périphérique (PBMC). Les mêmes sérums se sont montrés capables de favoriser la formation de syncytia entre cellules HeLa $CD4^+$ $CCR5^+$ exprimant la gp160 BX08 à l'aide d'un réplicon recombinant dérivé du virus de la forêt de Semliki (SFV). Ces observations soulèvent la possibilité que des vaccins anti-VIH à base de particules virales entières pourraient induire des anticorps facilitants qui, au lieu d'être protecteurs, faciliteraient l'infection des vaccinés [27-29].

MATÉRIEL ET MÉTHODES

Virus

La souche VIH-1 BX08 a été isolée à Bordeaux du patient BX08 quelque temps après sa séroconversion [30]. Elle n'a été cultivée que sur PBMC de donneur sain [31, 32]. La séquence de la boucle V3 de la gp120 de ce virus est très proche de la séquence consensus des virus isolés en France au début des années 1990 [33, 34]. Le virus a été purifié par centrifugation sur coussin de saccharose puis inactivé soit par action de la bêta-propiolactone (1/400, 1 heure à 4°) soit par action du formol (1/1000, 16 heures à 4° C).

Test de neutralisation et facilitation

Le test de neutralisation du VIH-1 en culture de PBMC (Moog 97) combine, de façon matricielle, des dilutions séquentielles de série 4 du stock de virus BX08 avec des dilutions de série 2 du sérum à tester. On met 50 µl de chaque dilution du virus dans 4 puits d'une plaque Durapor DV 0,65 m (Millipore, Molsheim, France). On ajoute ensuite 50 µl de chaque dilution du sérum. Le mélange est incubé 1 heure à 37° C, après quoi on ajoute, dans chaque puits, 10^5 PBMC préalablement incubés pendant 3 jours en présence de 2 µg/ml de phytohémagglutinine A (Sigma). Après 2 heures d'incubation à 37° C, on lave 3 fois les cellules en RPMI 1640 et on les resuspend dans 200 µl de RPMI 1640 contenant 10 % de sérum de veau fœtal et

20 U d'interleukine 2 (IL-2) (Boehringer, Mannheim) par ml. La multiplication virale est révélée au 7e jour d'incubation à 37° C par la détection de l'antigène p24 par ELISA (Innogenetics ou Dupont) dans le surnageant de culture.

Le titre du virus est calculé par la méthode de Reed et Muench en doses infectieuses 50 % (TCID50), en absence et en présence des diverses dilutions du sérum. Le titre neutralisant du sérum est défini comme la dilution du sérum pour laquelle le titre infectieux du virus est diminué de 90 %. Dans le cas de la facilitation, le titre du virus est augmenté en présence du sérum facilitant. L'effet facilitant n'est considéré comme significatif que lorsque le titre infectieux du virus est augmenté d'un facteur au moins égal à 5. Dans ce cas, l'effet facilitant est exprimé par la formule X (D), où X est le facteur d'augmentation du titre infectieux du virus et D la dilution du sérum pour laquelle on observe cette augmentation.

Immunisation des singes

On a utilisé, pour l'expérience, des singes macaques rhésus (*Macaca mulatta)* mâles, d'origine chinoise, pesant entre 3 et 4 kgs et séronégatifs pour le SIV, le rétrovirus de type D, le virus de la leucémie T du singe (STLV-1) et le virus de l'herpès B. Les animaux ont été anesthésiés à la kétamine (10 mg/kg) avant chaque inoculation ou saignée. On les a immunisés 9 fois de suite par voie intramusculaire, puis 3 fois par voie sous-cutanée. Trois adjuvants ont été successivement utilisés, le QS21, l'adjuvant RIBI et l'adjuvant incomplet de Freund (ISA51, Seppic, France). Trois des singes (N° 202, 253 et 303) ont été immunisés avec du VIH-1 BX08 pleinement infectieux et les trois autres (N° 055, 087 et 121) avec des lots de VIH-1 BX08 inactivé, d'abord à la bêta-propiolactone, puis au formol (Tableau 1). On a suivi chaque mois l'immunisation des animaux en mesurant leurs taux d'anticorps anti-VIH par tests ELISA (kit ELAVIA, Sanofi Diagnostic Pasteur) et Western blot (Sanofi Diagnostic Pasteur). Les activités neutralisante et facilitante ont été déterminées, comme décrit ci-dessus.

Production des recombinants SFV-*env*BX08

Le gène *env* du VIH-1 BX08 a été exprimé en utilisant comme vecteur le virus de la forêt de Semliki (SFV) [35]. Le principe de ce système d'expression est basé sur la complémentation d'un génome recombinant dans lequel les gènes des protéines structurales du SFV ont été remplacés par le gène *env* du VIH, par un sous-génome SFV « helper » contenant uniquement les gènes des protéines structurales du SFV et délété des signaux d'encapsidation. Le recombinant SFV-*env* BX08 est donc un réplicon défectif, car il lui manque les gènes des protéines de structure du SFV, mais il en a conservé la réplicase et il exprime à haut titre la gp160 de l'isolat BX08 dans les cellules qu'il infecte [15, 37]. Pour le générer, l'ARN hybride SFV-*env*BX08 (1 µg) et de l'ARN du virus défectif SFV helper 2 (1 µg) ont été mélangés à des cellules BHK-21. Les cellules ont été soumises à des chocs électriques (25 µF, 830 Volts)

TABLEAU I — Schéma d'immunisation. Trois macaques rhésus (singes N° 205, 253 et 303) ont été immunisés aux dates indiquées avec du VIH-1 BX08 infectieux, et trois autres (singes n° 055, 087 et 121) avec du VIH-1 BX08 inactivé émulsifié dans l'adjuvant indiqué. Pour les six premières injections, le virus a été inactivé à la bêta-propiolactone (BPL), et pour les six suivantes au formol.

Date (*mois*)	Dose (*µg p24*)	Adjuvant	Agent inactivant
0, 1, 3	20	QS21	BPL
6, 7, 9	175	QS21	BPL
14, 15, 17	250	RIBI	Formol
20, 21, 22	200	ISA 51	Formol

dans une chambre de 0,4 cm (Electroporateur Gene Pulser II, Biorad), puis mises en culture. Après 24 heures d'incubation à 37° C, le surnageant de la culture a été clarifié par centrifugation (20 min à 2 000 tours/min), puis centrifugé pendant 1h 30 à 26 000 tours/min à 4° C dans le rotor Beckman SW28. Le culot de virus est repris dans 1/100 de volume de tampon Tris-HCl 50 mM pH 7,4, NaCl 100 µM, EDTA 0,5 mM et réparti en échantillons de 20 µl qui sont conservés à - 80°C.

Avant usage, le virus doit être activé par traitement à la chymotrypsine. A cette fin, on ajoute aux 20 µl de suspension virale 1 µl d'alpha-chymotrypsine (Boehringer) à 10 µg/ml et 0,4 µl de CaCl2 50 mM. On arrête le traitement après 30 min d'incubation, par addition de 9 µl d'une solution d'aprotinine (Sigma) à 2 mg/ml. D'une préparation à l'autre, le titre des stocks de SFV-*env*BX08 mesuré sur cellules BHK-21 a oscillé entre 10^8 et 10^9 particules infectieuses/ml.

Test d'inhibition ou d'augmentation de la formation de syncytia

Les cellules utilisées pour la formation de syncytia sont des cellules HeLa [36, 37]. La lignée P4 exprime les récepteurs CD4 et CXCR4, la lignée P4P exprime de surcroît le récepteur de chimiokines bêta CCR5. Les cellules sont ensemencées, à raison de 2×10^5 cellules par puits en plaques 24 puits en milieu DMEM supplémenté de 10 % de sérum de veau fœtal, 500 µg/ml de G418 (Gibco), et 150 µg/ml d'hygromycine B (Sigma). On les infecte avec le recombinant SFV-*env*BX08 à la multiplicité de 3,5 particules infectieuses par cellule, puis, après 30 min d'adsorption, on les lave et on les recouvre de milieu DMEM contenant 5 % de sérum de veau fœtal et le sérum ou l'inhibiteur à tester. Après 12 heures d'incubation à 37° C, on lave deux fois les cellules au PBS et on les fixe au glutaraldéhyde à 0,5 %, avant de les colorer au Giemsa. Les syncytia sont comptés au microscope optique à l'aide d'une grille de 8 carreaux (1/7e de la surface totale du puits). Le nombre de syncytia par puits est compris entre 1 500 et 2 000. On calcule le pourcentage d'inhibition de la formation des syncytia selon la formule P = (1 - (NI : NT)) × 100, où NI représente

le nombre de syncytia comptés en présence du sérum immun, et NT le nombre obtenu en présence du sérum pré-immun de référence. L'augmentation de la formation des syncytia se traduit par une valeur négative de P. Les valeurs finales sont les moyennes de trois expériences indépendantes.

Anticorps

On s'est procuré auprès du NIH les anticorps monoclonaux anti-CD4 [38] ; 2G12 [39] ; IgG1 b12 [40] ; 670D [41] ; 694/98-D (42,43) ; 2F5 [44] ; et le (CD4-IgG)2 tétramérique [45].

RÉSULTATS

Mise au point d'un test de fusion intercellulaire médiée par la glycoprotéine du VIH BX08.

Nous avons précédemment mis au point un test de fusion intercellulaire basé sur l'infection de cellules HeLa CD4$^+$ CCR5$^+$ (lignée P4P) par des réplicons SFV exprimant la glycoprotéine d'enveloppe (gp160) de diverses souches du VIH-1 [37]. Nous avons ainsi montré que les cellules P4P infectées avec le recombinant SFV-*env*BX08, qui exprime la gp160 de l'isolat primaire VIH-1 BX08, forment des syncytia en culture. Ce phénomène n'est observé qu'avec des cellules CCR5$^+$. Il est bloqué par les anticorps monoclonaux anti-CD4 et par le CD4 soluble sous sa forme tétramérique (CD4-IgG)2 [45], de même que par les chimiokines bêta RANTES et MIP-1bêta, qui sont des ligands du CCR5, mais pas par le ligand du CXCR4, SDF-1 (Fig. 2). A l'inverse, l'infection de cultures de cellules P4P avec un recombinant SFV-*env*LAI génère l'apparition de syncytia dont la formation ne dépend pas du récepteur CCR-5. Elle est bloquée par le SDF-1, mais pas par RANTES ni MIP-1bêta (Fig. 2). On sait que la souche VIH-1 LAI est le prototype des souches de VIH adaptées à la multiplication sur lignées lymphocytaires T humaines immortalisées et qu'elle utilise le CXCR4 comme co-récepteur [46-48]. Le système de formation de syncytia en cellules P4P reproduit donc avec fidélité les premières étapes de l'interaction du VIH-1 avec sa cellule cible.

Nous avons utilisé ce système pour tenter de mettre en évidence une activité anti-fusion dans le sérum du patient BX08. Nous avons utilisé comme témoin des anticorps monoclonaux humains ou murins anti-gp120 qui montrent un large spectre de neutralisation vis-à-vis du VIH (anticorps 2G12, IgG1b12, 670 D, et 694/98-D). Tous ont montré une activité inhibitrice prononcée sur la formation de syncytia dans le système P4P/SVF-*env*BX08. Il en est allé de même avec l'anticorps monoclonal anti-gp41 2F5. Cependant, les anticorps 670D et 694/98-D ne neutralisent pas l'infectivité du VIH-1 BX08 [32]. Cela suggère que l'inhibition de la fusion pourrait être due à des anticorps différents de ceux mis en évidence dans les tests de

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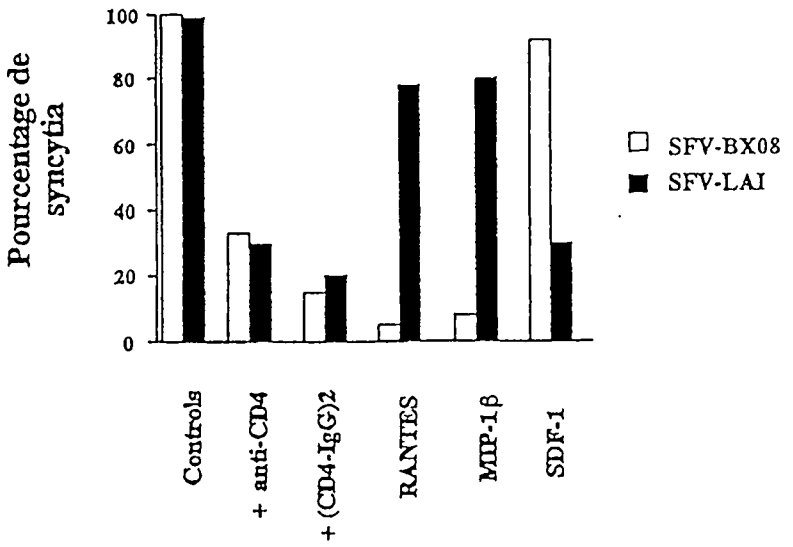

FIG 2. — Caractérisation du test de fusion.
Le nombre de syncytia formés par les recombinants SFV-*env*BX08 et SFV-*env*LAI a été mesuré dans des cultures de cellules P4P en présence du milieu de culture (contrôles) ou d'un anticorps anti-CD4 (liquide d'ascite dilué au 1 : 5), de la molécule (CD4-IgG)2 (100 µg/ml), ou des chimiokines RANTES, MIP-1bêta ou SDF-1 (4 µg/ml chacune). Le virus BX08 utilise les récepteurs CD4 et CCR5, le virus LAI les récepteurs CD4 et CXCR4 (voir texte). Les résultats sont présentés en % du nombre de syncytia dans des cultures infectées non traitées.

neutralisation du pouvoir infectieux, ou, plus vraisemblablement, que le test d'inhibition de la formation de syncytia est plus sensible que le test de neutralisation.

Nous avons ensuite analysé des échantillons de sérum provenant du patient duquel le virus VIH-1 BX08 a été isolé. Utilisés au 1 : 10, tous les sérums testés ont provoqué une inhibition de 66 à 94 % de la formation des syncytia par le recombinant SFV-*env*BX08 en culture de cellules P4P (Tableau 2). Aucune inhibition n'a été observée avec divers sérums humains séronégatifs utilisés comme témoins. Les mêmes échantillons de sérum ont été étudiés en parallèle dans un test de séroneutralisation classique du virus sur PBMC. Le sérum BX08 prélevé environ six mois après la séroconversion du patient (11/12/92) n'a montré aucune activité neutralisante vis-à-vis du virus. Une activité neutralisante autologue a, en revanche, été détectée dans les sérums plus tardifs à la dilution 1 : 20 (Tableau 2) [31]. Il est intéressant d'observer que le sérum précoce du patient BX08 présente une activité inhibitrice de la fusion, mais est dépourvu d'activité neutralisante (Tableau 2). Cela suggère que les anticorps bloquant la fusion apparaissent plus précocement que les

TABLEAU 2. — Analyse longitudinale du sérum du patient BX08. L'activité inhibitrice des échantillons de sérum prélevés aux dates indiquées a été mesurée vis-à-vis de la formation de syncytia en cultures de cellules P4P infectées avec SFV-*env*BX08, en utilisant les sérums dilués au 1 : 10. L'activité neutralisante des mêmes sérums a été mesurée vis-à-vis du VIH-1 BX08 sur PBMC (voir Matériel et Méthodes). Les concentrations de bêta-chimiokine RANTES dans les sérums ont été mesurées à l'aide du kit Quantikine Human RANTES Immunoassay (Pharmacia).
NT : non titré

Date du prélèvement	Inhibition de la formation de syncytia (%)	Titre neutralisant du sérum	Concentration de RANTES (ng/ml)
11.12. 92	94	< 10	50
09. 09. 94	85	20	45
10.10.95	66	NT	41
23.04.96	88	22	45

anticorps neutralisants chez les individus infectés et/ou que le test d'inhibition de la formation de syncytia est plus sensible que le test de séroneutralisation. Il sera intéressant de vérifier cette hypothèse et de déterminer la spécificité des anticorps bloquant la fusion en analysant des sérums provenant d'autres individus séropositifs.

On sait que les chimiokines bêta RANTES, MIP-1alpha et MIP-1bêta sont les ligands du CCR5, et à ce titre des facteurs d'inhibition de la pénétration des souches de VIH-1 à tropisme monocytaire [49-51]. Nous avons donc mesuré la quantité de RANTES dans chacun des échantillons de sérum du patient BX08. Comme le montre le Tableau 2, la concentration détectée dans les échantillons analysés varie de 40 à 50 ng/ml, soit près de 1 000 fois plus que ce que l'on trouve dans les sérums VIH-négatifs (25 à 85 pg/ml), mais en accord avec les concentrations habituellement retrouvées chez les séropositifs [52-54]. Nous avons donc répété l'expérience d'inhibition de la formation de syncytia par un échantillon de sérum du patient BX08 en présence d'un mélange de 10 ng/ml d'anticorps monoclonaux anti-RANTES, anti-MIP-1alpha et anti MIP-1bêta. Ce mélange est capable de neutraliser l'effet inhibiteur de 160 ng/ml de chacune des 3 chimiokines bêta [37]. En présence des anticorps monoclonaux, l'activité du sérum BX08 a été réduite de moitié. On en conclut que l'activité inhibitrice des échantillons de sérum du patient BX08 résulte en partie de l'effet des chimiokines, mais pour 50 % seulement. Nous pensons que les anticorps anti-VIH présents dans ces échantillons sont responsables de l'autre moitié de l'effet.

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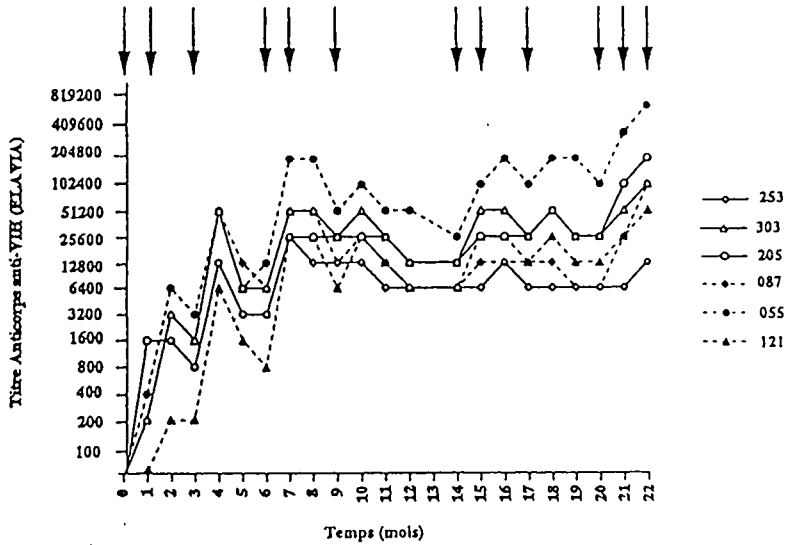

FIG. 3 — Évolution des anticorps anti-VIH au cours de l'immunisation des singes.
Les taux d'anticorps ont été mesurés par ELISA (kit ELAVIA, Sanofi Diagnostic Pasteur) dans les sérums des singes n° 205, 253 et 303, immunisés avec du virus infectieux, et des singes n° 055, 087 et 121, immunisés avec du virus inactivé (voir Tableau 1). Les flèches indiquent les dates d'injection.

Mise en évidence d'anticorps facilitant la fusion

Dans la perspective d'induire des anticorps capables de neutraliser l'infectivité des isolats primaires du VIH-1, deux groupes de trois macaques ont été immunisés de façon répétée, l'un avec du VIH-1 BX08 pleinement infectieux (le VIH-1 ne se multiplie pas chez le singe rhésus), l'autre avec du virus inactivé à la bêta-propiolactone ou au formol (Tableau 1). Les animaux ont développé des anticorps anti-VIH en réponse aux 4 premières injections de virus, mais les injections suivantes n'ont été suivies que de peu d'effet si l'on en juge d'après l'évolution des titres d'anticorps mesurés par ELISA (Fig. 3). Paradoxalement, les sérums des animaux immunisés, loin de neutraliser le stock de VIH-1 BX08, se sont au contraire avérés capables d'augmenter jusqu'à plus de 90 fois le titre infectieux du virus mesuré sur PBMC (Tableau 3).

Nous avons étudié l'effet de ces sérums dans le test de fusion induite par le SFV-*env*BX08 en culture de cellules P4P. Chaque sérum a été testé à la dilution du 1 : 10 en présence du mélange d'anticorps monoclonaux anti-RANTES, anti-MIP-1alpha et anti-MIP-1 bêta à 10 µg/ml. Comme le montre le Tableau 3, les sérums des macaques immunisés amplifient de façon considérable la formation des syncytia. Le nombre de ceux-ci augmente de 70 % en présence du sérum de plusieurs des

TABLEAU 3 — Comparaison de l'effet de facilitation de plusieurs sérums de macaques sur la fusion induite par la gp160 du virus BX08 en cellules P4P et sur l'infectivité d'un stock de VIH-1 BX08 en PBMC.

[a] : mois d'immunisation,

[b] : Facteur d'augmentation du titre infectieux du virus à la dilution du sérum indiquée entre parenthèses,

[c] : Pourcentage d'inhibition de la formation de syncytia mesuré en cellules P4P infectées par SFV-*env*BX08 en présence du sérum indiqué dilué au 1/10.

Sérums	Dates[a]	Neutralisation	Facilitation[b]	Inhibit ion de la fusion[c]
303	0	<10	-	-13
	17	<10	70 (1/80)	-73
	18	<10	10 (1/10)	+13
253	0	<10	-	-13
	17	<10	70 (1/10)	-27
	18	<10	60 (1/20)	-33
087	0	<10	-	-13
	17	<10	40 (1/10)	-67
	18	<10	90 (1/10)	-100
055	0	<10	-	-6
	17	<10	40 (1/10)	-80
	18	<10	20 (1/10)	-113

animaux. L'augmentation atteint et dépasse même 100 % pour deux des sérums prélevés en fin d'immunisation (macaques 055 et 087). Tous les sérums immuns étudiés montrent donc une activité facilitant la formation de syncytia médiée par la gp160 du VIH-1 BX08 et une activité facilitant l'infection des PBMC par le virus. Il est cependant intéressant de noter la discordance entre l'effet facilitant du sérum du singe 303 (saignée M 18) sur l'infectivité du VIH BX08 et son absence d'effet marqué sur la formation de syncytia. Par ailleurs, ce phénomène n'est pas restreint à l'isolat BX08 car les mêmes sérums ont démontré aussi une activité facilitante sur la multiplication de deux autres isolats primaires, BX17 et BX26, à des taux de 15 fois et de 5 fois, respectivement (Résultats non présentés).

DISCUSSION

Le phénomène de facilitation de l'infection virale par des anticorps a été décrit pour plusieurs virus, et notamment celui de la dengue [55], où l'on admet qu'il serait responsable du syndrome de la dengue hémorragique avec choc (*DHSS*). On a associé le phénomène à la présence, chez l'individu infecté, d'anticorps antiviraux de

faible affinité qui se fixeraient à la surface de la particule virale sans la neutraliser et l'attireraient au contact de la membrane de la cellule cible, notamment des macrophages, par interaction directe de leur portion Fc avec les récepteurs FcR de la cellule, ou par interaction indirecte avec le complément et le récepteur de ce dernier. L'augmentation d'infectivité du virus de la dengue qui en résulte, ou ADE (« *antibody-dependent enhancement* ») se traduit dans les tests de séroneutralisation par l'augmentation du titre du virus en présence de certaines dilutions du sérum du patient [56]. Il semble que la facilitation de l'infection des macrophages par des anticorps spécifiques soit une constante dans la famille des flavivirus [57]. Ainsi, dans les modèles murins de l'infection par certaines souches de virus de la fièvre jaune, le transfert passif d'anticorps monoclonaux se traduit par une évolution plus rapide de la maladie [58]. Cependant, on pense aujourd'hui que les mécanismes physiopathologiques qui conduisent au développement de la dengue hémorragique sont aussi en partie sous la dépendance de facteurs viraux déterminant la dynamique de réplication du virus ou l'émergence de variants particulièrement agressifs [59, 60] et sous celle de facteurs de l'hôte tels que la production massive de TNF-alpha [61]. Par ailleurs, bien qu'une activité facilitante ait été retrouvée *in vitro* chez les vaccinés, aucun phénomène délétère majeur n'est apparu après la vaccination de millions d'individus contre la fièvre jaune ou l'encéphalite japonaise [62].

Des phénomènes de facilitation attribuables à une immunisation spécifique préalable ont également été décrits pour d'autres familles de virus. La vaccination des enfants contre le virus respiratoire syncytial, avec du vaccin inactivé au formaldéhyde, augmente le risque de développer une infection sévère [63]. Comme pour le virus de la dengue, c'est la facilitation de l'infection du macrophage par des anticorps spécifiques qui semble ici en cause [64]. Le phénomène de facilitation se retrouve dans l'infection du chat par le virus de la péritonite infectieuse féline, et on a incriminé là encore la facilitation de l'infection du macrophage par les anticorps spécifiques [65-67].

La facilitation de l'infection par les anticorps et/ou la réponse immune a été clairement mise en évidence pour les lentivirus des animaux. Une augmentation de la sévérité de la maladie induite chez le cheval par le virus de l'anémie infectieuse équine (EIAV) a été rapportée chez les animaux vaccinés. En fait, si l'immunisation par du virus inactivé protège de l'infection expérimentale avec des souches virales homologues et hétérologues, en revanche, la vaccination à l'aide d'une protéine recombinante de l'enveloppe produite dans le système baculovirus aboutit, après l'injection d'épreuve, au développement d'une maladie plus sévère chez les animaux vaccinés que chez les animaux témoins [68].

Des phénomènes similaires ont été décrits chez la chèvre, lors de la vaccination contre le virus de l'arthrite et encéphalite caprine (CAEV) [69] et dans le cas du virus VISNA du mouton. De même, la vaccination des chats contre le virus de l'immunodéficience féline (FIV) par des virus inactivés ou des protéines recombinantes ou par de l'ADN nu codant pour les protéines d'enveloppe du FIV se traduit, après injection d'épreuve, par une augmentation de la charge virale [70].

Le phénomène de facilitation a aussi été décrit dans le cas de l'infection *in vitro* à VIH-1 [20, 21]. On a pu le reproduire avec des anticorps monoclonaux, ce qui a permis de montrer qu'il pouvait être engendré aussi bien par des anticorps anti-gp41 [26, 71] que par des anticorps anti-gp120, notamment les anticorps dirigés contre la boucle V3 de la gp120 [27-29, 72]. Le même anticorps monoclonal peut d'ailleurs s'avérer neutralisant vis-à-vis d'une souche de VIH-1 donnée et facilitant pour d'autres [27, 29]. Les résultats décrits dans cet article montrent que nous avons détecté le phénomène de facilitation avec des sérums des macaques immunisés de façon répétée avec les virions purifiés de l'isolat primaire VIH-1 BX08. Les sérums des singes immunisés avec le VIH-1BX08 ont montré en effet une activité de stimulation de l'infectivité du virus BX08 pour les PBMC pouvant atteindre 90 fois. Les mêmes sérums ont été capables d'augmenter significativement le nombre de syncytia formés en cultures de cellules HeLa $CD4^+$ $CCR5^+$ par la gp160 du VIH-1 BX08. Après purification sur colonne de protéine A, l'effet facilitant a été retrouvé dans la fraction du sérum contenant les immunoglobulines (résultats non publiés), ce qui montre que l'activité facilitante est bien due à des anticorps.

La corrélation entre facilitation de l'infection virale et facilitation de la formation de syncytia n'est pas absolue, puisque certains sérums ont montré une activité facilitante nette sur l'infectivité du VIH-1 BX08 mais sont restés sans effet sur la formation de syncytia (singe 303 par exemple). Dans l'ensemble néanmoins, les sérums des macaques qui se sont avérés facilitants dans le test de séroneutralisation se sont avérés aussi facilitants dans le test de formation de syncytia.

Dans notre étude, le virus pour lequel la facilitation paraît la plus marquée est l'isolat primaire BX08, le même que celui utilisé comme immunogène, mais un effet facilitant a aussi été retrouvé vis-à-vis de 2 autres isolats primaires de séquence V3 différente [34]. Kostrikis et *al.* [73] ont montré que les sérums de patients infectés par le VIH-1 pouvaient être facilitants vis-à-vis de certains isolats primaires, neutres vis-à-vis d'autres isolats et neutralisants vis-à-vis d'un 3^e groupe. Ces résultats laissent présumer que les activités neutralisantes et facilitantes pourraient être virus-spécifiques plutôt que sérum-spécifiques [72].

Il est intéressant de constater que le test d'inhibition de la formation de syncytia en cellules P4P paraît plus sensible que les tests de séroneutralisation de l'infectivité du VIH. Ainsi, la formation des syncytia peut être bloquée par des anticorps monoclonaux non neutralisants [37]. Par ailleurs, les anticorps bloquant la fusion semblent apparaître plus précocement que les anticorps neutralisants au cours de l'infection chez l'homme (Tableau 2) et il semble en aller de même au cours de l'immunisation du lapin ou du singe avec des préparations de glycoprotéines d'enveloppe (F. Verrier, D. Brand et B. Rovinski, résultats non publiés). L'explication de cette différence de sensibilité pourrait être d'ordre technique. En effet, les anticorps du sérum à tester restent présents dans la culture de cellules pendant toute la durée du test de formation des syncytia, alors qu'il ne sont en contact avec le virus que pendant une heure dans le test de séroneutralisation. A supposer qu'un anticorps entraîne une diminution de 2 fois (50 % d'inhibition) du nombre de syncytia formés en culture de

cellules P4P, on comptera 750 syncytia par puits au lieu de 1 500 dans les puits témoins, ce qui se détectera aisément et représentera une inhibition significative, alors que dans le test de séroneutralisation, une diminution de 2 fois du nombre de particules infectieuses dans l'inoculum de départ n'aura pas d'effet significatif sur le titre du virus qu'on calculera d'après la positivité des puits de culture en antigène p24 au bout de 7 jours d'incubation.

Le test de fusion intercellulaire médiée par des réplicons SFV-*env* est donc un test intéressant. Il permet, non seulement de détecter des anticorps potentiellement neutralisants, mais aussi des anticorps facilitants. La mise au point d'un test quantitatif pouvant être automatisé est en cours.

Notons pour terminer que l'activité facilitante des sérums de macaques immunisés avec les virions BX08 a été étudiée seulement *in vitro*. Trois des animaux de cette étude ont été ultérieurement éprouvés par injection intraveineuse d'un virus hybride d'immunodéficience simienne-humaine (SHIV) non pathogène pour le macaque (SHIVSbg). Les animaux n'ont montré aucun symptôme particulier et leurs charges virales n'ont pas été différentes de celles des témoins (article en préparation). Le rôle délétère que les anticorps facilitants pourraient éventuellement jouer *in vivo* dans la rapidité d'évolution de l'infection à VIH ou dans la pathogenèse de la maladie n'a donc pas été mis en évidence dans cette expérience. Il nécessitera toutefois d'être évalué très rigoureusement.

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DISCUSSION

M. Charles PILET

Je me réjouis de ce qu'un ancien élève de l'École d'Alfort soit devenu l'un des principaux responsables de la recherche sur le vaccin sida. Je souhaiterais vous demander quels sont les meilleurs adjuvants actuellement utilisés avec les vaccins VIH d'une part, et quels sont, d'autre part, les résultats obtenus chez l'individu infecté par le VIH grâce aux immunostimulants ?

Les seuls adjuvants autorisés dans les vaccins à usage humain sont, comme vous le savez, les sels d'aluminium. Ils ont tendance à favoriser les réponses de type Th2 (immunité humorale). D'autres adjuvants n'ont pas ce défaut (dérivés de saponines, émulsions huile dans l'eau...). On les a expérimentés avec succès chez des volontaires dans des études de phase I.

M. Michel BOUREL

Le macaque demeure-t-il le primate non humain le plus adapté aux études préliminaires d'un processus de vaccination anti-VIH ? Existe-t-il d'autres animaux-modèles et quelle expérience découle des vaccinations sur volontaires humains ? Les anticorps facilitants dont il a été question s'accompagnent sans doute de l'émergence de molécules d'adhésion ; quelle action ont-ils sur les cultures de moelle et l'éventuelle formation de colonies ?

Le seul animal sensible au VIH est le chimpanzé, mais il se comporte comme un porteur sain asymptomatique et ne développe pas de sida. Le macaque est sensible au virus de l'immunodéficience simienne (SIV) qui est assez proche du VIH-2, et aux virus hybrides simiens-humains (SHIV) qui sont des virus simiens dans lesquels on a substitué le gène de l'enveloppe du SIV par celui de l'enveloppe d'un VIH. Le macaque développe un sida en tous points identique à celui de l'homme et constitue donc un modèle de choix. Le chat infecté par le virus de l'immunodéficience féline développe, lui aussi, un sida. Les souris SCID humanisées constituent un autre modèle intéressant. Nous n'avons pas étudié le rôle éventuel des anticorps facilitants dans l'émergence de molécules d'adhésion, ni leur effet sur des cultures de moelle.

M. Jacques-Louis BINET

Qu'en est-il des anticorps facilitants chez les patients atteints d'infection à VIH ? Leur taux est-il en rapport avec l'évolutivité et la gravité de la maladie ? Le taux d'anticorps varie-t-il sans trithérapie ?

Il existe plusieurs descriptions de mise en évidence d'anticorps facilitants chez les patients atteints d'infection à VIH, mais il ne semble pas que leur taux varie de manière systématique avec l'évolution de la maladie. Leur rôle dans la pathogenèse du sida n'est donc pas, en fait, démontré.

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M. Jacques FROTTIER

Quelle serait, à ce jour, en fonction de vos recherches et de votre grande expérience en matière d'immunisation vis-à-vis du VIH, la meilleure approche pour aboutir, à moyen terme, à cette vaccination préventive chez l'homme ?

L'idéal, pour un vaccin, serait d'induire des anticorps neutralisants actifs contre les souches sauvages (« isolats primaires ») du VIH et une réponse cytotoxique (CTL) pluriépitopique contre plusieurs antigènes du virus (Env, Gag, Pol, etc.). C'est ce qu'on cherche à faire avec les régimes de vaccination combinés où l'on injecte successivement un vaccin vivant recombinant, utilisant un poxvirus pour vecteur, pour induire une réponse cellulaire, puis un vaccin à base de glycoprotéine virale (gp 120) purifiée pour induire la réponse humorale. Malheureusement, les anticorps que l'on obtient jusqu'ici, ne sont pas capables de neutraliser les souches de virus sauvages, et l'on n'enregistre de réponses CTL que contre un petit nombre d'antigènes viraux, chez un nombre limité (30 à 50 %) de volontaires. On cherche donc à modifier la conformation de la glycoprotéine pour qu'elle expose efficacement ses sites critiques de neutralisation et on cherche à mettre au point de nouvelles formulations vaccinales (lipopeptides, nouveaux vecteurs viraux ou bactériens, ADN vaccins) pour induire des réponses cellulaires plus efficaces. La combinaison ADN — poxvirus recombinant semble, par exemple, assez prometteuse. On voit mal cependant comment on pourra utiliser, en pratique courante, des combinaisons de plusieurs vaccins, surtout pour des campagnes de vaccination dans les pays en développement. Une toute nouvelle approche a été décrite récemment. Elle consiste à utiliser pour vaccin des protéines non structurales du VIH, notamment la protéine transactivatrice Tat. Cependant on manque encore de recul pour juger la portée réelle de cette observation.

M. Jacques EUZÉBY

Le phénomène de facilitation que vous avez observé en matière d'immunité à médiation humorale existe aussi en matière d'immunité à médiation cellulaire : le fait a été signalé dans le cas des essais de vaccination du chien contre Leishmania infantum.

On peut tout à fait concevoir l'existence de phénomènes de suppression, par exemple de délétions clonales du répertoire, pour expliquer cela. Dans le cas que nous présentons, nous avons montré qu'il s'agissait d'immunoglobulines G induites en réponse à l'hyperimmunisation des animaux avec le virus entier.

M. Guy de THÉ

Pourriez-vous résumer, pour nos confrères, les données expérimentales principales et les espoirs chez l'homme, des vaccins ADN ?

Les ADN vaccins ont pour avantage la simplicité de leur conception, de leur production et de leur purification. Ils sont stables et bon marché. Leur immunogénicité est excellente chez les petits rongeurs, surtout avec certains antigènes (hémaglutine du virus grippal, par exemple). Cela est lié, en partie, au rôle adjuvant de certaines séquences nucléotidiques (CpG) qui jouent le rôle de signal d'alarme pour le système immunitaire. Cependant, lorsque l'on passe aux primates, on s'aperçoit que l'efficacité des ADN vaccins est

nettement moins marquée. Ainsi, les réponses CTL chez le macaque sont d'intensité faible et de courte durée et les taux d'anticorps sont souvent à la limite de la détection, même si l'on utilise des doses d'ADN de plusieurs milligrammes. Plusieurs procédés sont à l'étude pour tenter d'augmenter l'immunogénicité de l'ADN chez les primates, par exemple, en en humanisant les codons, ou en lui ajoutant des gènes de cytokines (GM-CSF, IL-12, IFN-γ) qui vont jouer le rôle d'adjuvant immunologique, ou en le formulant sous forme de plasmides dans des bactéries (*Salmonella*) à multiplication intracellulaire. On peut aussi coupler la présentation ADN avec l'emploi d'un système viral autoréplicatif (réplicons Sindbis ou Semliki, par exemple), ce qui va permettre l'amplification du message au sein des cellules transfectées. L'intérêt des vaccins ADN est leur propension marquée à induire des réponses de type Th1 (immunité cellulaire), mais ils ne semblent pas présenter, à l'heure actuelle, d'avantages particulièrement décisifs par rapport à bien des vaccins vivants recombinants.