The Oncolytic Properties of Two Newcastle Disease Virus Strains.

By Nienke-Nanje Kriek

Submitted for the degree M.Med.Sc. in the Department Hematology and Cell Biology in the Faculty of Health Sciences at the University of the Free State.

November 2003

Promotor: Prof. G.H.J. Pretorius.
Co-promotors: Prof. R.R. Bragg & Prof. H.S. Cronje.

For Evan.

For your patience and grace.

TABLE OF CONTENTS

ABBREVIATION LIST

ACTH	adrenocorticotropic hormone
AFP	alpha feto protein
ASI	active specific immunotherapy
ASIR	age standardized incidence rate
ATV	autologous tumour cell vaccine
BMI	body mass index
CAM (project)	complementary and alternative medicine project
CC	cervical cancer
CDV	canine distemper virus
CIN	cervical intraepithelial neoplasia
CR	complete regression
CRT	chemoradiotherapy
dsRNA	double-stranded RNA
DTH	delayed-type-hypersensitivity
EAC	esophageal adenocarcinoma
EBRT	external beam radiotherapy
EC	esophageal cancer
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ESCC	esophageal squamous cell carcinoma
F	fusion protein
FDA	Food and Drug Administration
FFWI	fusion from within
FFWO	fusion from without
GCP	good clinical practice
GERD	gasto-esophageal reflux disease
HAD	high antigen density
HDR	high dose rate
HIV	human immunodeficiencyvirus
HN	hemagglutinin neuraminidase protein
HPV	human papillomavirus
HSV	herpes simplex virus
ICPI	intracerebral pathogenicity index

IFN	interferon (-α & -β or -A & -B)
ILBT	intraluminal brachytherapy
IP	intraperitoneal
IRES	internal ribosome entry site
IRF	interferon regulatory factor (genes / proteins)
IT	intratumoural
IU	infective unit
IVPI	intravenous pathogenicity index
L	large protein
LAD	low antigen density
M	matrix protein
MAb	monoclonal antibody
mCa	micro-invasive cervical carcinoma
MDT	mean death time
MHC	major histocompatibility complex
MSI	microsatellite instability
MTP	microtitre plate
MVM	minute virus of mice
N	nucleocapsid protein
NCI	National Cancer Institute (of the United States of America)
NDV	Newcastle disease virus
P	polymerase-associated / phosphoprotein
Pap	Papanicolau (smear test)
PAP	pokeweed antiviral protein
PBL	peripheral blood leukocytes
PBMC	peripheral blood mononuclear cell
PDV	phorcine distemper virus
PFU	plaque forming unit
PKR	protein kinase RNA
PMV	plasma membrane vesicle
pRb	retinoblastoma tumour suppressor protein
PSA	prostate specific antigen
RT-PCR	reverse transcription polymerase chain reaction
SA	South Africa(n)
SCC	squamous cell carcinoma
SIL	squamous intraepithelial lesion

TAA	tumour associated antigen
TF	transcription factor
TNF	tumour necrosis factor
VSV	vesicular stomatitis virus
wt	wild-type

CHAPTER 1

1.1. INTRODUCTION

1.1.1. General

Treatment of cancer patients with live attenuated viral vaccines, resulting in tumour regression, is well described (*Csatary & Gergely, 1990*; *Csatary et al., 1999 (b)*; *Cassel & Garrett, 1965*; *Nemunaitis, 2002*; *Pecora et al., 2002*). Several authors have also described cases of tumour regression while the patients had viral infections such as mumps or measles (*Russel, 2002*). A recent renewal of interest, as well as optimism in the field of oncolytic virology occurred due to a confluence of ideas from molecular oncology and virology. This has lead to new research into the development of novel virus-based therapeutics for the treatment of cancer (*Norman et al., 2001*).

Considering that viruses have spawned their fair share of misery throughout the history of mankind and the fact that cancer has an equally grim track record, it seems almost unbelievable that we can recruit one scourge against another (*Pennisi, 1998*).

1.1.2. A brief history of oncolytic virology

The beneficial effects of bacterial and / or viral infections on the progress of malignant disease have been demonstrated repeatedly in the past. The Italian physician De Pace made one of the earliest recorded observations in 1912: regression of cervical carcinoma in patients undergoing Pasteur's treatment for rabies. In the 1950's it became apparent that, *in vitro*, many viruses infect and lyse tumour cells more readily than normal cells.

The NCI produced some of the most illustrative results in a study that was conducted in 1954: 30 patients suffering from locally advanced cervical carcinoma were treated with ten different serotypes of wild-type human adenovirus to which the patients, where possible, had no neutralizing antibodies. The virus was administered directly intra-tumourally (IT) or intra-arterially (systemically), or in a combination of these two methods. Corticosteroids were co-administered in roughly half of the cases in order to suppress the patients' immune systems. The results were very promising: two thirds of the patients responded well with

liquefaction and ulceration of the treated tumour mass, while none of the control patients (treated with either virus-free tissue culture supernatant or heat-inactivated virus) responded. No serious side effects were observed. Up to 1975, a large number of oncolytic viruses were studied in cancer patients. These include adeno-, mumps-, measles-, bovine entero-, Egypt 101 -, West Nile -, Ilhéus - and Bunyamwera viruses, as well as NDV and attenuated HSV (*Nemunaitis, 2002*).

The reasons why virotherapy was abandoned as a cancer treatment, despite some good research results, include the following:

(i) Only a few clinical responses were ever reported. In addition, most of the patients in these clinical trials had end-stage cancers with life expectancies of less than three months.

(ii) Effects of the viruses on the patients' bodies were unpredictable.

(iii) The development of more active chemotherapeutic agents excelled and replaced virotherapy.

(iv) There were, in the early stages of virotherapeutic research, insufficient methods for large-scale production of high-titre purified virus and / or quantitation of its biological activity.

However, renewed interest in this field was sparked recently by experiments that showed genetically modified lytic viruses to be cancer-selective (*Wildner, 2001*).

Virotherapy was demonstrated as one of the potential new colorectal cancer therapeutics that are in the early stages of clinical testing in response to promising preclinical data. These demonstrations also included treatments such as immune system stimulation and specific gene therapy (*Chen et al., 2001*).

The treatment of human cancer with a live virus is, however, still constrained by issues of efficacy, safety, public health and the potential risk(s) of germ-line transmission. This leads us to preferably use viruses with low pathogenicity that are already prevalent in the human population, while still able to replicate efficiently in the target tissue (*Wildner, 2001*). Table 1.1. summarizes the characteristics that an oncolytic virus preferably should and should not have.

Table 1.1.

The ideal characteristics of an oncolytic virus: safety *vs.* oncolytic potency.

SAFETY	ONCOLYTIC POTENCY
Minimal mutagenicity, teratogenicity and carcinogenicity. [a]	Short lytic life cycle. [a]
Availability of clinically approved antiviral treatment or incorporation of prodrug-suicide system allowing termination of viral replication and spread. [a,b]	High burst size. [a]
Minimal genetic instability and likelihood of generating wild-type revertants. [a]	Efficient spread throughout the tumour, as well as efficient lateral spread. [a,b]
Already prevalent in the human population. [a]	No pre-existing immunity in the human population. [a]
A preference for replication in tumour cells *vs* normal cells. [a,b]	High infectivity in a wide range of tissues. [a]
Disease caused by the parental wild-type virus should be mild. [a]	Capacity of avoiding early detection and eradication by the immune system. [a,b]
Genetic structure of the virus and the functions of its gene products should be well characterized. [a]	High physical stability. [a]

a = (Wildner, 2001)

b = (Fueyo et al., 1999)

1.1.3. Basic ways of employing viruses in cancer treatment

Several different ways of using viruses in the targeting and treatment of cancer have already been developed so that virotherapy seems to represent a new avenue of potential treatment (*Pennisi, 1998*).

1.1.3.1. Naturally oncolytic viruses

An oncolytic virus can be defined by its selective infection and destruction of cancer cells, while not infecting or affecting normal cells (*Stanziale & Fong, 2003*). For most oncolytic RNA viruses, tumour specificity is either a natural characteristic of the virus, or it could be a serendipitous consequence of the virus adapting to propagate in human tumour cell lines when cultured in this manner (*Russel, 2002*). In contrast with viral vectors, the secret of a naturally oncolytic virus is precisely their ability to replicate and spread, killing off the cancer cells (*Pennisi, 1998*).

The virus can operate according to one of two principles:

(i) either by directly attacking and lysing tumour cells, or

(i) by indirectly triggering a host immune response against the tumour cells (*Pennisi, 1998*).

Most of the excitement around this concept is not even so much the fact that studies show tumour shrinkage and improved results when used in conjunction with other therapies, but simply the fact that the cell-killing agents are so curiously selective and do not seem to cause any serious side-effects (*Pennisi, 1998*).

1.1.3.2. Genetically altered oncolytic viruses

Advances in virology and molecular biology techniques allow the manipulation of viral genomes to attenuate their pathogenicity and modify their life cycles in order to allow tumour specific viral replication. Currently, adenovirus and Herpes Simplex virus (HSV) type I mutants are the most commonly employed altered oncolytic viruses, since clinical trials have shown them to be safe and efficient. The most popular way of employing these viruses in practice, however, is in combination with radio- or chemotherapy in order to achieve an additive antineoplastic effect (*Suzuki & Curiel, 2001*). Table 1.2. summarizes the viral strains (natural and altered) that are currently being used as oncolytic agents, as well as their mechanisms of tumour-selective propagation.

1.1.3.3. Viral vectors

Oncolytic viruses, whether they are natural or genetically manipulated, are not to be confused with the viruses that are employed in another type of cancer therapy where the virus itself is only used as a vector for the transfer of therapeutic genes into cancer cells. In that kind of therapy, the transformed cells' genetic errors are corrected by masking the underlying uncontrolled cell growth signals that result from cancerous mutations. Here it is important that the virus vector is "disarmed" to keep it from replicating and spreading to normal cells, where no mutation has to be masked and overexpression of a correct gene could be disastrous (*Pennisi, 1998*).

Table 1.2.

Virus strains currently employed as oncolytic agents.

Oncolytic agent	Basis of tumour-selective propagation	Therapeutic traits
Reovirus		
Reovirus	Replication is dependent on activation of the Ras signaling pathway.	Oncolysis.
Autonomous parvovirus		
B19, H-I, MVM	None: parvovirus replication depends on cellular function during the S phase.	Oncolysis.
NDV derivatives		
73 T	Unknown mutation generated by serial passage on tumour cells.	Oncolysis.
MTH-68	Unknown mutation: attenuated veterinary NDV vaccine.	Oncolysis.
Poxvirus		
Vaccinia	None.	Oncolysis (and expression of cytokines or recombinant tumour vaccines).
Poliovirus derivatives		
PV1(RIPO)	Attenuated neurovirulence as a result of exchange of IRES.	Oncolysis.
VSV		
VSV-Indiana	Preferential replication in tumour cells that have lost interferon responsiveness.	Oncolysis.
Adenovirus derivatives		
*dl*1520	E1B-55K-deletion abrogates p53 binding.	Oncolysis.
FRG	E1B-55K-deletion abrogates p53 binding.	Oncolysis and suicide gene therapy (CD + TK).
Ad.TK^{RC}	E1B-55K-deletion abrogates p53 binding.	Oncolysis and suicide gene therapy (TK).
AdvE1AdB-F/K20	E1B-55K-deletion abrogates p53 binding.	Oncolysis with enhanced infectivity.
Ad.OW34	None.	Oncolysis (enhanced compared with Ad.TK^{RC} as a result of the expression of E1B-55K) and suicide gene therapy (TK).
AdD24	E1A-deletion abrogates pRb binding.	Oncolysis.
CV706	Regulation of E1A under the PSA promotor.	Oncolysis.
CN787	Regulation of E1A under rat probasin promotor . Regulation of E1B under human PSA promotor.	Oncolysis.
AvE1a041	Regulation of E1A under the AFP promotor.	Oncolysis.
dl337	None.	Oncolysis (enhanced as a result of E1B-19K deletion).
*dl*316	Complete deletion of E1A makes this mutant dependent on intrinsic IL-6-induced E1A-like activity.	Oncolysis.
HSV-1 derivatives		
*D*lsptk	Replication-restricted, thymidine kinase-negative mutant.	Oncolysis (lacking sensitivity to acyclovir and ganciclovir).
hrR3	Mutation of ribonucleotide reductase.	Oncolysis.
HSV-1716	Neuroattenuated ICP34.5 gene mutant.	Oncolysis.
G207	Lacking ICP34.5 and ribonucleotide reductase minimizing generation of wild-type revertants.	Oncolysis.

(Wildner, 2001)

1.1.3.4. Combination

Viral infection where the virus not only lyses the tumour cells, but also makes these cells more susceptible to radiation or chemotherapy, thereby delivering a "double blow" to the tumour, is a further development in viral oncolytic therapy. One such example involves a recent event in the development of HSV as an oncolytic virus, where a rat cytochrome P450 gene was added to the genome of a herpes virus. The resulting enzyme converts cyclophosphamide (a drug that is currently used for cancer chemotherapy) to its active form. This means that, as the virus spreads through the tumour, it does not only kill the cells directly, but also makes them susceptible to additional chemotherapeutic drugs, increasing the chance that all cancer cells will be destroyed. This research has been applied in pre- and clinical testing (*Eiselein et al., 1978*).

1.1.4. General human anti-viral immune response

Viruses have long been notorious for causing persistent infections in man. Some viruses have developed various clever strategies in order to evade attack and elimination by the immune system. One good example can be found in the type C adenoviruses Ad2 and Ad5: the early region (E3) encodes a 19 K glycoprotein that associates with the host's class I MHC heavy chain in the ER, thus preventing the transport of class I MHC protein products to the cell surface. The host's CD8 T cell mediated immunity is therefore decreased, increasing the chances of virus survival. It has also been shown that one or more gene(s) within this E3 region can protect the infected cell against cytokine-mediated apoptosis (*Wildner, 2001*). As an avian virus, NDV has no such escape mechanisms in mammalian cells (*Washburn & Schirrmacher, 2002*).

In order to control the replication of a large variety of lytic and non-lytic viruses upon infection of a cell, the cell produces a spectrum of early inflammatory proteins including **interferons** (**IFNs**). These proteins fight viral infection by activating immune cells and directly inhibiting viral replication (*Barnes et al., 2001*).

1.1.5. Anti-viral immune response in cancer

The ability to propagate selectively in tumour cells is any oncolytic virus' most important characteristic. For DNA viruses, this specificity is often determined at gene transcription level, where it is dependent on the interactions between host cell nuclear transcription factors (TFs) and its own viral promotor / enhancer elements. However, RNA viruses have alternative mechanisms for "choosing" tumour cells to replicate in and to control the spread of its progeny virions:

(i) specific receptors for viral entry or cell-to-cell fusion may play a role,

(ii) the specific activity of a viral IRES,

(iii) dsRNA stimulates the protein kinase PKR, which inhibits cellular protein synthesis and promotes apoptosis, and finally,

(iv) dsRNA also stimulates the production if IFNs, which activate PKR in neighboring cells, protecting them from viral infection.

Since tumours are often defective in their PKR signaling pathway, they are top candidates for hosts of RNA virus infections (*Russel, 2002*).

It has been shown that, if the host has prior immunity to replication competent adenovirus or HSV, the level of gene transfer and expression within a tumour may be altered. This prior immunity does not, however, affect the overall antitumour effects of these viruses when administered intraneoplastically. Various methods for xenogenization were suggested based on the assumption that generally, under normal circumstances, tumour cells have a limited ability to trigger an immune response. Some of these methods involve the infection of tumour cells with viruses. Confirmative evidence that intratumoural injection of an oncolytic virus can result in regression of untreated lesions and protection from delayed rechallenge with tumour cells, suggested that treatment of a local tumour with such an oncolytic virus might be a suitable "*in situ* vaccination" strategy for the induction of a specific antitumour immunity (*Wildner, 2001*).

1.2. CANCER OF THE CERVIX

1.2.1. Cervical cancer in SA

Roughly 80% of the 500 000 cases of cervical cancer (CC) diagnosed worldwide each year come from developing countries such as ours (*Jain, 1996*). One SA study suggests that SCC of the cervix is found in 90% of our CC patients (*Lomalisa et al., 2000*).

While the total ASIR (*Hao et al., 1999*) for cancer in South Africans is lower in the Black population than in the White population, the CC incidence rate in Black SA women is higher than in White SA women (35.0 *vs* 11.7 per 100 000) (*Walker et al., 2002*). CC also seems to manifest at a younger age in Black women when compared to White women: the mean age of Black women from Durban diagnosed with CC is 52, while for White women it is 58 (*Walker et al., 2002*).
Interestingly, in the USA, African-American women (who are originally from mainly West Africa) have an ASIR of twice that of White US women (*Walker et al., 2002*).

CC is one of the leading causes of death among female SA cancer patients and is the cause of very low quality of life and high psychiatric morbidity (*Nair, 2000*): in the 1980's in the Transkei, the second most common cancer diagnosed among females was cervical cancer (*Jaskiewics et al., 1987*).

1.2.2. Risk factors

Risk factors elucidated for Black patients are mostly similar to those for White patients: **early age at first intercourse** and **numerous sexual partners** are major risk factors, while a previous history of **sexually transmitted diseases**, **high parity** and a **lack of knowledge** are less consistent risk factors (*Walker et al., 2002*). **Human Papillomavirus** (HPV) associated cancers are generally more prevalent in developing countries. Of these, CC is most strongly associated with HPV. This calls for the development of an inexpensive HPV vaccine (see section 1.2.3) for women who do not have access to screening programmes and are therefore at increased risk of developing CC (*Williamson et al., 2002*).

It has also been found that **Human Immunodeficiency virus** (HIV) -positive patients present with invasive CC 10 to 15 years earlier than their HIV-negative counterparts (*Lomalisa et al., 2000*; *Moodley et al., 2001*). A very low CD4 cell count is also strongly associated with advanced-stage CC (*Lomalisa et al., 2000*). Invasive CC is also found more frequently in HIV-positive patients than in HIV-negative patients (*Moodley et al., 2001*).

A study conducted in Lesotho showed that Black women from that area who consumed **indigenous alcohols** have an even more significant risk of developing CC than those who consume **tobacco** (in the form of cigarettes, pipe or snuff) (*Martin & Hill, 1984*).

Statistics suggest that a small number of CC patients have a familial susceptibility for CC and probably other HPV-related neoplasms, but concrete evidence of specific genetic polymorphisms has yet to be established (*Horn et al., 2002*). One genetic factor that might predispose an individual to CC development in the absence of a high-risk HPV strain infection, is the Arg allele of p53 codon 72, although its significance and role in CC and CC development are not yet clarified (*Pegoraro et al., 2002*).

1.2.3. Cervical cancer: from prevention to treatment

A number of HPV genes can manipulate cell cycle control to promote viral persistence and replication, but E6 and E7 encode the virus' main transforming proteins, which are responsible for host cell immortalization and neoplastic or malignant transformation (*Brenna & Syrjänen, 2003; Finzer et al., 2002*). Viral integration into the host genome leads to disruption of the HPV E2 gene, which lifts its suppression from the expression of the E6 and E7 genes, leading to unregulated increases of these proteins' concentrations. These two oncoproteins in turn inactivate products of the tumour suppressor genes p53 and Rb. These tumor suppressors would usually regulate the cell cycle and cellular response to DNA damage, initiation of DNA repair, apoptosis induction and cell differentiation (*do Horto dos Santos Oliveira et al., 2003*). *P. Finzer et al* discussed the different ways that E6 and E7 can modulate the cell's natural apoptotic antiviral host defense, ensuring survival of the virus (*Finzer et al., 2002*). Figure 1.1. shows the pathway by which HPV infection could cause cancer.

In recognition of the causal role of HPV infection in CC, prophylactic strategies that are currently under investigation focus on the induction of effective humoral and cellular immune responses in the form of **HPV vaccines**, that would potentially be protective against subsequent HPV infection. Application of such vaccines could diminish the costs of existing CC screening programs and reduce morbidity and mortality associated with these neoplasias and their conventional treatments (*Steller, 2002*).

Early cellular changes that lead to CC development can be detected with **a Papanicolaou smear test**, so that these early lesions could be treated. In more developed countries, this is a successful screening method, but resource constraints (and other factors) in our country cause CC to be generally diagnosed at advanced stages, which are synonymous with untreatability (*Jain, 1996*). **HPV-DNA testing** has been found to be as sensitive as, or perhaps even more so than cytological screening. This method may be easier to implement in low-resource settings and should be considered for primary screening in countries such as ours (*Kuhn et al., 2000*). **Direct visual inspection** of the cervical cells after application of a 5% acetic acid solution and HPV-DNA testing were found to identify similar numbers of high grade disease as the Pap smear test. However, both classify considerably more women without cervical disease as false positives (*Denny et al., 2000*).

Despite calls for screening, there are several implementation difficulties, e.g. shortages of both funds and skilled personnel. It is also speculated that within certain areas of our country, the high ASIR of African women with CC could well be rising (*Walker et al., 2002*). A recent multicentre study suggested that improvements in the functioning of the health system, a uniform national cytology reporting system, clear guidelines on the actions that are to be taken based on these cytology reports, as well as linkage between the screening sites and treatment centers are needed in order to decrease the mortality rates that are currently associated with CC in South Africa (*Fonn et al., 2002*).

An optimal **radiation** regimen combined with a cisplatin-based **chemotherapy** is the modern standard for treatment of advanced CC patients (*Witteveen et al., 2002*). In cases where CC is locally advanced or the patient has distorted anatomy, adequate treatment can be accomplished by means of **interstitial brachytherapy**. Patients treated with this method have been shown to achieve excellent locoregional control and have a reasonable chance of cure with acceptable morbidity (*Syed et al., 2002*).

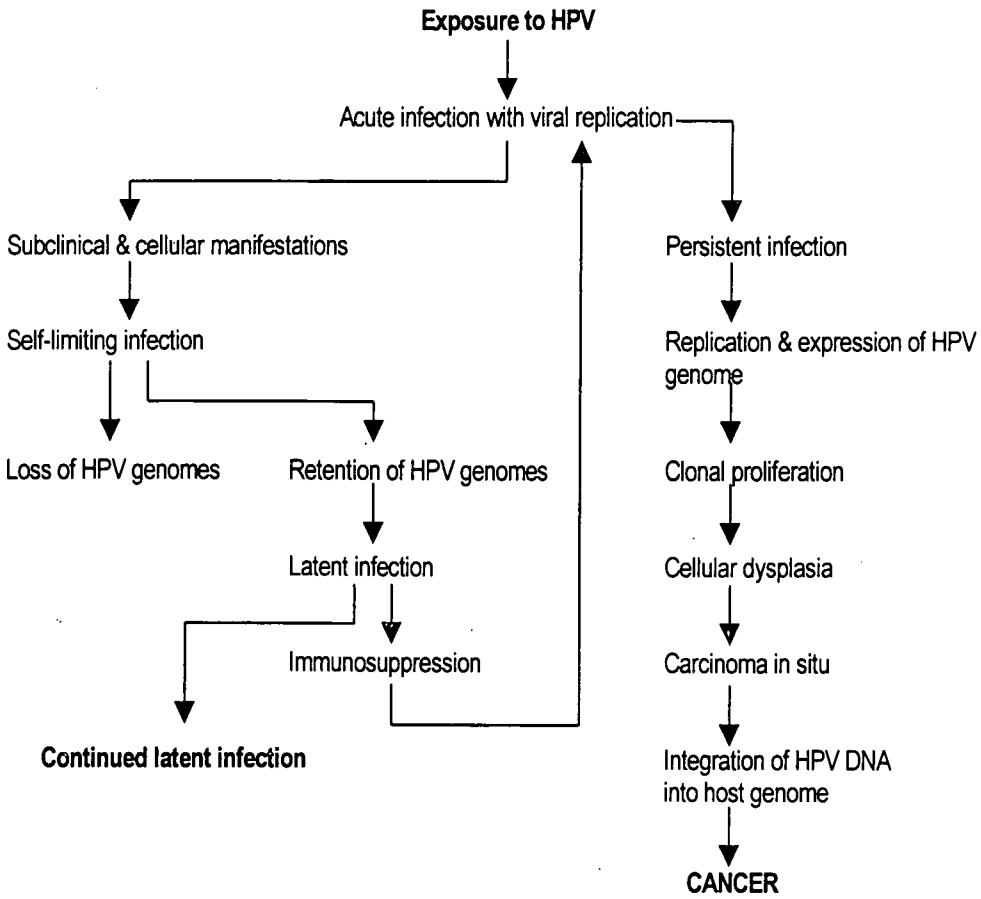

Figure 1.1.
Flow diagram of the natural history of HPV infection and the biological pathway followed up to carcinogenesis (*Burk R.D., 1999*). After the initial infection, HPV genomes can be lost from the host cells, or retained as latent infections. These latent genomes can either stay latent, or can start replicating again, producing progeny virions and making the infection worse. When the HPV DNA integrates into the host genome, the resulting mutation of host genes may lead to cancerous transformation of the host cells. (*Author's Interpretation*)
*The literature is inconsistent as to exactly at which point of carcinogenesis HPV integrates into the host genome. While some believe this point to be somewhere between the transition from CIN II or III to CIN III or mCa (*Hopman A.H.N., et al, 2004* & *Evans M.F., et al, 2004*), others believe it to be in the progression from low-grade to high-grade dysplasia of the cervical mucosa (*Ueda Y., et al., 2003*).

1.3. CANCER OF THE ESOPHAGUS

1.3.1. Esophageal cancer in SA *vs* the world

Esophageal cancer (EC) in humans occurs worldwide in males and females. It ranks eighth in the order of cancer occurrence, varying with geographical distribution. Two main types of this malignancy exist, each with its own distinct etiological and pathological characteristics: **esophageal squamous cell carcinoma** (ESCC) totals 90% of OCs world-wide and **esophageal adenocarcinoma** (EAC) has a higher prevalence in the USA.

ESCC is thought to develop from a precursor esophageal lesion (or accumulation of atypical cells) through a progressive sequence from mild to severe dysplasia, carcinoma *in situ* and finally to invasive carcinoma. These tumours frequently present as fungating, ulcerating or infiltrating lesions in the esophageal epithelium. They can range from well-differentiated keratinizing tumours with moderate nuclear atypia and minimal necrosis to poorly differentiated tumours with a high mitotic index and large areas of necrosis. A large majority of EC patients present with advanced metastatic disease, rendering the prognosis poor, with a very short survival term.

ESCC shows marked variation in its geographical distribution and occurs at very high frequencies in certain parts of China, Iran, SA, Uruguay, France and Italy (*Stoner & Gupta, 2001*). The incidence of EAC has been reported to be rising in the USA, Australia, New Zealand, as well as in certain parts of Europe, including Norway, Denmark, Sweden, the Oxford area of England and the Swiss Canton of Vaud. This cancer seems to generally occur more frequently in males. At the same time, the incidence of ESCC seems to have declined in US White males after the mid-1970's and in US Black males after the mid-1980's (*Levi et al., 2001*).

In the 1980's in the Eastern Cape (former Transkei), the most frequently reported cancer was EC, representing 45,8% of all reported cancer cases, recurring more frequently in males than in females. The second most common cancer among males and females, respectively, was liver and cervical cancer (*Jaskiewics et al., 1987*). The former Ciskei region of the Eastern Cape is another area with a high EC incidence. The short survival

period is in contrast with other high incidence areas e.g. in China, since their mass cytological screening leads to early detection and treatment (*Lazarus & Venter, 1986*).

Because of its aggressive clinical behaviour and poor prognosis, sporadic EC still is one of the leading causes of death among Black South African males. EC incidence is lower in White and Asian South Africans than in Black and Coloured SA populations. ESCC is more common among Blacks whereas EAC occurs more frequently among Whites (*Du Plessis et al., 1999*).

1.3.2. Risk factors

Human ESCC has a multifactorial etiology that involves several environmental and / or genetic and / or molecular components (*Stoner & Gupta, 2001*). (The molecular elements are summarized in Table 1.3.) ESCC risk factors apparently differ, at least in part, from those associated with EAC: **alcohol drinking** and **tobacco smoking*** seem to account for over 80% of ESCCs in developed countries. The association between tobacco smoking and EAC development is less strong and alcohol consumption can not be consistently related to EAC development. **Overweight and obesity** have been consistently related to EAC, but not to ESCC: in fact, measures of body-mass index (BMI) seem to be inversely related to the risk of ESCC. This obesity factor for EAC may in turn be related to increased **gastro-esophageal reflux**, since the risk of EC is strongly related to Barrett's esophagus[N] (*Levi et al., 2001*). More risk factors include **vitamin and trace mineral deficiencies** and consumption of **hot beverages** such as tea (*Stoner & Gupta, 2001*). **Social class indicators** tend to be inversely related to ESCC and EAC[ϕ] in developed countries (*Levi et al., 2001*). The consumption of **salt-pickled and salt-cured foods** have also been implicated in the pathogenesis of EC, since some of these products are frequently contaminated with *N*-nitrosamine carcinogens and / or fungal toxins (*Stoner & Gupta, 2001*).

---

* The tobacco constituents related to EC formation include nitrosamines, polycyclic aromatic hydrocarbons, aromatic amines, various aldehydes and phenols (*Stoner & Gupta, 2001*).

[N] Barrett's esophagus, or gastro-esophageal reflux disease (GERD) occurs very rarely in SA. This disease may be prevented by the ubiquitous infection of *Helicobacter pylori*, which has a prevalence of 61 to 100% in sub-Saharan Africa and is believed to be protective of the esophagus (*Segal, 2001*).

[ϕ] On a positive note, however, it is suggested that a diet high in fruit and vegetables may decrease the risk of EAC (*Chen et al., 2002*).

Fumonisins are mycotoxins (produced by the fungi *Fusarium moniliforme* and *F. proliferatum*) that contaminate maize-based foods and feedstuffs throughout the world. They cause a variety of toxicity-related diseases in animals and humans. Fumonisin B_1 has been shown to be carcinogenic in mice and rats, although most environmental toxic insults are known to involve complex exposures both to other toxins and to infections (*Turner et al., 1999*).

In 1996, Gelderblom *et al* reviewed several potential mechanisms of fumonisins' hepatotoxicity and carcinogenesis:

(i) Interruption of the biosynthesis of sphingolipids, important cell membrane lipids that are involved in regulatory processes.

(ii) Disruption of cellular lipids.

(iii) Fatty acid accumulation and cell proliferation.

(iv) Oxidative stress and lipid peroxidation.

(v) Peroxisome proliferation (*Gelderblom et al., 1996*).

Physical fungal invasion of esophageal tissues may also play a role in carcinogenesis, since such infections cause localized inflammation and irritation. **HPV infection** may play a contributory role in the formation of EC (see section 1.2.3.). HPV-16 and HPV-18 positivity occurs at a low but significant frequency in EC tumour samples, but the exact role of this virus in EC is yet to be elucidated (*Stoner & Gupta, 2001*).

The molecular factors that are associated with esophageal cancer are summarized in Table 1.3. There are still some gray areas concerning certain molecular predisposing factors, for instance the association between a specific TP53 codon 72 polymorphism and its association with HPV infection and EC (*Kawaguchi et al., 2000; Guimaraes et al., 2001*).

Table 1.3.

Molecular alterations in human ESCC.

Loss of heterozygosity on the following chromosomes or chromosome arms: [a,b] 1p, 3p, 4p, 5q, 9, 11q, 13q, 17q, 18q, 19p, 19q and 22q. **DNA gains and / or MSI often occur at on the following chromosomes or chromosome arms:** [b,c] 1q, 2q, 3q, 5p, 7p, 7q, 8q, 18q and Xq.
Loss of function of the following tumour suppressor genes: [a] × *p53* mutation × Methylation and / or loss of *p16MST1* and / or *p15.* × Reduction in the expression of functional *Rb.* **The loss of the following genomic DNA in males may provide clues as to male predisposition in acquiring EC:** [b] × 8p × Xp
Amplification of the following genes: [a] × *Cyclin D1* × *HST-1* × *EGFR* × *INT-2* **High level amplification of the following genomic loci:** [b] × 2q24 – 33 × 6p21.1 – q14 × 7p12 – q21 × 7q11.2 – 31 × 8q22 – 24 × 8q13 – qter × 13q21 – 34 × 13q32 – 34
Increased expression of the following genes: [a] × *iNOS* × *hTERT* × *BMP-6* × *COX-2* × *c-myc* × β-catenin

a = (*Stoner & Gupta, 2001)*

b = (Du Plessis et al., 1999)

c = (*Naidoo et al., 1999*)

1.3.3. Esophageal cancer: from prevention to treatment

Currently available therapies for EC offer poor survival and / or cure rates. A number of approaches could be undertaken in order to reduce ESCC occurrence: these include **changes in lifestyle** and **improved nutrition**, but this type of preventing approach is not easily implemented. **Chemoprevention** offers a viable alternative that has a greater chance of being effective against EC. Several tumour initiation or carcinogen inhibitors have been identified: these include diallyl sulfide, isothiocyanates and a couple of polyphenolic compounds. The identification of single agents that inhibit the progression of already existing dysplastic lesions has, however, proven difficult. Results from a food-based approach suggest that the use of freeze-dried berry preparations can affect both the initiation and progression of ESCC in an animal model.[♣] This is valuable information that can easily be applied in clinical chemoprevention programmes (*Stoner & Gupta, 2001*).

Because EC prognosis is generally poor, the main aim of palliation is improved dysphagia-free survival. Various methods of palliation have been used in attempts to improve the patients' quality of life and to provide near normal swallowing until death occurs, which would then be due to progressive systemic disease. These methods include **surgical bypass**, **laser**, **chemotherapy**, **intubation**, **external beam radiotherapy** (EBRT) or a **combination** of these methods. Despite these efforts, prognosis continues to be dismal, with a survival of 2.5 to 5 months from any of these techniques alone, or a marginal improvement with combination therapy (*Sur et al., 2002*).

New data show that the use of **chemoradiotherapy** (CRT) as definitive treatment, or in combination with **surgery** may improve locoregional control and survival, when compared to radiotherapy or surgery alone. Problems do arize, e.g. acute treatment-related toxicity is increased with CRT (*Geh, 2002*).

[♣] *Freeze-dried strawberries and black raspberries are proposed to inhibit tumour formation and / or proliferation by means of inhibition of DNA adduct formation and inhibition of post-initiation events (Stoner & Gupta, 2001).*

It has recently been established that fractionated **HDR brachytherapy** or **intraluminal brachytherapy** (ILBT) as sole treatment gives the best results in terms of palliation and survival in advanced EC patients in South Africa. This treatment surpasses the results of any other modality of treatment that is currently available: this is a technique that allows the delivery of a very high radiation dose to the luminal aspect of the tumour, which is thought to be relatively hypoxic and radioresistant. Also, the risk of injuring the surrounding tissue structures is minimal, because the dose falloff is rapid. This type of treatment seems to be relatively efficient in inducing tumour shrinkage and restoring swallowing. High dose rate (HDR) ILBT is also a quick procedure, minimizing patient discomfort.

Furthermore, fractionating the HDR-ILBT has been shown to give even better results in terms of dysphagia-free and overall survival compared to a single fraction dose alone. It is speculated that the addition of EBRT to HDR-ILBT may improve results in advanced EC patients (*Sur et al., 2002*).

1.4. Newcastle Disease virus (NDV)

1.4.1. The ND virus

NDV is an avian virus with a wide host range: it attacks 27 of the 50 orders of bird species (*Seal et al., 2000 (b)*), including domestic chickens and turkeys. Such an infection can cause up to 100% mortality in poultry, depending on the virulence of the infective strain.

Some mammals, including humans, may also be infected. NDV infections in humans present as granular conjunctivitis (which has symptoms like pain and photophobia and may lead to some lasting vision impairment), lymphadenitis, headache, malaise and chills. It has been found that generally, most such infections occur among laboratory workers and in the poultry vaccine production industry (*Gallili & Ben-Nathan, 1998*). Human-to-human transmission has never been reported (*Alexander, 2000*).

NDV outbreaks were first reported in poultry from Java, Indonesia (*Seal et al, 2000 (b)*) and the virus was first isolated from domestic chickens in 1926 in Newcastle-upon-Tyne, England (*Wildner, 2001*). NDV is still an economic problem worldwide, causing severe losses to farmers and governments (*Gallili & Ben-Nathan, 1998*). One of the most recent outbreaks occurred in 2002 in China (*Liang et al., 2002*). A long-term plan for managing such outbreaks effectively involves radical stamping out of infected flocks, quarantine of suspected poultry and vaccination of all flocks in the absence of the disease (*Smagner, 1999*).

1.4.2. Classification

1.4.2.1. NDV can be ***taxonomically*** classified as shown in Figure 1.2. (*adapted from De Leeuw & Peeters, 1999; Seal et al., 2000 (b)).*

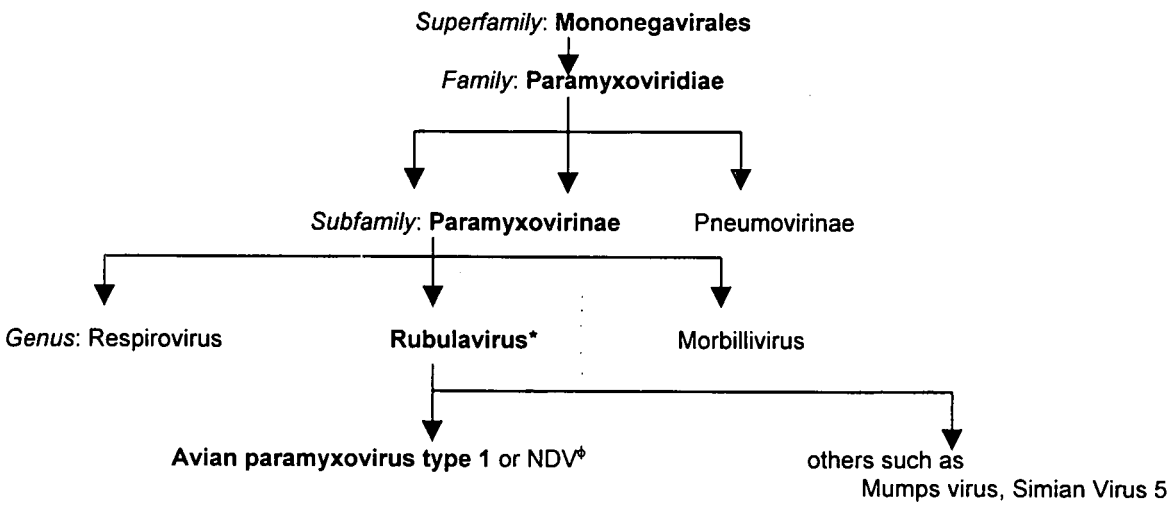

Figure 1.2.

Taxonomical classification of NDV. (*Author's assembly*)
* NDV was classified as a Rubulavirus by the International Committee on the Taxonomy of Viruses in 1993 (*Seal et al., 2000 (b)*).
$^{\phi}$*De Leeuw & Peeters, 1999*

The paramyxoviridiae family also includes the following viruses:

(i) Canine Distemper Virus (CDV) that infects dogs, foxes, coyotes, wolves, raccoons, pandas, etc.

(ii) Porcine Distemper Virus (PDV) that infects seals.

(iii) Rinderpest Virus infecting ruminants, especially cattle.

(iv) Parainfluenza Virus that infects dogs, cattle and humans.

(v) Measles virus (rubula virus) that causes infection of the mucosa and skin epidermal cells in humans (*Ulane et al., 2003*).

(vi) Unclassified viruses such as the Hendra and Nipah viruses (*Morrison, 2001*).

After the entire NDV *La Sota* strain genome was sequenced in 1999, sequence comparison showed that NDV is only distantly related to its fellow genus members. This may be due to a separate evolution as a result of an early host switch from mammals to birds. It was then suggested that NDV should not, in fact, be classified as a Rubulavirus, but should instead be considered as a member of a new genus within the subfamily Paramyxovirus (*De Leeuw & Peeters, 1999*). Individual amino acid sequence analyses of the matrix (*Seal et al., 2000 (a)*) and nucleocapsid proteins of other NDV isolates also clustered NDV in a distinct group apart from the *Rubulaviruses* (*Seal et al., 2002*).

1.4.2.2. The NDV species can be further classified according to ***pathogenicity in chickens*** *(De Leeuw & Peeters, 1999)*, as is summarized in Table 1.4.

Table 1.4.

Pathogenic forms of ND viruses: pathotypes and pathogenicity indices.

Pathotype	Pathogenicity			Virus strains
	MDT[a]	ICPI[b]	IVPI[c]	
Velogenic (viscerotropic)	<60	2.0-3.0	2.0-3.0	Herts 33.N.Y, Parrot 70181, CA2089/72.
Velogenic (neurotropic)	<60	1.5-2.0	2.0-3.0	Texas GB.
Mesogenic*	60-90	1.0-1.5	0.0-0.5	Komarov, Roakin, Mukteswar, H.
Lentogenic*	>90	0.2-0.5	0.0	LaSota, Hitchner B1, Clone 30.
Asymptomatic*	>90	0.0-0.2	0.0	V4, MC110, Ulster 2C.

[a] Mean death time in embryonated eggs in hours.
[b] Intracerebral pathogenicity index in 1-day old chicks.
[c] Intravenous pathogenicity index in 6-week old chickens.
* Strains of these groups are used in producing commercial vaccines.

(Gallili & Ben-Nathan, 1998; De Leeuw & Peeters, 1999)

Velogenic NDV: strains show high virulence in birds of all ages. The group is comprised of *viscerotropic* (that cause hemorrhagic intestinal lesions) and *neurotropic* strains (that cause acute respiratory and nervous disorders). The disease caused by velogenic strains is so

severe that it induces mortality in embryos in less than 60 hours (*Gallili & Ban-Nathan, 1998*). Highly virulent NDV isolates are List A pathogens and reports of its isolation should be made to the Office of International Epizootes (*Locke et al., 2000*).

Mesogenic strains are less virulent and produce a mild disease that is fatal only to young chickens, although acute respiratory disease and nervous signs appear in some cases. Some selected strains have been developed as live vaccines in certain countries (*Gallili & Ben-Nathan, 1998*).

The **lentogenic** strains are even less virulent and induce mild respiratory infections, while **asymptomatic** strains cause virtually no symptoms at all. These two strain types are very popular in the production of live NDV vaccines (*Gallili & Ben-Nathan, 1998*).

1.4.3. NDV in birds

1.4.3.1. General

ND is considered to be one of the most serious infectious diseases of birds, infecting commercial and wild flocks alike (*Alexander, 2000*).

Clinical diagnosis (after a two to fifteen day incubation period *(Arnoldi et al., 1998)*) of birds with NDV is as follows:

(i) Respiratory signs: gasping and / or coughing and / or

(ii) Nervous signs: drooping wings, dragging legs, twisting of the head and neck, circling, depression, inappetence and / complete paralysis (*Saville, 1996*; *Arnoldi et al., 1998*).

(iii) Partial or complete cessation of egg production.

(iv) Greenish watery diarrhea.

(v) Tissue swelling around the eyes and in the neck (*Arnoldi et al., 1998*).

Interestingly, the persistence of the virus has not yet been clearly defined: research results are always inconsistent, which may be due to factors such as reinfection. Viral shedding is a very common phenomenon where a recovered or asymptomatic bird completely loses the ND virus (*Seal et al., 2000 (b)*).

1.4.3.2. Diagnostic techniques:

(i) The **differential diagnosis** (identification) of NDV involves hemagglutination inhibition with polyclonal NDV specific antisera or an enzyme-linked immunosorbent assay (ELISA). Limited success has been achieved with oligonucleotide probes and viral genomic RNA fingerprint analysis in the identification and differentiation of NDV strains. Monoclonal antibodies are more often used to identify antigenic groups, but pathotyping procedures are still very labor intensive (*Seal et al., 2000 (b)*). Newcastle disease can also be identified by means of PCR or sequencing (*Cavanagh et al., 1997; Barbezange & Jestin, 2002; Aldous et al., 2001*).

(ii) **Pathotype / pathogenicity prediction** initially involves inoculation of embryonated eggs with the virus in order to determine the MDT of the embryo. Further testing entails the inoculation of chickens to determine the ICPI, as well as the IVPI, as summarized in Table 1.4. Viscerotropic or velogenic NDV strains can be differentiated from neurotropic velogenic strains with the intracloacal inoculation pathogenicity test. (This test is used in the USA.)

Another characteristic that can be exploited for differentiation purposes is virulent NDV strains' ability to replicate in most avian and mammalian cell types without the addition of trypsin: all NDV isolates will replicate in chicken embryo kidney cells, but lentogens require trypsin for replication in avian fibroblasts or any mammalian cell type (*Seal et al., 2000 (b)*).

1.4.3.3. Molecular basis of pathogenicity

NDV strains' varying pathogenicities are, on a molecular level, dependent on the F protein cleavage site amino acid sequence, as well as on the ability of the host cell proteases to cleave the specific NDV strain's F protein. Lentogenic NDV isolates have fewer basic amino acids in the F protein cleavage site compared to meso- and velogenic isolates (which have similar cleavage site sequences). Classification by reverse transcription (RT) -PCR

amplification followed by restriction enzyme digestion of various NDV strains' F gene sequences used to be difficult, since the results obtained were inconsistent. That was until M.S. Collins managed to amplify a portion of the F protein gene and deduct the cleavage activation site from nucleotide sequences of the amplification product (*Seal et al., 2000 (b)*).

1.4.3.4. *La Sota*

Infectious virus transmission between individual birds by ingestion and / or inhalation has been exploited to such an extent that these are now the basic methods used with great success in mass poultry vaccination procedures. Both inactivated and live-virus vaccines are available for NDV control in poultry. The mildly virulent *La Sota* strain is currently one of the most widely used efficacious live-virus NDV vaccines marketed worldwide. The immune response induced by this type of vaccine involves overall elevated levels of IgA, IgY and IgM antibodies, as well as a local antibody response such as IgA production in the Harderian gland and lacrimal IgM production in bird (*Seal et al., 2000 (b)*). This issue is further discussed in section 1.4.6.

1.4.4. Structure, morphology & mechanics

NDV is an enveloped RNA virus with a negative-strand single-stranded genome of 15 kilobase pairs (*Seal et al., 2000 (a)*). This genome codes for six proteins as indicated in Figure 1.3. (*Seal et al., 2000 (b)*).

The mechanism of infection, in short, is believed to proceed as follows: the virus binds to the host cell membrane through HN protein attachment to certain host cell surface proteins; the two membranes are then fused via the F protein (*Chen et al., 2001 (a)*). NDV may use the endocytic pathway as a complementary way of entering cells by direct fusion with the plasma membrane (*San Román et al., 1999*).

The following functions are ascribed to the various viral proteins:

M - matrix protein:

This non-glycosylated protein is located underneath the lipid bilayer of the viral particle (*Muñoz-Barroso et al., 1997*). Upon infection, it localizes to the host cell's nuclear membrane (*Coleman & Peeples, 1993*) and nucleolus independently of any other viral protein (*Peeples et al., 1992*).

F - fusion protein:

This transmembrane protein (*Cobaleda et al., 2001*) enables the viral and host cell membranes to fuse. Paramyxovirus membrane fusion is thought to involve a series of structural transitions of the F protein: from a metastable prefusion state to a very stable postfusion state. The complete atomic mechanism for this transition is not yet known, although the three-dimensional structure of the F protein suggests that a novel molecular mechanism is involved (*Chen et al., 2001 (a)*).

F protein molecules are initially synthesized as single-chain precursor F_0's, which assemble into trimers within the ER (*Chen et al., 2001 (a)*). Proteolytic cleavage activation results in the formation of two chains, which are covalently linked via disulfide bonds. This cleavage always occurs during viral multiplication in eggs, but in tissue culture extracellular cleavage by an added protease (such as trypsin) is sometimes necessary. Virulent strains of NDV encode readily cleaved fusion proteins, while less virulent strains have an uncleaved fusion protein when grown in tissue culture. The ability of this protein to be activated by cleavage correlates with the presence of two pairs of basic amino acid residues at the cleavage site (*Morrison, 1990*). The resulting fragments are termed F_1 (C-terminal) and F_2 (N-terminal). A hydrophobic segment at the F_1-terminus anchors the protein in the viral membrane. The newly created N-terminus of F_1 is also hydrophobic and is called the fusion peptide (*Chen et al., 2001 (a)*).

The F protein mediates fusion not only between the viral and host cell membranes, but also between the membranes of an infected cell and its neighboring or adjacent cells (*Morrison, 1990*).

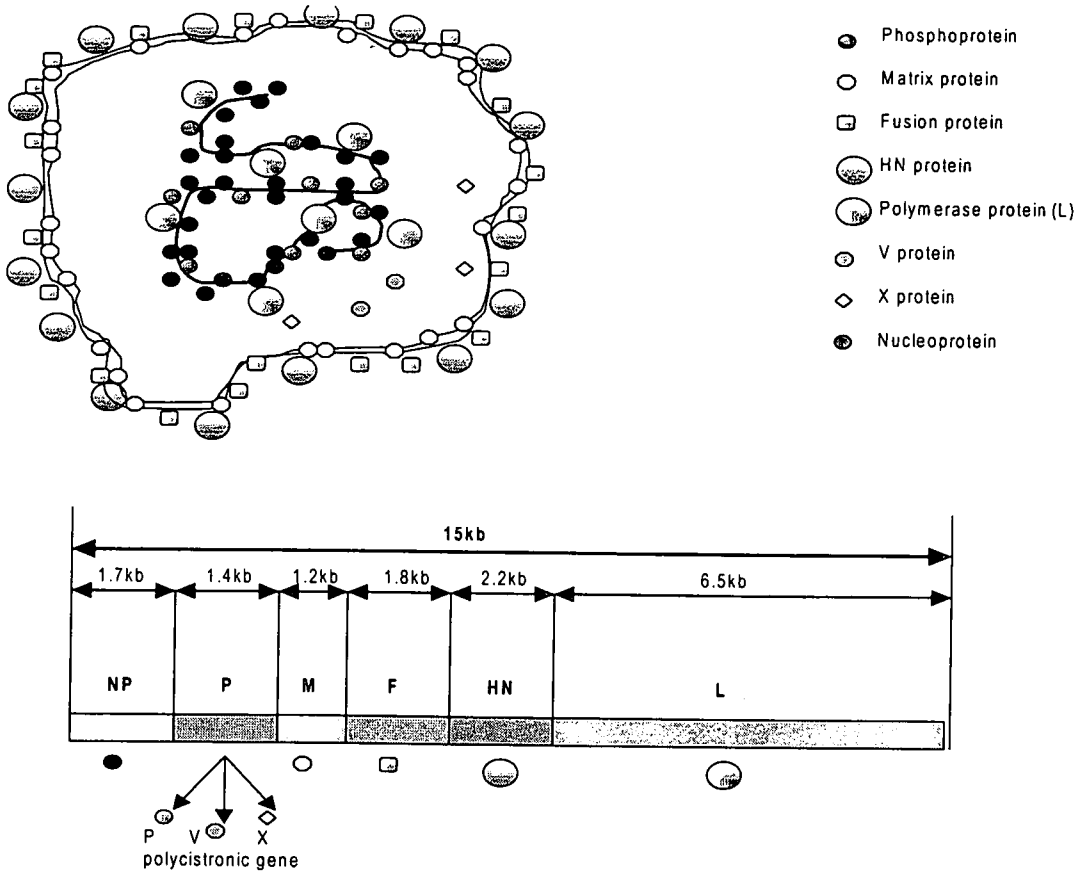

Figure 1.3.

NDV structure and genomic organization: (b) schematic representation of the genes transcribed into proteins from the NDV genome, the approximate locations of these genes on the genome relative to each other, and (a) the relative sizes of the resulting proteins. The enveloped virus has two surface glycoproteins (F & HN). The F and HN proteins are the main agents in triggering the host organism's protective immune response. The M protein is juxtaposed between the envelope and the interior nucleocapsid structure. The N, P and L proteins make up the transcriptase complex and are in close contact with the viral genome. Transcription occurs in the 5'-3' direction with decreasing amounts of protein transcribed from each subsequent gene. The L protein also transcribes the intermediate positive-sense RNA genome into DNA, from which mRNA is then transcribed and translated. P is polycistronic due to insertion of at least one additional guanosine during transcription and utilization of potential alternative transcription start sites (*adapted from Seal et al., 2000 (b)*).

HN - hemagglutinin neuraminidase or **attachment protein:**

This is a transmembrane protein (*Cobaleda et al., 2001*) that enables the virus to attach to the host cell membrane. The HN and F proteins form 'spikes' on the surface of the viral envelope: these proteins elicit the immune response in the host organism.

During viral infection, the HN protein plays a number of roles (Figure 1.4.): firstly, in targeting the viral particle to the host cell (*Chen et al., 2001 (a)*), HN binds the sialic acid-containing receptors on the cell surface (**hemagglutinating activity**). It also displays receptor-destroying activity, termed **neuraminidase** or **sialidase activity**. Both of these functions are dependent on the surrounding viral membrane's lipid composition (*Cobaleda et al., 2001; Muñoz-Barrozo et al., 1997*). This second (neuraminidase) function becomes active later on during viral maturation when the newly formed viral particles bud from the host cell: HN removes the sialic acid components from the viral envelope's surface to prevent self-attachment of such particles. Inhibiting either of these functions of HN would disrupt the viral infection cycle. X-ray crystallography revealed that a single active site on the HN protein is responsible for both of these functions: stable binding to sialic acid during targeting, as well as sialic acid cleavage during maturation. Switching between these functions could involve conformational change within the protein. This could be the basis for structure-based drug design against NDV or even all the paramyxoviruses (*Crennell et al., 2000*). HN's third activity is **fusion promotion**. The mechanism is not known, but the action is mediated by the middle or stalk part of the HN protein. The presence of two homotypic membrane glycoproteins in the same bilayer is an absolute requirement for such fusion induction (*Cobaleda et al., 2001*).

L - large protein:

The largest structural protein acts as the viral transcriptase and replicase in association with P (*Seal et al., 2002*).

N - nucleocapsid (envelope) protein:

This protein is the major constituent of the ND virion's nucleocapsid, with about 2600 N proteins per virion (*Seal et al., 2002*). N binds to P as a required factor for encapsidating the newly synthesized viral genomic RNA. Phosphoprotein complexes with N:RNA and L form the minimal transcriptional unit of paramyxoviruses, which absolutely requires phosphorylation for activity (*Locke et al., 2000*).

P - polymerase–associated protein (or **polysistronic phosphoprotein** (*Locke et al., 2000*)):

P forms part of the transcriptase complex and subsequently plays major roles in genome replication and transcription. The P protein inhibits the formation of N self-assembly, thus acting as a chaperone to prevent uncontrolled encapsidation of non-viral RNA by N. Transcriptional modification of this P gene mRNA allows for the potential expression of two smaller putative proteins, designated V and W. The V protein produced by NDV in birds binds zinc and contains a highly conserved motif homologous to DNA-binding proteins (*Locke et al., 2000*).

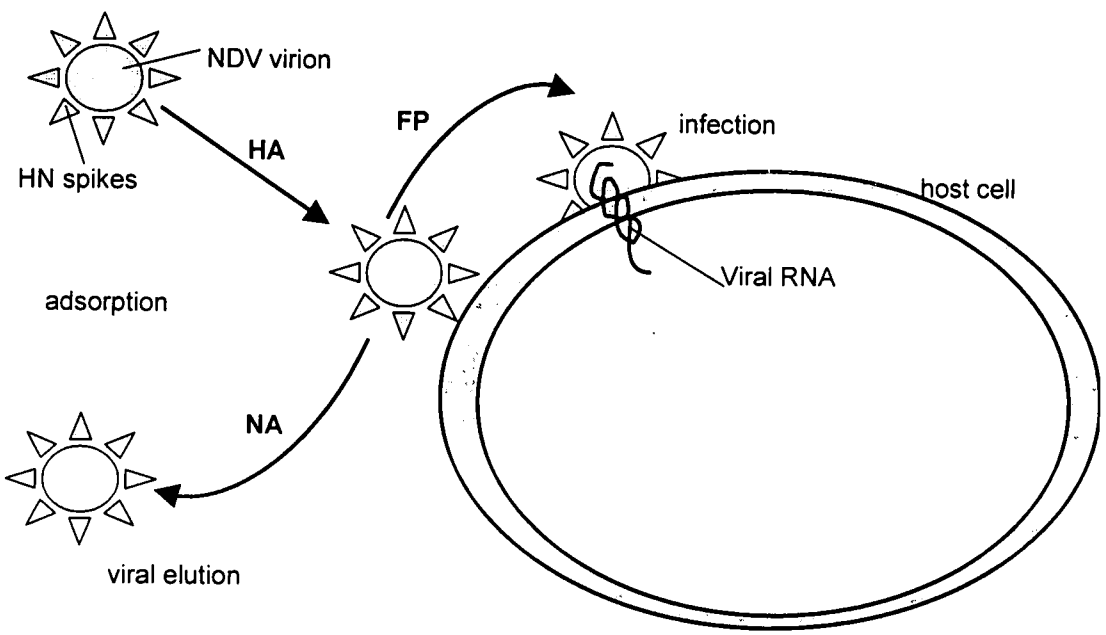

Figure 1.4.
The three activities of paramyxovirus attachment proteins. (1) HA = hemagglutination = binding of sialic acid-containing receptors on the tagret cell surface. (2) FP = Fusion promotion = subsequent promotion of fusion between the target cell membrane and the virus membrane. (3) NA = neuraminidase = viral elution from the cell surface after unsuccessful infection initiation, or in the release of progeny virions budding from the host cell surface. (Adapted from *Morrison, 2001.*)

1.4.5. NDV in the treatment of cancer

1.4.5.1. Naturally oncolytic strains

Strain differences are substantial in respect to virulence, syncytium formation, replication, host immune responses and oncolysis. The most oncolytic strain recognized so far is the **Cassel's 73 T** strain. This strain was derived from Lederle's NDV by 73 passages *in vitro* and 13 passages *in vivo* in murine Ehrlich ascites carcinoma cells. 73 T has proven to be a very effective oncolytic agent in numerous melanoma and colon cancer patients, without causing harmful side effects. It has also been found to be non-neuropathogenic after intracranial injection into rodents (*Cassel & Garrett, 1965; Wildner, 2001*). Other human tumours treated successfully with NDV 73 T include large cell lung, breast and prostate carcinomas (*Phuangsab et al., 2001*); neuroblastomas, fibrosarcomas (*Lorence et al., 1994 (a); Lorence et al., 1994 (b)*). (See Xenografting: section 1.5.)

The antineoplastic effects of NDV may be induced by several possible mechanisms:

(i) *In vitro*, the virus particles can infect and directly lyse a variety of human tumour cells, while not significantly affecting normal human fibroblasts.

(ii) NDV can induce tumour necrosis factor -α (TNF-α) production in human mononuclear cells.

(iii) NDV-infected cells can be more sensitive to TNF-α's cytolytic effects than uninfected cells.

(iv) The results of tumour vaccination trials with a live virus mixture that contains an oncolysate, suggests that NDV can serve as an immune adjuvant.

It has been shown that for NDV, the direct effect can be limited in time, scope and specificity, where the use of viral oncolysates to augment antitumour immunity can be more effective (*Shoham et al., 1990*).

NDV is a fast growing virus that can produce detectable progeny virions within 3 h post-infection. Plaque formation is a macroscopic means of analyzing cytolysis and, according to Phuangsab *et al* (1999), can be observed in tumour cell monolayers as early as 18 h post infection. Sialic acid, the cellular receptor that NDV recognizes and binds to, is found on diverse cells including human cancer cells of neuroectodermal, mesenchymal and epithelial origins. Addition of a high multiplicity of the virus to cultured tumour cells can result in rapid

cell-to-cell fusion (in less than 1 h). At the same multiplicity of virus, this does not occur in fibroblasts, suggesting that NDV preferentially recognizes tumour cells. Oncogenic transformation increases the sensitivity of malignant cells to NDV cytolysis. [Fibroblasts that were transfected with the N-ras oncogene and thus made to be tumourigenic, were 1000 times more sensitive to 73 T infection after compared to before transfection (*Lorence et al., 1994 (a)*).]

The molecular basis for these tumour selective properties of NDV is currently under investigation (*Phuangsab et al., 2001*).

1.4.5.2. NDV as a vector

NDV can be engineered to express a foreign gene in a stable manner and has a whole spectrum of potential for future clinical use as a recombinant vaccine vector (*Krishnamurthy et al., 2000; Nakaya et al., 2001*): a complete cDNA clone of the avirulent vaccine strain Hitchner B1 was used to construct an infectious recombinant virus expressing influenza virus hemagglutinin. The resulting virus induced a strong humoral antibody response against influenza virus and complete protection against a lethal dose of influenza virus challenge in mice. NDV has been shown to be a safe and effective vaccine vector for use in avian and mammalian species (*Nakaya et al., 2001*).

Most cancer-directed gene therapy applications that make use of replicating viruses are directed to *in vivo* gene therapy of the cancer. However, non-virulent strains of NDV are used for *ex vivo* tumour cell infection and for the generation of autologous tumour cell vaccines (ATV's), as discussed in section 1.4.5.4. (*Galanis et al., 2001*).

1.4.5.3. NDV plasma membrane vesicles

The therapeutic potentials of many drugs are limited by selective intracellular delivery of large and / or membrane-impermeable molecules and some aqueous solutions like peptides, proteins and oligonucleotides that have difficulty crossing the cell membrane. There are many possible ways of overcoming this obstacle, e.g. employing liposomes or lipidic bilayers that have small volumes of the therapeutic agent enclosed. Specific surface properties give these vesicles the ability to selectively target and deliver their contents to

specific cells. An elaboration of this approach involves the insertion of viral envelope proteins into a plasma membrane vesicle (PMV), in order to achieve delivery of the entrapped molecules to specific cells by means of the fusogenic properties of the viral proteins.

Taking this idea further, in 2000, Trigiante *et al* employed the whole NDV virus inside a 'bubble' of host cell membrane as such a delivery agent. This kind of host membrane vesicle has been shown to fuse with viral envelope proteins and to display functional viral receptors by means of fusion from within (FFWI), giving the vesicle strong tissue-specificity; while it elicits a weaker response from the host's immune system than any non-host lipid bilayer would. The logic behind this novel idea involves the fact that enveloped viruses may (under appropriate conditions) promote simultaneous fusion between two cell membranes, causing the formation of multinucleated polykaryocytes or syncytia, which are routinely observed in NDV and many other kinds of viral infections,

The results proved very successful: the enclosed virus caused the PMVs to fuse with the target cells, forming syncytia. The viral infection, as well as the syncytia generation are both responsible for death of the infected cells. Further studies by this group showed that the addition of an RNA-hydrolyzing agent and potential inhibitor of viral RNA replication (e.g. RNase A which is cytotoxic, but also a membrane-impermeable molecule) to the virus-containing PMV, caused rapid and extensive death of the infected cells and a concomitant significant decrease in the amount of virus produced. These results confirmed the proposed mechanism of solute delivery, showed that solute delivery is selective for the NDV-infected cells and that the RNase does not affect cells that are unrelated to the targeted cells (*Trigiante & Huestis, 2000*).

This primary research employed NDV and one human cell line, but the principles and techniques are applicable to a large number of virus/cell systems. Techniques such as UV-irradiation can be used to abolish viral infectivity while preserving the fusogenic potential (*Trigiante et al., 2000*).

1.4.5.4. NDV tumour vaccines

General

A tumour vaccine is a substance that contains a specific component that represents a tumour-associated antigen (TAA) against which the T-lymphocytes of a cancer patient should become activated. These T-lymphocytes need a co-stimulatory signal that is represented in the vaccine by adjuvant components, which trigger non-specific immune activation (*Washburn & Schirrmacher, 2002*).

How tumour vaccines work

The specific components of the vaccine are autologous tumour cells from the patient. The cells can be taken from the primary tumour or metastases, because these are the closest approximation to the individual disease. The live tumour cells are isolated from freshly operated tumour material according to a very specific standard operating procedure (*Adam et al., 2003*).

The tumour cells are inactivated by gamma-irradiation (*Schirrmacher et al., 1999; Haas et al., 1999*) and infected by an apathogenic non-virulent substrain of NDV, such as the Ulster strain (*Schirrmacher et al., 1999*). The cells can additionally be co-incubated with bispecific antibodies that recognize both the viral HN-molecule that is displayed on the infected tumour cells, and the CD28-activating epitopes on the surfaces of T-lymphocytes (*Galanis et al., 2001*) in order to enhance the resulting immunostimulation (*Haas et al., 1996*; *Haas et al., 1999*). The other viral proteins also stimulate the host cell genes and local production of cytokines and chemokines, recruiting a broad antitumour response *in vivo*, when the treated cells are injected back into the patient. This whole new modality of employing the cancer patient's own immune system to treat his disease is called active specific immunotherapy (ASI) (*Schirrmacher et al., 1995*).

NDV has the following properties that make it potentially useful in ATV therapy:

(i) Pleiotropic immune stimulatory properties.

(ii) Good cell-binding.

(iii) Selective proliferation in specific cells.

(iv) It can introduce T-cell co-stimulatory activity & induce cytokines like INF-α, INF-β & TNF-α, that affect T-cell recruitment and (*Galanis et al., 2001*).

A great number of human tumour cells can be effectively infected by NDV with viral replication independent of tumour cell proliferation. This, together with its discussed immunological properties make NDV a suitable agent for modification of noncultured freshly isolated patient-derived tumour cells (*Schirrmacher et al., 1999*).

ATV-NDV in practice

Experimental ATV-NDV treatment seems to cause very mild side effects in the patient in comparison to the long-lasting ulcers and more serious side effects caused by comparable tumour vaccines (*Schirrmacher et al., 1995*). In long-term follow-up studies, NDV-treated patients have repeatedly been shown to be much less prone to tumour recurrence than patients that have only undergone tumour resection. These clinical trials should stimulate further prospective randomized studies (*Schlag et al., 1992*). Limiting factors are the number of tumour cells that can be reliably infected, as well as tissue selectivity and safety of the viral strain used (*Schirrmacher et al., 1999*).

Previous and current clinical trials evaluating NDV as an ATV showed encouraging results in cancer patients suffering from a variety of tumour types (*Kirchner et al., 1995*) such as breast (*Washburn & Schirrmacher, 2002*), colon (*Galanis et al., 2001*), metastasized liver cancers (*Schlag P., et al, 1992*), and advanced renal-cell carcinoma (*Kirchner et al., 1995*). Such trials often involve NDV ASI treatment given after surgical resection (sometimes in combination with low-dose cytokines (*Kirchner et al., 1995*)) of the primary and / or metastasized tumour(s) (*Schlag et al., 1992*).

This therapy is usually given to patients with a risk of developing metastases. ASI has the potential to become a treatment for complementing standard treatments such as tumour resection, radiotherapy and chemotherapy (*Adam et al.,2003*). Side-effects include mild toxicity manifesting as flu-like symptoms, fevers of up to 38°C (*Kirchner et al., 1995*) and DTH-like reactions (*Stoeck et al., 1993*). Figure 1.5. shows a schematical representation of the major effects that NDV infection has on cancer cells.

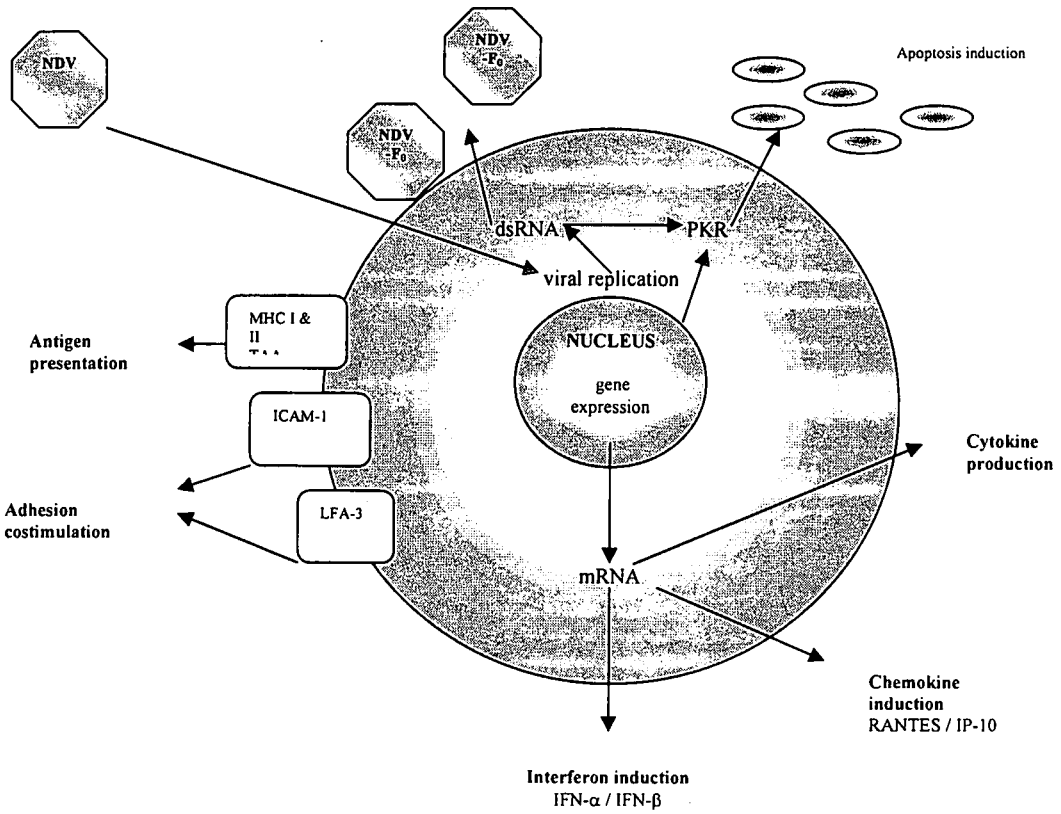

Figure 1.5.

Schematic representation of the main effects of NDV infection on tumour cells: introduction of viral surface antigens HN and F, secretion of cytokines, chemokines and type I interferons, modulation of surface molecules (HLA and CAM) and apoptosis induction (*Washburn & Schirrmacher, 2002*).

1.4.5.5. Clinical trials with NDV

A Phase II trial in Budapest produced encouraging results from using NDV as a nasal spray. In most cases the treatment caused either complete tumour regression, or symptomatic relief to advanced cancer patients (*Csatary et al., 1993*). Table 1.5. summarizes NDV strains that have been evaluated in clinical trials.

Conclusions resulting from clinical trials often differ in their degree of positivity for NDV as a cancer treatment. One criticism is that, although virus inoculations and viral oncolysates often induce cancer remissions, reduce tumour burdens, decrease relapse rates and prolong the lives of some patients, it still falls short of curing human cancers. In another article in 2000, Sinkovics *et al* proposed that the immune response against replicating NDV virions in the form of 'haphazard cytokine production in the inoculated host' could be replaced with transfection of single genes of well-defined, limited but selective efficacy (*Sinkovics & Horvath, 1995* & *Sincovics & Horvath, 2000*).

Although NDV-treatment is relatively inexpensive, non-toxic and developed in the USA, this treatment is not readily available to Americans, because it does not have FDA approval (*Moss, 1994*).

Table 1.5.

NDV used in clinical trials.

NDV type and strain	Clinical phase	Tumour target(s)
Live virus PV701	I	Advanced solid tumours.
Live virus – MTH68/H	-	Advanced renal, colon and breast tumours.
Viral oncolysate – Cassel's 73 T	II	Stage II malignant melanoma.
Viral oncolysate – Ulster	II	Advanced renal, colon and breast tumours.
ATV – Ulster	I	Colorectal carcinoma (Dukes stage B2, C., D).
ATV – Cassel's 73 T	-	Renal carcinoma.
ATV – La Sota IV	-	Advanced tumours of the digestive tract.
ATV – PV701	III	Breast carcinoma.

(*Omar et al., 2002*)

1.4.5.6. NDV vaccine strains' clinical applications

MTH-68

The commercially available vaccine that is used for poultry protection against fowl plaque, MTH-68, (see Table 1) has been used as an oncolytic agent in a large number of advanced cancer patients. The story of this strain's discovery was published in *Lancet* in 1971 by Laszlo Csatary: a chicken farmer with metastasized stomach cancer suddenly and miraculously underwent a complete and lasting disappearance of his advanced tumour while his flocks were devastated by NDV. Csatary's subsequent dogged pursuit of an NDV-based cure for cancer eventually led to the production of an attenuated non-pathogenic NDV strain (derived from the original highly virulent strain) that we now know as **MTH-68/N** (*Moss, 1994*).

The mechanism of MTH68's oncolytic action is still poorly understood, although it appears to trigger a specific signaling pathway in the target cell, which leads to internucleosomal DNA fragmentation, the most characteristic feature of programmed cell death. Major mitogen-activated protein kinase pathways (like the stress-inducible c-Jun N-terminal kinase pathway), or mechanisms regulated by reactive oxygen species appear to play no role in this virus-induced apoptosis.

The MTH-68/N strain also showed very promising results in a placebo-controlled clinical trial on advanced cancer patients. The vaccine was given at a dose of 4000U/day, twice weekly by inhalation (*Csatary et al., 1993; Csatary et al., 1999 (b)*).

Experimental results suggest that **MTH-68/H** might disrupt a growth-factor stimulated survival pathway, since direct stimulation of protein kinase A-catalysed phosphorylation events (treatment with cyclic AMP) bypass this NDV-induced block and thus partially protects the cells from virus-induced cell death (*Fabian et al., 2001*).

Dr. Csatary's work eventually led to the treatment of a small number of terminal cancer patients, who have exhausted conventional treatments, with his attenuated vaccine strain MTH-68/N. By 1993 the promising data from his ongoing clinical studies were published in the journal *Cancer Detection and Prevention*. In short, it showed that NDV treatment:

(i) slowed or halted tumour growth

(ii) lessened pain and / or

(iii) improved the quality of his patients' lives (*Csatary et al., 1993; Moss, 1994*).

By 1996 several dozen papers have confirmed these preliminary findings.

In one of these recent experimental treatments, the MTH-68/H strain gave spectacular results: it was used in conjunction with conventional (initially unsuccessful) chemotherapy on a 14-year old boy with a fast-growing grade IV glioma. The combined therapy started in 1996 and resulted in ~95% tumour regression. By 1999 he was still receiving NDV, as sole therapy. He fully recovered to perform above average in a public school (*Csatary et al., 1999 (a)*).

In 1999, more promising results came from an experimental study, in which patients with advanced neoplastic diseases were given MTH-68/H after non-efficient tumour-destructive treatment. This study lead to the proposition of a phase III trial in accordance with good clinical practice (GCP) for confirmation of the results (*Csatary et al., 1999 (b)*).

Csatary followed these results up and conducted clinical trials in a private Budapest clinic on a larger patient population. The results were very positive: a total of 33 patients suffering from various cancers were given inhalations that contained MTH68. Eighteen of these (55%), mostly those with colorectal cancer, responded to this treatment (*Wildner, 2001*).

MK 107

This vaccine strain is naturally attenuated and was tested on advanced cancer patients with solid tumours that were unresponsive to standard therapy in a phase I clinical study. The strain that was used for this study was named PV 701. Only two patients out of 79 showed partial to complete response. Importantly, however, it was determined that viral infections did not persist and were cleared within 3 weeks of viral administration. Again no other side effects than flu-like symptoms were observed (*Russel, 2002*). This trial warranted PV 701's further investigation as a potential oncolytic agent (*Pecora et al., 2002*).

Ulster

An incompletely replicating NDV mutant strain, **Ulster,** (see Table 1.4.), has been used in oncolysates for immunization against melanoma, as well as colon and kidney carcinoma (*Wildner, 2001*).

Concluding remarks

It has not been determined whether NDV oncolysates or repeated inoculation of NDV is more effective and which strains are the most effective ones. It also still needs to be determined how NDV therapy will interact with chemo- and radiation therapy and / or cytokines. One of the major concerns is, however, that we have no antiviral agents available to treat NDV infections in humans if any of the mentioned symptoms arise (*Wildner, 2001*). Potential new anti-NDV treatments are discussed in section 1.4.7.

1.4.6. Immune response to NDV infection

1.4.6.1. The avian response

Chickens have two kinds of IFN namely ChIFN1 and ChIFN2, which are homologous to mammalian IFN-α and IFN-γ respectively. Both of these ChIFNs are induced by NDV in chicken macrophages. Macrophages from NDV-infected turkeys have been found to exhibit depressed phagocytic and bactericidal capabilities. The ND virus can replicate in chicken macrophages, but peripheral blood lymphocytes and heterophils do not support such viral propagation and undergo apoptosis upon infection (*Seal et al., 2000 (b)*).

1.4.6.2. The murine response

NDV infection triggers a non-specific immune response: cultured murine macrophages produce IFN-α and -β, while resident peritoneal macrophages produce mostly IFN-β with little or no IFN-γ. The IFN genes are under transcriptional control of the *If-1,high* and *If-2,low* alleles. These alleles are expressed into IFN-inducible proteins, which in turn suppress cytokine expression in response to NDV infection (*Seal et al., 2000 (b)*).

1.4.6.3. The human response

The proposed molecular mechanism of the human immune response against NDV involves a lectin-carbohydrate recognition (without enzymatic function) between the HN glycoprotein (*Zeng et al., 2001*) presented on the virion surface or expressed on infected antigen-presenting cells (*Ertel et al., 1993*) and sialic acid expressing cellular receptors on human cells that naturally express INF-α (*Zeng et al., 2001*).

There appears to be three major immunological pathways that can be followed from antigen recognition to infected cell (or virion) destruction: one on cellular level and two on molecular level: (*Author's interpretation.*) Please refer back to Figure 1.5.

(i) **Cellular response:** ND virions or viral HN expressed on antigen-presenting cells or tumour cells have been found to exert a T cell co-stimulatory function (*Ertel et al., 1993*). These T lymphocytes in turn regulate IFN response (including IFNα, and -β (*Suzuki & Pollard, 1982*)), which triggers macrophage stimulation (*Hamburg et al., 1980*). A definite correlation exists between the virulence and the IFN producing ability of the strain, e.g. the nearly avirulent *La Sota* strain is a much poorer IFN inducer than the virulent *Herfordshire* strain (*Toneva, 1977*).

(ii) **IFN dependent pathway:** The type I IFNs are encoded by a cluster of genes situated on human chromosome 9p22. Most cells do not express these genes all the time, but transcription can be induced by many DNA and RNA viruses via the **transcription factors of the IRF family**. IRFs are apparently selective in their responses to different viruses: IRF-5 is activated by phosphorylation in response to NDV infection and induces transcription of an IFN profile that differs from the profile induced by other viruses. (*Weaver et al., 2001*).

(iii) **IFN independent pathway:** NDV (and other paramyxoviruses) can also induce **apoptotic pathways** in its host cell that are **independent of IFNs and / or p53**. One of these alternative routes involves the binding of interferon regulatory factor –3 (IRF-3) to DRAF-1 (not an IFN) forming an active transcription factor that induces a subset of genes whose products stimulate apoptosis. It is thought that this apoptotic response to the virus has evolved as a mechanism that limits viral dissemination (*Weaver et al., 2001*).

NDV-triggered immune response in cancer patients

NDV is a known potent IFN-α inducer in humans and is used safely as such in medical research fields e.g. immunology. In one such study, it was shown that cancer patients (and especially those with advanced disease) have no or very little natural cytotoxicity. Treating peripheral blood leukocytes (PBLs) with NDV normalized this immune response in virtually all patients that did not have disseminated malignancies. The same effect is observed when the PBLs were pretreated with IFN-α (*Kadish et al., 1981*). NDV is also a potent inducer of **TNF-production** (mainly TNF-α, which has cytolytic effects on neoplastic cells) by the infected individual's PBMCs. It has further been found that NDV also confers TNF susceptibility to certain TNF-resistant human cancer cells *in vitro* (*Lorence et al., 1988*).

Investigation of the effects of vaccinating renal cell carcinoma patients with irradiated autologous or allogenic tumour cells and NDV as adjuvant on the patient's cellular and humoral immunity proved interesting: in most cases, antibodies were formed against NDV proteins, but not against tumour cell related antigens. NDV-infected target cells were lysed more effectively than non-infected targets (*Zorn et al., 1997*).

Infection of tumour cells with NDV has further been shown to lead to changes in tumour cell surface adhesiveness and tumour immune costimulatory function. The virions adsorb to the tumour cell surfaces shortly (10 minutes) post exposure, which, within 5 – 24 hours leads to viral antigen expression on the cell surfaces at low antigen density (LAD). Further viral replication in the cytoplasm leads to high antigen density (HAD) viral antigen expression on the tumour cell surface. These cells then show an increased adhesiveness for erythrocytes and lymphocytes with increased lysis of the virus infected target cells. This virus-mediated cell adhesion is dependent on and a function of the amount of HN protein expressed in the cell. These NDV-modified tumour cells also cause the up-regulation of T-cell activation markers CD69 and CD25 (*Haas et al., 1998*).

In 1990, Csatary *et al* postulated that the regression of tumours and subjective improvement by vaccine therapy of patients could be the result of three events:

(i) The infection of tumour cells by the virus enhances the cells' immunogenicity.

(ii) Activation of the immune system, increased production of interferons, TNF-α and other cytokines.

(iii) Increased production of ACTH and β-endorphin by monocytes and lymphocytes, resulting in general well being (*Csatary et al., 1990; Sinkovics et al., 2000*).

Before we can apply our knowledge of any oncolytic virus to humans, we need to clarify issues such as those related to the human antiviral immune response (*Smith & Chiocca, 2000*). Our current knowledge of our bodies' immune responses against therapeutically administered oncolytic viruses is very basic. Both systemic and intratumoural routes of administration have shown similar results: toxicity is limited to injection site pain, transient fever and tumour necrosis. Administration of crude materials elicits a response that is short in duration, while more recent and advanced trials with genetically attenuated viruses suggest responses of longer duration (*Nemunaitis, 1999*).

It has been suggested, but not yet proven, that NDV's tumour cell activity is based on cancer-specific defects in the interferon signaling pathway *(Russel, 2002).*

1.4.7. Potential anti-NDV treatments

As specified in Table 1.1., a clinically approved treatment against NDV would be essential in order to safely treat a person with such a live virus. Several potential treatments are currently under investigation:

(i) A **20-amino acid peptide** (ADLKLEESNSKLDKVNVKLT) has been found to inhibit the formation of syncytiae after NDV infection. This inhibitory effect is exerted prior to the cleavage-activation of the F protein in the viral cycle. This peptide is conformationally stable and is thought to interact with the F protein in a very specific mechanism (*Young et al., 1997; Young, et al., 1998*). It therefore has much potential in the development of an anti-NDV treatment.

(ii) Other fusion-inhibiting peptides have also been identified: The **GST-HR2** is one that has additional solubility characteristics that allow large-scale production of the peptide as a paramyxovirus fusion inhibitor (*Yu, et al., 2002*).

(iii) **PAP** (pokeweed antiviral protein) has anti-NDV properties when used in combination with guanidine or ribavirin:

- PAP & guanidine are synergistic at low, medium and high concentrations.
- PAP & ribavirin also produce synergistic effects at low and medium concentrations, but are antagonistic at high effective concentrations (*Weaver & Aron, 1998*).

(iv) The majority of **neutralizing MAbs** (monoclonal antibodies) to the HN protein prevents the virus from attaching to the cellular receptors and inhibits virion-induced FFWO and FFWI. This fusion-inhibition is thought to be the result of preventing HN-mediated bridging of fusion partners. Certain MAbs only neutralize FFWO by a mechanism other than attachment prevention, of which the nature still has to be established (*Iorio et al., 1992*).

1.5. XENOGRAFTING IN IMMUNE COMPROMIZED MICE

In experiments where human tumours were xenografted to immune compromized mice, it has been shown that a single intratumoural injection of the NDV strain 73 T causes tumour regression, which is sometimes complete and / or lasting. These results were successful with both human neuroblastomas and human synovial sarcomas. It has also been found that oncogene expression may contribute to the long-lasting effect of the NDV treatment (*Lorence et al., 1994 (b)*).

Various trials involving a wide spectrum of tumours xenografted in athymic mice proved certain strains of NDV to be very effective in tumour treatment when administered either IT or IP (*Phuangsab et al., 2001*).

A single local (IT) 73 T injection has been shown to cause durable, complete regression of human neuroblastoma and fibrosarcoma xenografts, while inhibiting the growth of epidermoid, colon, lung, breast and prostate carcinoma xenografts. IP dosage also proved to be effective against subcutaneous neuroblastoma xenografts (*Russel, 2002*).

Some variability in the response to treatment within each experiment was observed: while most of the carcinomas showed pronounced tumour growth inhibition, others underwent durable complete regression (CR). With some tumours there seems to be a dose-effect when administered either IT or IP: where the normal dose (10^7 PFU) of 73 T had no effect, a higher dose (ranging between 10^8 PFU and 5×10^9 PFU) caused durable CR. The mechanism of this dose-effect is not known, but numerous possibilities exist:

(i) viral binding to normal cells or tissues,

(ii) virus inactivation before it reaches the tumour site (with systemic administration) and

(iii) regrowth of virus-resistant tumour cells after the initial NDV challenge.

Contrary to the last possibility, additional NDV injections have been shown to be effective after tumour progression in response to initial NDV treatment: resistance does therefore not seem to develop after the first virus dose. Sometimes even UV-inactivated NDV had a small but significant effect on tumour growth (*Phuangsab et al., 2001*).

In summary:

(i) NDV 73 T administered to nude mice either IT or IP is an **effective antitumour therapy** in that system.

(ii) **Replication competency is necessary** for maximal effect.

(iii) **Multiple NDV doses** may be **more effective** than a single dose (*Phuangsab et al., 2001*).

However, to be able to employ this virus as a safe and clinically useful therapeutic agent, we need to identify the mutation(s) responsible for certain strains' oncolytic activity, as well as the mechanisms responsible for its specific tumour cell killing properties (*Wildner, 2001; Pecora, et al., 2002*).

Such a model seems ideal for evaluating the effect of previously untested NDV strains on tumours *in vivo*, since similar tests have been done successfully. In order to justify testing a virus in a living organism, *in vitro* tests would first have to be done.

1.6. AIM OF STUDY

The aim of this project was to evaluate the oncolytic efficiencies of two strains of the Newcastle disease virus. First, in order to determine whether either virus had any effect on tumour cells, initial experiments were done *in vitro* on two different types of cancer cell culture. For control purposes, the same experiments were done on a human embryo lung cell line, to assess whether either virus strain would have any effect on normal human tissue.

It was then determined whether intratumoural injections of either virus strain had any effect on tumour growth *in vivo* in a nude mouse xenograft model.

The ultimate aim is of course the development and implementation of a novel cancer therapy that is safe and cost-effective.

CHAPTER 2

2.1. MATERIALS & METHODS

2.1.1. MATERIALS

Cell lines

1. The MRC-5 human embryo fibroblast cell line was a gift from Dr. Wilma Vergeer from the *SA National Control Laboratory*.
2. The SNO1 esophageal carcinoma and the HeLa cervix uteri carcinoma cell lines were acquired from *Highveld Biological (P.O. Box 1456, Lyndhurst, 2106)*.

Cell culture

1. 15 cm^2 and 75 cm^2 cell culture flasks (*Nunclon*).
2. 24 - well microtitre plates (MTP's) (*Nunclon*).
3. 0.1 μm disposable tissue culture filters (*Nalgene*).
4. Lab-Tek chamberslides (*Nalge Nunc International, 2000 North Aurora Road, Naperville, IL 60563*).
5. Neubauer cell counter.
6. RPMI 1640 growth medium (*Highveld Biological*).
6. Minimal Essential Medium (MEM) (*Highveld Biological*).
7. Bovine Fetal Serum (BFS) (*Highveld Biological*).
8. Trypsin (0.25 % in distilled water) (*GIBCOBRL, Life Technologies*).
9. EDTA-free trypsin (0.25 % in growth medium) (*Highveld Biological*).
10. Penicillin / Streptomycin / Fungizone (100 U / ml) (*Highveld Biological*).
11. DMSO (*Merck, P.O.Box 6803, Bloemfontein, 9300*).
12. Crystal Violet (*Merck*).
13. Iodine (*Merck*).

14. Safranine (*Merck*).
15. Annexin-V-FLUOS Staining Kit (*Roche*).
16. Phosphate Buffered Saline (PBS) (*Oxoid*).
17. 15 ml Falcon tubes (*Becton Dickinson*).
18. Screw-top cryopreservation tubes (*Nunclon*).
19. Cryo 1 °C freezing container (*Nalgene*).
20. Absolute ethanol (*Merck*).

Virus strains & antiserum

1. *La Sota* is a vaccine virus that was cultured from the TAD NCD vaccine (*Immuneovet Services, P.O.Box 6035, Halfway House, 1685*).
2. *Texas GB* was imported from Dr. Denis Alexander (*Central Veterinary Laboratory, Weybridge, England*).
3. The antiserum was imported from *SPAFAS, USA*.

Microscopy

1. Phase contrast microscopy was done on a *Nikon TMS* inverted phase contrast microscope.
2. Fluorescence microscopy was done on a *Nikon Eclipse E400* equipped with a 100 W excitation system and a filter slider for observing green, blue and orange light.

Immune compromized mice

1. Immune compromized (athymic) mice of the MF-1*nu* strain (*Central Animal Service, University of the Witwatersrandl*).
2. Virukill *(ICA International Chemicals, P.O.Box 2312, Stellenbosch)*.
3. Soluble Vitamins and Electrolytes for Poultry (*Solvay Animal Health, P.O.Box 1785, Kempton Park, 1620)*.
4. Formalin / Formaldehyde (37 % isotonic solution) (*SIGMA, P.O. Box 4853, Atlasville, 1465*).

2.1.2. METHODS

2.1.2.1. General cell culture techniques

Cryopreservation

The growth medium was removed from the culture and the cells washed with 5 or 10 ml PBS per 50 cm^3 or 250 cm^3 flask, respectively. Cells were detached from the culture flask bottom by trypsinization (5 ml or 10 ml per 50 cm^3 or 250 cm^3 flask) for 2 – 4 min until 90 % of the cells were detached. Trypsin was used for SNO1 and HeLa cells, while EDTA-free trypsin was used for the more sensitive MRC-5 cells. Each flask's cell suspension was transferred to a 15 ml Falcon tube and pelleted in a *Hettich Universal II* centrifuge at 1800 rpm for 3 - 4 min. The trypsin was discarded and the cells resuspended in 10 ml washing medium (20 % BFS and 80 % RPMI 1640 for SNO1; 20 % BFS and 80 % MEM for HeLa and MRC-5 cells. The resuspension / centrifugation – cycle was repeated twice in order to wash all the trypsin and debris from the cells. The pellets were then resuspended in 3 ml medium consisting of 70 % RPMI 1640 or MEM, respectively, 20 % BFS and 10 % DMSO to keep the medium from crystallizing and puncturing cells. This 3 ml cell suspension was quickly divided into three 1 ml screw-top cryopreservation tubes and transferred to a Cryo 1 °C Freezing container and frozen at –70 °C.

Thawing frozen cells

The cryopreserved tube was submerged in a 37 °C waterbath for 40 – 50 s, until the outer liquid started to melt. The tube was then quickly washed with 70 % ethanol and the frozen pellet in the center of the tube, together with the melted liquid, transferred to a 15 ml Falcon tube with 10 ml medium containing 20 % BFS. Careful resuspension was followed by centrifugation at 1800 rpm for 3 min. The cells were then washed clean of any DMSO (which is toxic) by two centrifugation / resuspension cycles with 10 ml fresh medium (20 % BFS and 80 % medium). Finally, the cells were resuspended again in 10 - 15 ml 20 % BFS - containing medium, 100 U / ml penicillin / streptomycin / fungizone was added as preventative antibiotic and the suspension was transferred to a 50 cm^3 culture flask and incubated at 37 °C and 5 % CO_2.

Subcultivation

All medium was discarded from the confluent culture and the cells rinsed once with 5 ml PBS. Cells were trypsinized for 2 - 4 min and the suspension transferred to a 15 ml Falcon tube. The cells were pelleted for 3 min at 1800 rpm and resuspended in 10 ml 20 % BFS – containing medium. This washing cycle was repeated twice with more of the same medium and the cells finally resuspended in 15 ml 20 % BFS - containing medium containing 100 U / ml antibiotic mixture. The 15 ml cell suspension was then divided between three new culture flasks and incubated as before.

When using the 250 ml culture flasks, the total medium volume was adjusted to 20 ml per flask after dividing the cell suspension.

Gram stain

Gram stains were done to detect and analyze bacterial contamination of cultures, media and incubators. ~ 1 ml of the suspect culture medium was spread onto a microscope slide and allowed to air dry in a sterile laminar flow cabinet and lightly fixed over a flame. Crystal violet was applied to stain Gram positive bacteria blue and rinsed off with tap water after 45 sec. The colour was fixed with iodine for 30 sec and the iodine also rinsed off with tap water. Destaining was done with 80 % ethanol for 15 sec and the slide again rinsed with tap water. Gram negative bacteria were then coloured red with Safranine for 45 sec and the slide finally rinsed with tap water and dried over a flame.

2.1.2.2. The effect of NDV on normal cells

The slow-growing MRC-5 cells were cultured for 5 days (until confluent) in a 24 - well MTP and given 1 ml fresh medium (10 % BFS and 90 % MEM) per well as needed. Cells were grown until the cultures in most of the wells have reached ~ 80 % confluence. On day 5 all medium was removed, cells were carefully washed twice with 1 ml PBS per well and incubated at 37 °C with 100 µl of 5 serial 10 × dilutions of either virus, or an equal amount of PBS as control, for 20 min. This ensures optimal viral infection. Four wells were infected with each dilution. 1 ml of 5 % BFS-containing MEM with 100 U / ml antibiotic (maintenance medium) was then added to all the wells and the progressive results observed with phase contrast microscopy and recorded daily for five days.

The medium was not replaced during the course of the experiment, in order to achieve the maximal accumulative effect of the virus on the cells.

2.1.2.3. The titration of NDV on cancer cells

SNO1 and HeLa cells were grown for two days in 24 - well MTPs on growth media (20 % BFS - containing RPMI 1640 and MEM respectively) until ~ 80 % confluence has been reached in most of the wells. On the second day the cultures had grown to confluence. All media was then removed, the cells were washed once with 1 ml PBS per well and then covered with undiluted virus, a 10 × dilution series (100 µl viral dilution per well) of either of the two virus strains or PBS as control. Each dilution was done in quadruplicate. Attachment and infection was allowed for 20 min at 37 °C, whereafter 1 ml of maintenance media was added per well. The media was not replaced during the course of the experiment, in order to get the maximal effects of the viruses. [The resultant lowering in pH would also represent the *in vivo* conditions more accurately, since cancer growth mostly occurs in an acidic environment (*San Romàn et al., 1999*).]

Visual observations of the cell status were made and recorded daily for five days and the Endpoint Infective Doses (EID_{50}) determined with the method described in *Reed & Muench, 1938.*

2.1.2.4. Viral neutralization

HeLa cells were grown for two days in four 24 - well MTPs on 10 % BFS MEM. Cell growth was allowed until the cultures in most of the wells have reached ~ 80 % confluence. Four - fold dilution series (up to 10^{-5}) of both virus strains were made with PBS as control. A dilution series (up to 10^{-3}) of sterile chicken antiserum was also made with PBS as control. [The antiserum was isolated from a chicken that lived in sterile isolation and was vaccinated with *La Sota* and later challenged with *Texas GB*. It should therefore have antibodies against both of these viral strains. It was not exposed to any other infectious agent(s) and should therefore have no additional neutralizing antibodies.] 100 µl of the antiserum series was added to each of the viral series dilutions so that one of each *viral concentration / antiserum concentration* combination was represented. The combinations were incubated at 37 °C for 30 min to allow the antibodies to bind to the virus particles. This mixture was then

added to the wells and incubated for 20 min at 37 °C to allow infection. 1 ml maintenance medium was added to each well.

The MTP's were incubated and observed daily for five days and the results were recorded daily.

2.1.2.5. Apoptosis analysis

In order to quantify the degrees of cell death caused by the two virus strains and also to differentiate between apoptosis and necrosis, apoptosis analysis was done with an Annexin-V-FLUOS Staining Kit.

During early apoptosis, several changes occur at the cell surface, e.g. phosphatidylserine (PS) is exposed on the cell surface as it is translocated from the inner part of the cell membrane to the outer part. Since Annexin V is a calcium - dependent phospholipid - binding protein with a high affinity for PS, it can be used as a sensitive probe for PS exposure on the outer leaflet of the cell membrane. Since necrotic cells also expose some PS due to loss of membrane integrity, they can be differentiated form apoptotic cells by the simultaneous application of a DNA stain (apoptotic cells do not expose DNA extracellularly, while necrotic cells do).

Culture preparation: MRC-5, HeLa and SNO1 cells were grown in coverslip chambers in 4.5 ml 20 % BFS MEM or RPMI 1640 with 100 U / ml antibiotic mixture per chamber for one day, until an ~ 80 % confluence monolayer culture was reached in most of the chambers. Each chamber was then washed twice with 2 ml PBS and treated with a 100 µl aliquot of a 10^7 IU of either virus or an equal amount of PBS as control. Infection was allowed for 20 min at 37 °C. 4 ml of the 5 % MEM or RPMI 1640 with 100 U / ml antibiotic was then added to maintain the cultures. The chamber slides were then incubated as usual and one of each was analyzed at the following time intervals post infection: 1 h, 24 h (one day), 48 h (two days), 72 h (three days) and 96 h (four days).

Apoptosis analysis: All media was removed from the chambers and each chamber washed twice with 2 ml PBS. Chambers and silicone borders were removed from the slides and each slide labeled with 104 µl per sample of the labeling solution that consisted of:

a) 100 µl HEPES buffer
b) 2 µl Annexin-V-Fluorescein (stains PS on apoptotic and necrotic cell membranes fluorescent green) and
c) 2 µl Propidium Iodide per sample (stains DNA leaking from necrotic cells fluorescent red).

The cells were covered with coverslips and incubated at 25 °C for 10 – 15 min. The slides were then analyzed by means of fluorescence microscopy at the specified wavelengths (excitation: 488 – 540 nm & emission 518 – 617 nm).

Observations were recorded and photomicrographs taken of representing areas of each slide with both signals, red signals only and green signals only in order to differentiate between apoptosis and necrosis and the results were compared. After the initial analysis, the fluorescently labeled slides were kept at 4 °C for comparison to the next days' results.

2.1.2.6. Oncolytic efficiency of NDV in immune compromized mice

Mice

12 immune compromized mice of the MF-1*nu* strain were kept in a sterile flexible film isolator at ~ 26 °C. The 6 - week old group consisted of 8 females and 4 males. Food was sterilized by autoclaving. Drinking water was sterilized by addition of 0.5 ml *Virukill* in 5 litres of water. Vitamins and electrolytes were added to the water in a concentration of 0.05 %. Containers, bedding and measuring instruments were sterilized with *Virukill* (1 %) in the inlet port of the isolator. Contact time of 30 min was allowed.

Tumour growth in immune compromized mice

HeLa cells were grown in 250 cm^3 flasks until ~ 80 % confluence was reached. Cells were trypsinized and washed three times by centrifugation at 1800 rpm and resuspension in 10 ml 20 % BFS - containing MEM. The pellet was then resuspended in 1 ml sterile PBS and a cell

count was done with a *Neubauer* cell counting slide. 100 µl aliquots were injected subcutaneously into all 12 immune compromized mice. Tumour growth was monitored twice a week for the duration of the trial: length (L), width (W) and height (H) were measured (in millimeters) for each tumour and the tumour volumes determined with the following equation:

$$\textbf{Tumour volume} = (\pi \times L \times W \times H) / 6 \quad (\textit{Lorence et al., 1994 (a\&b)})$$

Where one injection did not result in tumour growth within two weeks, a second was administered. Where a tumour still did not grow, tumour transplants were done from one donor (a female that was then excluded from the rest of the study) to several individuals.

Treatment of subcutaneous tumours

Before treatment, the mice were divided into three groups: the control group consisted of two females and one male and was kept in the same isolator. The second group also consisted of two females and one male and the group was transferred to a separate isolator. The third group consisted of two females and two males and was transferred to a third isolator. Separate isolators were used to avoid cross - infection of the virus strains.

The first group was injected intratumourally with 100 µl of PBS per mouse. The second and third groups were injected intratumourally with 10^7 IU in 100 µl PBS of the *La Sota* or *Texas GB* viruses respectively, (as determined from results obtained in 2.1.2.3.). Where a mouse had developed more than one tumour, only the largest tumour received a single dose of treatment. Tumour measurements were recorded twice a week for 4 weeks.

Euthanization

Mice were euthanized with formalin at the end of the trial, or when their tumour diameter exceeded 20 mm in any one dimension.

Histology

Before the tumours were treated, one tumour from the xenograft - donor mouse was sent for histological examination to confirm the tumour type. After the mice had been euthanized, one of the control group's tumours, two of *the La Sota* group's and two of the *Texas GB* group's tumours were dissected and sent for histological examination to confirm the tumour type once more and to see if the treatment altered the tumour histology in any way. The histology was done by Dr. V. Jaspec, Dept. Anatomical Pathology, UFS, Bloemfontein.

CHAPTER 3

3.1. RESULTS & DISCUSSION

3.1.1. The effect of NDV on normal cells

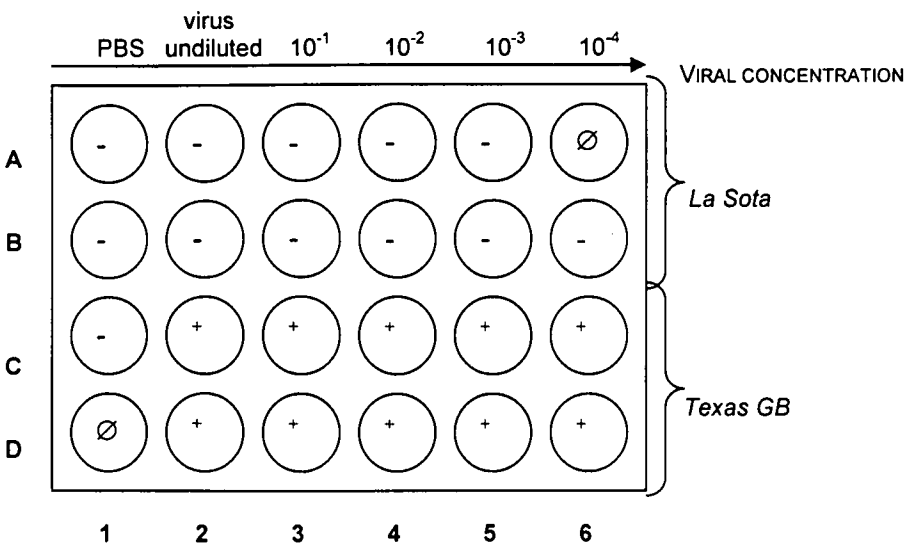

Figure 3.1.

The effect of NDV on MRC-5 cultures. Results are shown as observed three days post infection.

Legend:	+	positive for virus infection
	(+)	uncertain positive
	-	negative for virus infection
	∴	too much cell debris to be sure: results not considered
	∅	bacterial contamination visible: results not considered

In order to determine whether either ND strain would be harmful to non - cancerous human tissue, titrations were done on the human embryo lung cell line.

Figure 3.1. shows a shematical representation of the results obtained in response to the titrations on the MRC-5 cells as recorded three days post infection: it is clear that none of the *La Sota* dilutions showed any signs of deterioration, compared to the PBS - treated controls. No cytopathic effects (cpe) or additional cell death was observed. The small amount of cell death that did occur was also present in the PBS-treated control wells and

can therefore be ascribed to non-viral factors like metabolite accumulation and nutrient depletion. This shows that the *La Sota* strain is harmless to normal human fibroblasts. This suggests that it could also be harmless to other non-cancerous tissues in the human body.

In comparison with the controls, all dilutions of *Texas GB* caused severe cpe and cell death. One of the PBS-treated control wells was contaminated with bacteria, thus excluding it from the experiment. The other three control wells, however, differed so drastically from the *Texas GB*-infected wells, that the results are still clear. This could mean that the highly virulent *Texas GB* is harmful to human fibroblasts, suggesting that it may also be harmful to other non - cancerous tissues in the human body.

The results obtained with *Texas GB* infection could possibly be due to a higher virus titre, so that even the lower dilutions of the virus still have high viral multiplicities that can cause such cpe. However, even dilutions overlapping with that of the *La Sota* strain showed more pronounced effects, confirming *Texas GB* as a very virulent virus.
It has to be kept in mind, however, that MRC-5 is an embryonic cell line that may perhaps be more susceptible to infections than fully differentiated adult tissues. It is also derived from lung tissue, which is part of the respiratory pathway that is affected by more virulent NDV strains in birds. Also, even "normal" cell lines are not flawless: some mutation or adaptation would be necessary for any cells to proliferate in culture. The effect of oncogenic transformation of normal cells regarding NDV susceptibility has been shown (*Norman et al., 2001*).

Considering the above, these results suggest that *Texas GB* may possibly infect certain types of normal human tissue (most likely those affected by laryngitis and / or conjunctivitis as occurs in the infrequent event of human NDV infection) and may therefore not be safe for therapeutic use.

Additionally, although human - to - human infection of NDV has never been reported (*Alexander, 2000*), commercial distribution of a strain that is hazardous to wild birds and poultry alike has the potential to create a biological and economical disaster, even under the most controlled circumstances. It is therefore not recommendable to further develop *Texas GB* as a cancer therapeutic, however, for comparative purposes it is still included in the rest of this study.

3.1.2. Titration of NDV on cancer cells

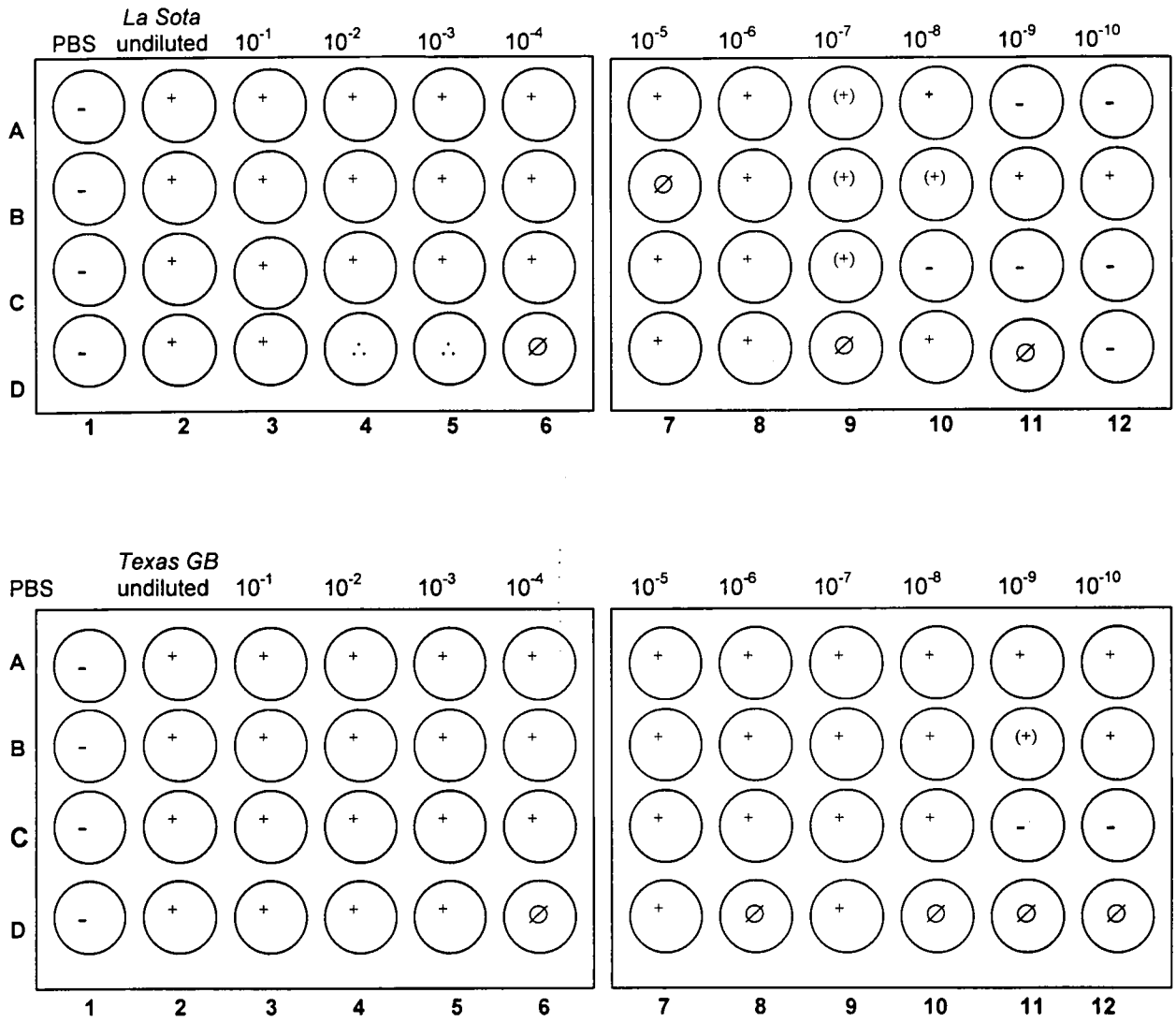

Figure 3.2.

The effect of *La Sota* (top panel) and *Texas GB* (bottom panel) on HeLa cells 3 days post infection. The results for the SNO1 cell line were similar and are not shown. The symbols are explained in Figure 3.1.

Titrations of both ND strains were done on both cancer cell lines in order to see if either strain induced any cpe in one or both cancer types. Representative results are shown in Figure 3.2.

La Sota

Infection of both the SNO1 and HeLa cell lines with the *La Sota* strain resulted in similar consequences in both cell lines: cell shape change, cell shrinkage and the formation of small (two to four - cell) syncytia within 24 hours (results not shown). After three days (Figure 3.2.), cell coverage appeared uneven compared to the evenly distributed appearance of the PBS - treated control cultures. Gradual worsening of the cpe over a period of another two days led to the formation of large (ten to fifteen cell) syncytia, cell shrinkage, cell compartmentalization, lysis and more than 50 % cell death.

Texas GB

The effect of *Texas GB* infection on both cell lines was much more defined by 24 hours (results not shown) than on the *La Sota* - infected cultures: more and larger syncytia, more and larger areas of morphologically deformed cells, as well as small foci of cell death were visible. By three days post infection (Figure 3.2.), the cpe was clearly more pronounced than that of the cultures infected with *La Sota* and the PBS-treated controls. Lower dilutions of this strain caused total destruction (100 % cell death) within five days, while higher dilutions only took a little longer to produce the same results.

La Sota vs. Texas GB

Based on the daily records of visual observations, the cpe were dose-dependent for both virus strains on both cell lines. With *La Sota*, this could clearly be seen in the relative amounts and severity of cpe and cell death over the whole seven - day period of incubation. In the case of *Texas GB*, the effects of all dilutions of the virions appeared to be much more aggressive and destructive and presented within a few hours post infection.

The visible effects of both viral strains became progressively more pronounced over time, suggesting that the virions actively propagated in the cells, releasing new progeny virions

that could then infect the next cells. Alternatively, this effect could be due to the lowering pH in the culture medium (as a result of the metabolic processes of the live cells) over the trial period: a more acidic environment has previously been shown to aid NDV infection of cultured cells (*San Román et al., 1999*).

The PBS-treated control wells (A1, B1, C1, D1) seemed to be largely unaffected by the treatment. An expected decline in cell density was apparent after a few days: this could be ascribed to accumulation of toxic metabolites in the medium, lowered pH due to metabolite- and CO_2-buildup and depletion of nutrients from the medium. This cell death was distinguishable from cell destruction due to viral infection as follows:
Virus-induced cell death caused foci of deformed and / or dead cells, leading to an uneven appearance of cell distribution with pieces of cell membrane and other debris still attached to the flask surface where the cells had died and the surrounding cells showing signs of deterioration in terms of morphological changes (cell shrinkage and deformation).
Where "normal" cell death occurred, there were no unevenly distributed, well-defined foci of cell death, rather individual cells disappeared from between their neighboring cells without disturbing or affecting them. The cultures still appeared even and the remaining cells healthy. Small openings between cells occurred evenly throughout the monolayer, not seemingly at random as with the viral infections. These openings were also more regularly shaped and had no cell debris still attached to the flask and / or surrounding cells.

Where *La Sota* was added at full concentration (A2, B2, C2, D2), some cells were still left on the fourth day post infection, although they showed severe morphological deformation: cells were either shrunken and partially detached from the flask surface, or appeared to be products of cell fusion, with multiple nuclei enclosed in large volumes of cytoplasm and irregularly shaped cell membranes. In the next ten rows, the cpe's were dose - dependent: low viral dilutions (e.g. A3, B3, C3, D3) correlated with lower cell density and more severe cpe's, while higher dilutions (A10, B10, C10, D10) resulted in less severe cpe's and the remainder of more cells, which were also more viable.

Because *La Sota* had no effect on the fibroblast cell line and a lytic effect on two cancer cell lines, it can be described as oncotropic and cytolytic, and therefore **oncolytic**. *Texas GB*, on the other hand, should then be described as **cytolytic**.

According to the method described previously by *Reed & Muench (1938)*, the endpoint infective dose (EID_{50}) of the *La Sota* stock was found to be 1.7×10^7 IU / 100 µl, while that of *Texas GB* was 2.4×10^9 IU / 100 µl. The difference in cpe caused in the cell cultures by the different virus strains could therefore be accounted for not necessarily only by the difference in virulence, but also by a difference in the virion concentration of the stock solutions.

In summary: titrations of both virus strains confirmed the dose-effect that was previously observed. At high concentrations, the effects of the viruses were very obvious and quite devastating. At lower concentrations, these effects took longer to appear and were more mild. Even at very low concentrations, some cpe were still visible. This suggests that the low multiplicities of virions might propagate in the cancer cells, eventually producing visible cpe. The stock concentration of *Texas GB* was higher than that of *La Sota*, possibly accounting for the more severe cpe observed in the cultures infected by *Texas GB*.

3.1.3. Viral neutralization

Neutralization experiments were conducted on the two NDV strains to ensure that the level of cpe seen on the cell lines can be contributed to the virus and not to some additional factor in the viral stocks.

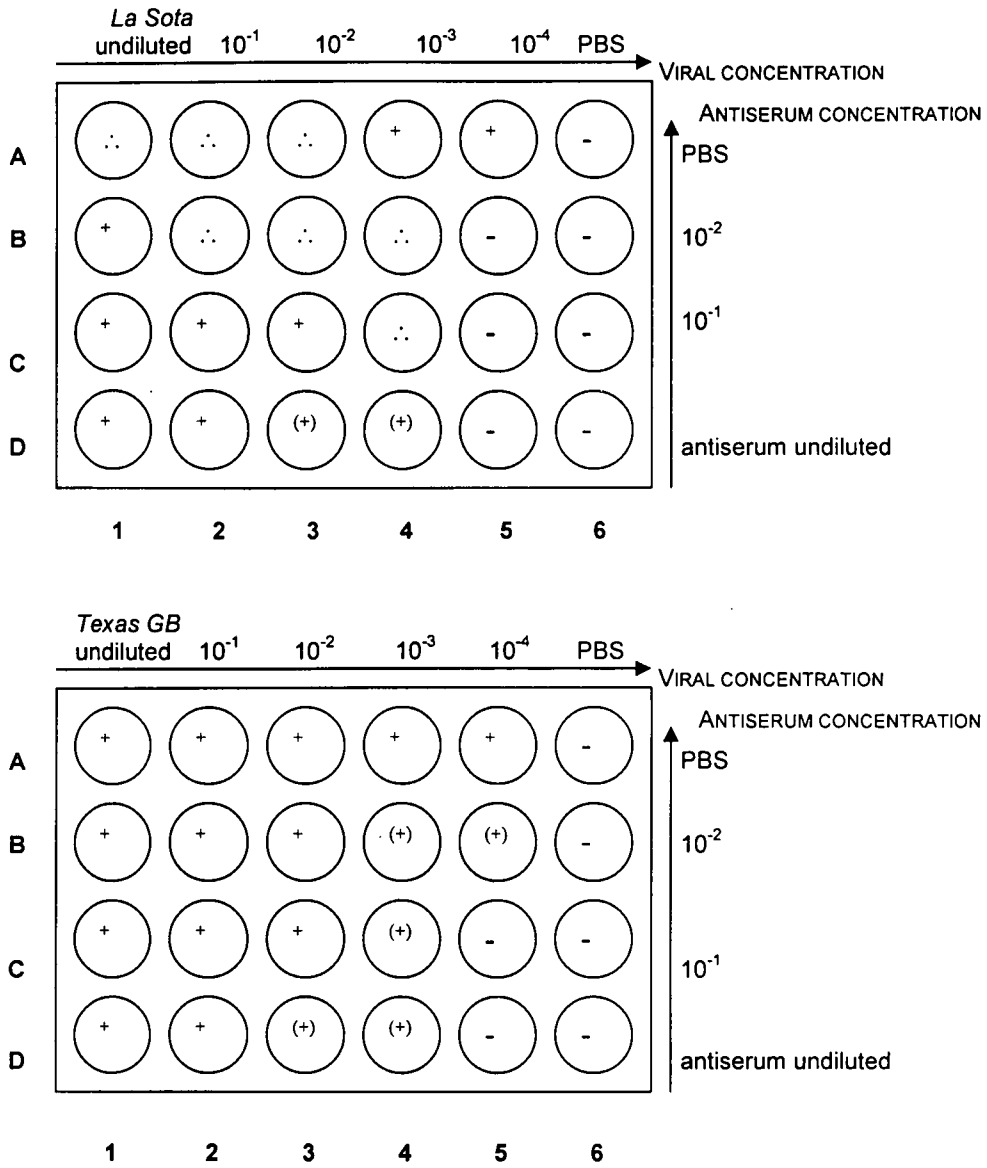

Figure 3.3.

Neutralization of *La Sota* (top panel) and *Texas GB* (bottom panel) by antiserum as observed in HeLa cell cultures 3 days post treatment. Symbols are explained in Figure 3.1.

La Sota

The following discussion is based on results as shown schematically in Figure 3.3.

- Where PBS as control replaced both the virus and the antiserum (A6), no adverse effects were visible on the cancer cells. This showed that no external factor was responsible for the results obtained.
- In the row of controls with PBS instead of antiserum (A1 – A5 horizontally), there was no protective effect and the results clearly showed the full effect of the viral dilution series on the cells.
- In the other control row (A6 – D6 vertically), where PBS replaced the virus and the cells only received the antiserum dilutions, there were no adverse effects on the cancer cells. This showed that the antiserum alone had no negative effect on the cells and that the cpe seen in the other wells can definitely be designated to the cytolytic effects of *La Sota*.
- In the wells with low *La Sota* dilutions and low antiserum concentrations (B5 & C5), the cells seem to have undergone no damage: no side effects were observed. The low antiserum concentrations therefore effectively neutralized the low virus concentration, rendering the virus ineffective and harmless.
- At low virus concentrations in combination with high antiserum concentrations (D5), little or no side effects were observed, confirming that the antiserum alone had no adverse effects on the cells. This means that all the virions have been neutralized by the antibodies in the antiserum.
- In the wells where both the virus and antiserum concentrations were high (D1 & D2), the antiserum seems to have had little or no protective effect on the cells. This is probably due to insufficient antiserum to inactivate all the viral particles. Unbound virions are therefore free to infect, propagate and reinfect the next cells.
- Where higher antiserum concentrations were combined with lower viral titres (D3 & D4), less cpe were observed, the cpe were also less severe. The small amount of cell death could also be explained by unbound virions, although initially present in smaller quantities than D1 and D2.
- Where low antiserum concentrations were combined with high *La Sota* concentrations (B2 & B3), only large amounts of cell debris were observed. It could therefore be assumed that all the cells had died. This means that the concentration of neutralizing antibodies in the antiserum was too low to protect the cells from the effects of the virus, allowing the virus to infect and kill the cancer cells.

- Although the results shown were recorded three days after treatment, over the five-day incubation period there was a slower onset of cpe in the wells that received high antiserum concentrations (C2 – C5, D2 – D5).
- Over the incubation period of five days, cultures with a higher serum concentration and lower viral titre contained normal-looking viable cells for up to the end of the incubation period, where the wells that received a high virus titre combined with a low serum concentration, had no normal cells left by day four and almost no viable cells left by day five.

In such a neutralization experiment, a 4 log reduction in virus titre is considered to be a positive result. The antiserum was therefore effective at neutralizing the adverse effects of *La Sota* on the cell culture.

Texas GB

It is clear from Figure 3.3. that the overall result for *Texas GB* is comparable to that of *La Sota*, although the higher viral titre is evident. The antiserum neutralized the cpe at low viral concentrations, but failed to do so at higher multiplicities of the virus. As with *La Sota*, the results are not clear-cut, but considered to be positive due to a sufficient reduction in the viral titre.

Since the chicken that the antiserum was isolated from was only exposed to the two viruses employed in this study, these results confirm that the cpe and subsequent cell death observed here can be attributed to *La Sota* and *Texas GB* and not to an additional agent present in the viral stock. This experiment was therefore successful in proving that the cpe observed is due to NDV infection and therefore that NDV causes cell death in cancer cell cultures.

3.1.4. Apoptosis analysis

In order to get insight into the mechanism of the cell death induced by NDV, infected cultures were studied for signs of apoptosis. If tumour cells could die off *in vivo* by apoptosis rather than necrosis, the organism hosting the tumour would be spared an inflammatory reaction to large - scale cell lysis. Tables 3.1., 3.2. and 3.3. compare the visual observations made during the course of the experiment. Figure 3.4. shows comparative photomicrographs of the results.

Table 3.1.
Annexin-V results with MRC-5 cells.

MRC-5			
time	**PBS**	**La Sota**	**Texas GB**
1 h	Few single red signals evenly distributed throughout culture. *Necrosis.*	Few single individual red signals, like in control. *Necrosis.*	Few single individual red signals, like in control. *Necrosis.*
24 h	Even distribution of single individual red signals. *Necrosis.*	No fluorescence. Phase contrast microscopy confirms the presence of healthy fibroblasts. *No cell death* detected.	Even distribution of individual red signals. Phase contrast microscopy confirms the presence of healthy fibroblast culture. *Necrosis.*
48 h	Hardly any signals. Mostly red. Phase contrast microscopy confirms the presence of healthy fibroblasts. Small amounts of *apoptosis* and *necrosis.*	Few red and few green signals, same as control. Even distribution. Little *apoptosis* and *necrosis.*	Single red green or bicoloured signals, same as control. Even distribution. *Apoptosis* and *necrosis* in small amounts.
72 h	Single red and green signals, more necrosis than previously. Some signals of both colours. *Increased necrosis. Little apoptosis.*	Individual signals of red or green or both colours, same as control. Little *apoptosis*, more *necrosis.*	Few red signals evenly spread throughout the culture. Single green signals and signals of both colours. *Necrosis* and a little *apoptosis.*

Table 3.2.
Annexin-V results with HeLa cells.

HeLa			
time	**PBS**	**La Sota**	**Texas GB**
1 h	Only a couple of signals visible. All signals are green and red and are from detached cells that coming loose from the growth surface. *Necrosis.*	In total more signals than on control slide. Few signals of both colours, but lots of green signals coming from cells that are still attached to the slide. *Little necrosis, more apoptosis.*	In total more signals than other slides. All signals are green and about half of the green signals are also red. Signaling cells are also still attached to the slide. *Same amount of necrosis and apoptosis.*
24 h	A few signals of either colour, in total more than were visible at 1 h post infection: *Apoptosis* and *necrosis.* Signals are evenly distributed throughout the culture.	In total more signals than on the control slide. Signals are mostly green. Red signals are also green. *Little necrosis, more apoptosis.*	Signals occur in small groups, suggesting plaque formation or syncytiae. These foci contain both signals while the surrounding areas contain only green. *Widespread apoptosis with necrosis in foci.*
48 h	Single cells of green or double signals on a black background. More cell death than in SNO1 equivalent slide. Signaling cells are well-defined single cells detatched from the slide surface. *More or less the same amount of apoptosis and necrosis occur evenly in single cells throughout the culture.*	A little more cell death than on the control slide. Cell death occurs in clumps of bright green signals with several well-defined red dots in the center of the foci. Background looks like a sea of tiny green specks, few single cells are distinguishable. Single cells are well defined on the edges of the foci, suggesting plaque formation rather that cell fusion. *Plaques of mixed apoptosis and necrosis on a background of early apoptosis.*	More cell death than on the control slide. Foci look like on the La Sota-treated slide, except that the background has more and brighter fluorescing green specks. *Plaques of mixed apoptosis and necrosis with more or more advanced apoptosis in the rest of the culture.*
72 h	Very few signals: about half of the signals are green and half are both colours. Signals are evenly spread and occur as single cells throughout the culture. Background now also has slight green specks. *Same amount of apoptotic as necrotic cells on an early apoptotic background.*	Much more bright green and double single-cell signals throughout culture. Green foci (with a fewer red dots in the centers than previous day) occur on a background of relatively bright green specks and whole-cell signals, suggesting *later-stage apoptosis with single cells already in full-blown apoptosis. Widespread apoptosis with foci of mixed apoptosis and necrosis.*	Background looks like La Sota-treated culture, while foci are generally larger and contain more red signals. *Widespread apoptosis as in La Sota-treated culture, with plaques of mixed apoptosis and necrosis, but with more necrosis than the La Sota-treated culture.*
96 h	A lot of *necrosis* (cells with both signals) in single cells is visible, cell death distribution is regular.	Widespread foci of both signals occur on a background of green (*apoptosis*). In total there is *more apoptosis than necrosis*, with *necrosis occurring mainly in foci.*	A very green background with necrosis occurring mainly in foci and in single cells on the *apoptotic background.*

Table 3.3.

Annexin-V results with SNO1 cells.

SNO1			
time	**PBS**	**La Sota**	**Texas GB**
1 h	Overall fewer signals were observed than on the HeLa control slide. Signals are also of single cells evenly distributed throughout the culture: couple of green signals, but most signals were both colours. *More necrosis than apoptosis.*	Overall more signals than on the control slide. Signals are mostly green, red ones that do show up are also green. *More cell death than on control slide. More apoptosis than necrosis, more apoptosis than on control slide.*	More or less the same as the La Sota-infected slide, more green signals than red ones and the ones that are red, are also green. *More apoptosis than necrosis, overall more cell death than on control slide. Same as La Sota-infected slide.*
24 h	Couple of signals visible. Background is black: no hazy green sea as in virus-treated slides. Single well-defined signaling cells are distributed relatively evenly throughout the culture. Very few red and both colour signals. Mostly green signals: *mostly apoptosis.*	Large areas of green background, cells are indistinguishable (early apoptosis?). Few red spots in a sea of green, also stronger green specks visible (individual cells in late apoptosis?) Overall more cell death than on control slide. Bicoloured foci of cell death (plaques) are visible. *Overall much early apoptosis with foci of late apoptosis and necrosis.*	Localized cell death in large foci: groups of cells show bright green in contrast to a lighter green background. Background appears as waves of light green fluorescence. Centers of these foci harbour several well-defined red spots. More plaques visible than in La Sota-infected culture. *Overall much apoptosis with more foci of mixed apoptosis and necrosis.*
48 h	Almost no signals. Single well-defined green and double signal cells on a black background suggest very little cell death and *amount of apoptosis more or less equal to amount of necrosis*	More signals than on control slide. Foci of cell death stand out on a background of faint green specks. Foci are bright green with well-defined red spots in the middle. *Plaques of mixed apoptosis and necrosis are surrounded by large areas of early apoptosis. Plaques contain more apoptosis than necrosis.*	Background shows more, brighter and larger green specks. Foci look like those in La Sota-treated culture, except that their occurrence is more frequent, sizes are slightly larger and foci contain more red signals. *More, more necrotic and larger plaques on an more advanced apoptotic background.*
72 h	Almost no signals. Tiny light green specks on black background, almost no whole cell signals. Virtually no red can be observed at all. *Few cells in early apoptosis.*	All sizes green foci with few red spots. Foci appear less solid: they contain black areas, presumably where cells have died. Slightly green-specked background again suggest early apoptosis in cells surrounding the foci. *Thinned-out plaques of more apoptosis than necrosis on an early apoptotic background.*	Large green foci with very few red dots and more black areas. More green specks on background. *Lots of apoptosis in thinned-out plaques, little necrosis. Early apoptosis in surrounding cells.*
96 h	Single weak green signals and single signals of both colours. *Same amount of apoptosis and necrosis.*	*Widespread apoptosis* on the background *with foci of necrosis.*	*Widespread apoptosis* with *single red cells signaling necrosis.*

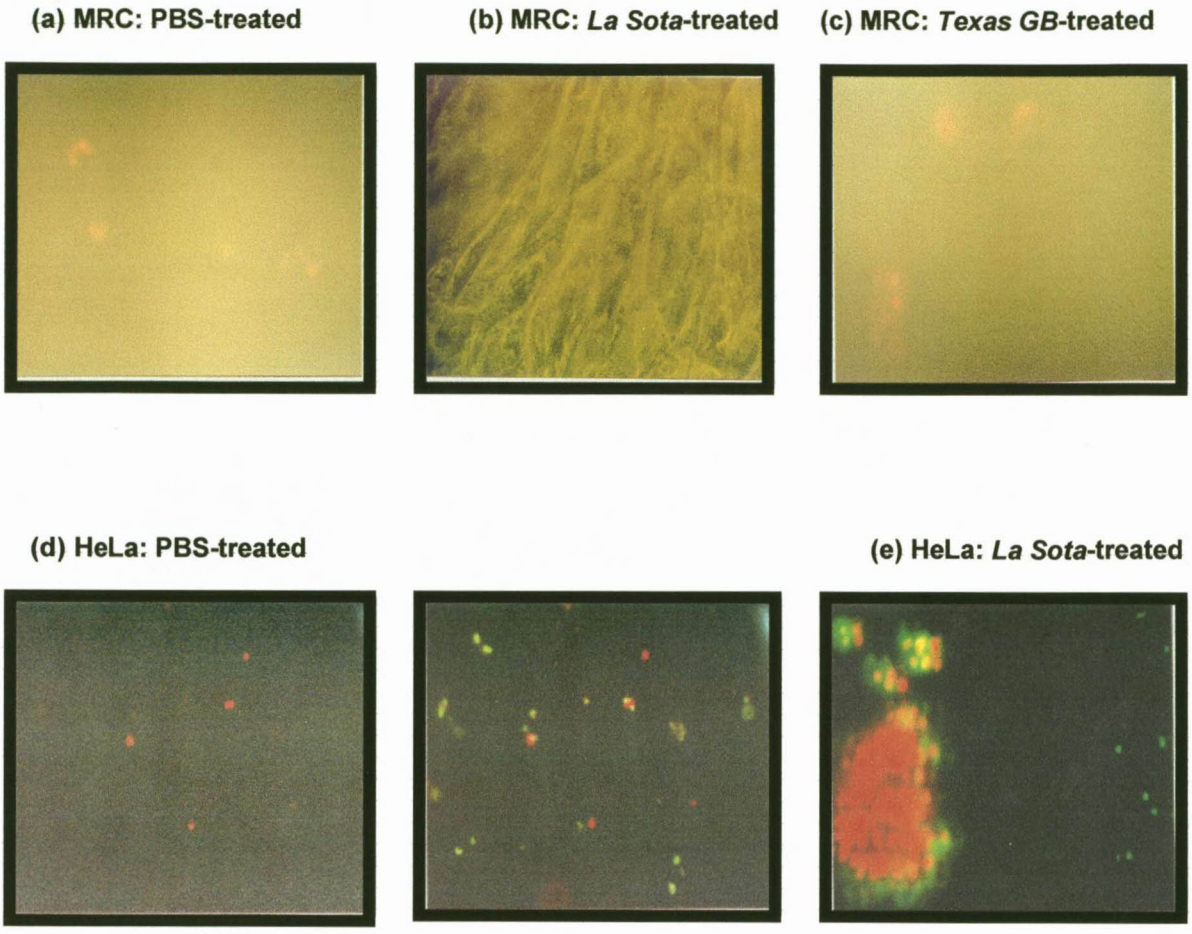

Figure 3.4.

Photomicrographs of the Annexin-V assay 48 h post infection. MRC-5 results are shown in the top panel and HeLa results in the bottom panel. SNO1 results were similar to HeLa results and are not shown. (b) shows the healthy growth of fibroblasts, due to the lack of fluorescence to photograph. (f) shows a focus of necrotic cells surrounded by apoptotic cells.

Controls

During the course of the experiment, the PBS - treated control cultures of all three cell lines seemed to gradually deteriorate as metabolite build - up, nutrient depletion and lowered medium pH caused cell death (Tables 3.1., 3.2. & 3.3.). Cell-death occurred mainly in the form of necrosis, with apoptosis presenting in the later stages of the experiment. The initial

necrosis could be ascribed to physical cell damage due to washing, handling, and the staining process and temperature changes at the beginning of the experiment.

La Sota

The results with both cancer cell lines were more or less the same, but both differed drastically from the results of the MRC-5 cells (Tables 3.1., 3.2. & 3.3.).

During the course of the experiment, the MRC-5 cultures that were treated with *La Sota* showed no significant difference to the PBS - treated controls. Initially there were small amounts of necrosis and later on apoptosis and necrosis occurred in more or less equal amounts.

In contrast, both cancer cell lines showed elevated amounts of apoptosis compared to the PBS controls just one hour post infection. During the course of the experiment, more and more apoptosis occurred throughout the *La Sota* - treated cultures and after 48 hours, foci (plaques) of mixed necrosis and apoptosis became visible. The formation of plaques could be explained as follows: an infected cell would start to go into apoptosis due to NDV infection. This infection would also cause it to fuse with its neighbouring cells, creating polycaryocytes (syncytiae), which would start to leak and eventually burst due to the large pressure exerted on the collective membrane. Therefore, the initial mode of cell death would be apoptosis, but fusion and leakage would present as necrosis. Viral progeny would then be released to infect the cells surrounding the plaque, spreading the infection.

Overall, there seemed to be more apoptosis than necrosis, as opposed to the PBS controls that had more necrosis or equal amounts of both types of cell death throughout the experiment.

Texas GB

MRC-5 cultures showed no significant difference to the PBS - or *La Sota* - treated cultures (Tables 3.1., 3.2. & 3.3.). Initially, both cancer cell types clearly showed increased amounts of cell death compared to either of the PBS controls, but the same amounts of cell death as the *La Sota* - treated cultures. Later on, however, more total cell death could be seen in the

Texas GB - treated cultures than in the *La Sota* - or PBS - treated ones. Cell death occurred in foci of mixed apoptosis and necrosis only 24 hours post infection. Towards the end of the experiment, the amount of cell death diminished. This could have been due to the fact that large amounts of cells have already been lysed, leaving fewer cells to cause an infection and eventual cell death in.

It would appear that both NDV strains that were tested induce both apoptosis and necrosis in two different cancer cell types, while not causing cell death in normal cells at the concentrations that were tested. However, we cannot rule out the possibility of higher viral concentrations having adverse effects on normal human cells.

Regarding the shortfalls of the study, some points need to be addressed. The experiments involving MRC-5 cells could not be repeated for confirmation of the results, due to the difficulties involving growth of such cells in culture. Minimal disturbance and / or medium replacement should be combined with exquisitely careful handling. Only a few passages are possible before the cells start to perish of their own accord (since essentially, *normal* cells should not be able to grow in culture at all) and cryopreservation yielded minimally viable cultures that did not seem to be able to reach confluence.

Incorporating the MRC-5 cell line as a control in all the other experiments would have been ideal, but these technicalities made the whole process rather difficult. The results that we did get were therefore taken as correct. The Annexin-V assay and *in vivo* experiment would also be regarded as confirmation of *Texas GB's* safety regarding infection of normal tissues.

Difficulties with quantifying cell death left a lot of the results up to human interpretation, which seems unscientific at the least. Cell lines that need trypsinization to remove cells from the flasks that they are grown in are unsuitable for flow cytometry and manual cell counts become tricky when polykaryocytes and multilayered cultures are involved. The Annexin-V assay came as close to quantifying the amounts of apoptosis and necrosis as was possible. This still did not give the comparable figures that were wanted, but did give a good idea of the amounts and proportions of cell death that were taking place.

3.1.5. The oncolytic efficiency of NDV in immune compromized mice.

After the initial success with the *in vitro* experiments, the effect of both NDV strains were assessed in HeLa tumours in a nude mouse model (Figures 3.5. and 3.6.) in order to see if the viruses had similar effects *in vivo*.

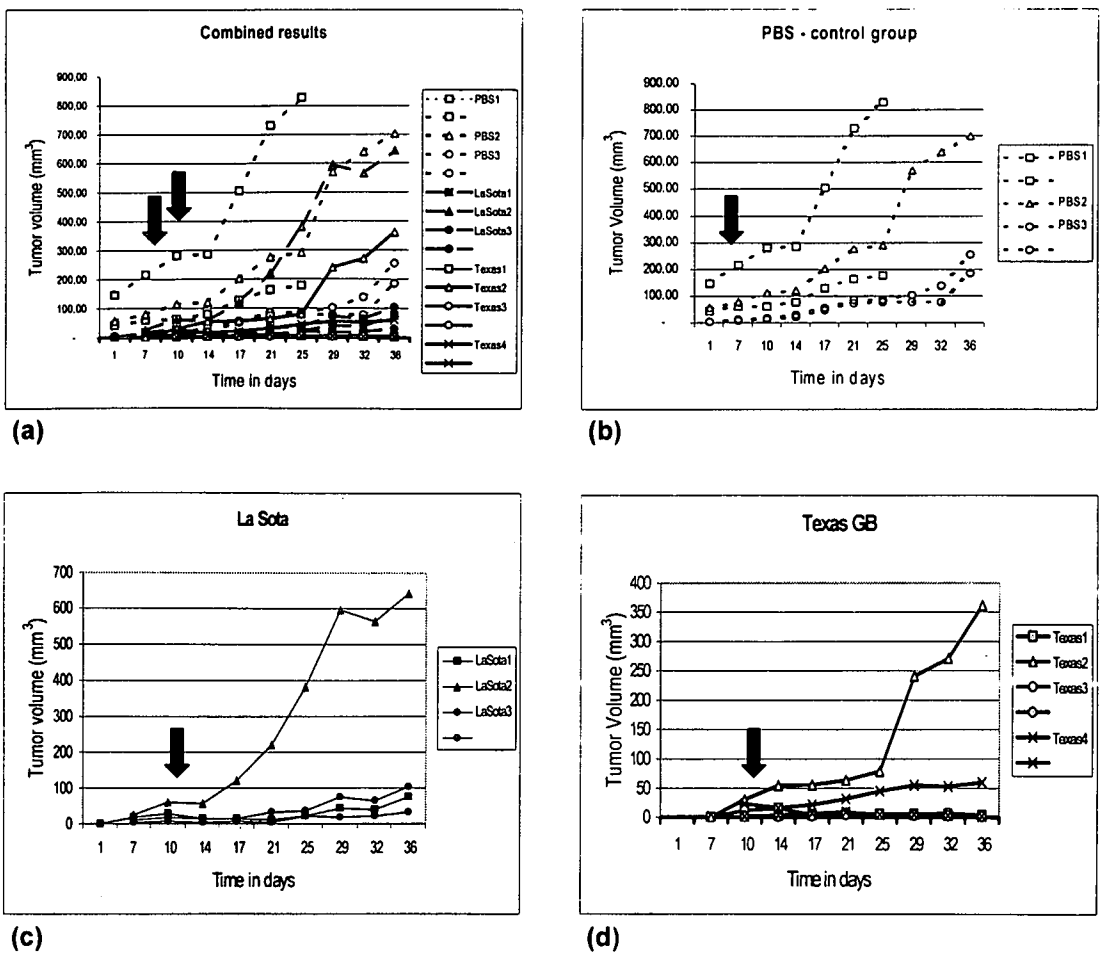

Figure 3.5.

Graphs of tumour volume against time over a period of 36 days. Each line represents one tumour. Where a mouse had two tumours, they are represented by two lines with the same symbols. In each case only the large tumour was treated.

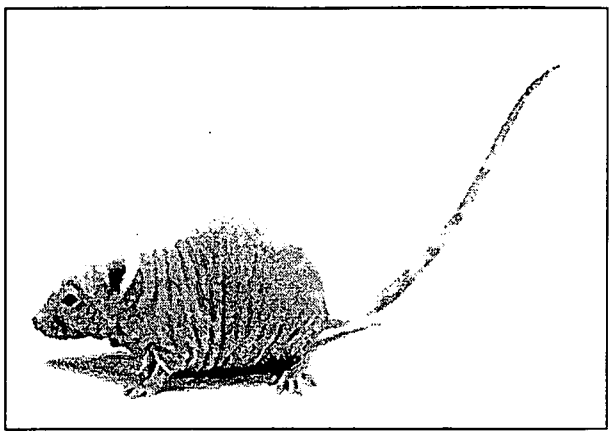

Figure 3.6.
Nude mouse strain MF-1*nu*.

Data analysis

The collective results are plotted together in Figure 3.5. (a): the control group's tumours grew irrespective of the treatment received on day 7. Overall, the treated groups seemed to undergo an initial tumour growth halt or growth rate reduction in response to the respective treatments given on day 10. Generally, the PBS - treated group had the largest tumours, the *La Sota* - treated group's tumours were middle-sized and the *Texas GB* - treated group seemed to contain the smallest collection of tumours.

When we look at the control group alone (Figure 3.5. (b)), we see tumour growth that seems to naturally occur in bursts of exponential growth. This is most probably the result of unrestricted tumour growth up to a certain point where the physical restraints of membranes and / or blood supply to the tumour causes a temporary reduction in growth rate, until membranes are stretched or torn or additional blood vessels are grown and the physical restraints overcome.

The curves of the PBS-treated control group show no sign of response to the PBS treatment given on day 7 in terms of growth rate. By the end of the trial period, one mouse has had to be euthanized due to ethical reasons when its tumour burden became too large (collective diameter of its tumours were 20 mm in one dimension), while another mouse's tumour volume reached almost 900 mm^3.

When we look at the *La Sota* - treated group separately (Figure 3.5. (c)), there seems to be an initial response (decline in tumour growth rate) to the treatment in all mice and in both the

treated and untreated tumours of *La Sota 3*. For *La Sota 2* and *3*, this response seems to wear off over time and tumour growth starts escalating again around day 25, about two weeks after the treatment. For *La Sota 2*, this tumour growth also seemed to be in a sequence of exponential phases, for which the explanation would be the same as for the PBS - treated control group. For *La Sota 3*, the growth rate was much lower and the growth curve linear rather than exponential. This could be due to a partial growth inhibition by the NDV.

This tumour growth could be explained by partial leakage of the viral inoculation from the tumour injection site, causing an attenuated response in the tumour, or possibly loss of the virus from the tumour tissue by viral shedding. (Shedding of the ND virus has been reported (*Seal et al., 2000 (b)*).) It could also be due to regrowth of NDV - resistant tumour cells, although the tumours were initiated from the same HeLa cell stock and should therefore not be too genetically diverse.

The largest tumour in this group reached just over 600 mm^3 by the end of the trial period.

In the Texas GB group (Figure V (d)), there also seems to be an initial response in all mice: some showed a decline in tumour growth rate (*Texas 2, Texas 4's treated tumour*), some halted tumour growth (*Texas 3, Texas 4's untreated tumour*) and one even showed tumour regression (*Texas1*). However, around day 25, about two weeks after the treatment, tumour growth rates of *Texas 2* and *4* started to escalate again. This could also be due to partial leakage of the viral inoculation from the treatment injection site, viral shedding, or regrowth of NDV-resistant tumour cells.

The largest tumour in this group reached a volume of just over 300 mm^3 by the end of the trial period.

Although there is a definite response to injection of equal amounts of either NDV strain in the growth rates of HeLa tumours in immune compromized mice, this difference is not statistically significant ($p = 0.0887$) according to the Krushkall-Wallis test. This could be due to the small sample size of the study ($n = 10$).

Generally, however, the results showed that intratumoural injection of either NDV strain caused a temporary carcinostatic effect not only in the treated tumour, but also in the

additional untreated tumours of the treated mice, even though these were not always adjacent to the primary tumour. We can therefore speculate that viral spread did occur and that a local NDV treatment could have had growth stunting effect on distant tumours.
The small number of mice made statistical analysis extremely difficult, but nonetheless proved the potential in this field that can now be further explored.

Histology

Histological analysis of tumours from each of the three groups identified both the PBS -, *La Sota* -, and *Texas GB* - treated tumours positively as HeLa cervical carcinoma. There was therefore no change in tumour histology after treatment with either strain of NDV. However, one of the very small *Texas GB* - treated tumours (the one that underwent regression after treatment) showed only fat - and epithelial tissues. This might indicate complete regression of the tumour, leaving only a remnant bump of scar tissue under the skin. It is also possible that the small cluster of cancer cells could have been lost in either the dissection or fixing processes.

General

Due to the fact that the mice treated with the NDV could not elicit an immune response, these results may only represent a part of the virus' actual oncolytic capability, since generally, immune stimulation seems to be at least partially involved in the mechanism of tumour lysis, at least for this virus.

Because it is not known whether the SNO1 cell line is tumourigenic and the difficulties and costs involved in working with nude mice and the small number of mice that were available for the study, this cell line was left out of the last part of the study. Ideally, it could have been included and the *in vivo* results compared.

3.2. CONCLUSION

In vitro, *La Sota* was shown to be an oncolytic virus with very favourable characteristics for human treatment, where *Texas GB* was shown to be cytolytic for two human cancer cell lines, but higher concentrations could possibly infect certain non - cancerous human tissues.

In vivo, a single dose of either of the two NDV strains was shown to exert a temporary carcinostatic effect on the treated and untreated tumours of an individual. This can only lead us to speculate on the possibilities of higher doses, multiple treatments or NDV in combination with surgery or other treatments. Generally, *Texas GB* seems to have slightly better oncolytic properties than *La Sota*, however, because of the hazards associated with *Texas GB* in the poultry industry, it would be inadvisable to continue studies with this NDV strain.

These results warrant further investigation into the oncolytic potential of the *La Sota* strain.

3.3. Future studies

The effect of NDV on tumours in immune competent mice should be explored in order to assess the oncolytic efficiency in a model that is physiologically closer to the patients that would receive such treatment. Follow-up studies could involve either the further basic or clinical testing of *La Sota* in order to patent it as a cancer therapeutic or the attenuation of the more virulent strain for the same ultimate purpose. This, however, would be difficult to pass by authorities, since *Texas GB* is so notorious in the poultry industry. *La Sota*, however, has been used safely in the poultry industry for years and should therefore pose no such problems.

Future studies could evaluate the effect of the *La Sota* strain of NDV on other cancer types *in vitro* and *in vivo*. The optimal viral dose(s) also still needs to be determined and standardized for body weight and tumour type.

CHAPTER 4

4.1. REFERENCES

Adam J.K., Odhav B., Bhoola K.D. (2003) Immune responses in cancer. *Pharmacol. & Therapeut.* **99**; 113-32.

Aldous E.W., Collins M.S., McGoldrick A., Alexander D.J. (2001) Rapid pathotyping of Newcastle disease virus (NDV) using fluorogenic probes in a PCR assay. *Vet. Microbiol.* **80**(3); 201-12.

Alexander D.J. (2000) Newcastle disease and other avian paramyxoviruses. *Rev. sci. tech. Off. int. Epiz.* **19**(2); 443-62.

Arnoldi J.M., Beard C.W., Bech-Nielsen S., Blackwell J.H., Bram R., Brown C., Buisch W.W., et al. (1998) Part IV: Velogenic Newcastle Disease. Foreign Animal Diseases: The Gray Book. [<http://www.vet.uga.edu/vpp/gray_book/FAD/index.htm> on 14/11/2003.]

Barbezange C., Jestin V. (2002) Development of a RT-nested PCR test detecting pigeon Paramyxovirus-1 directly from organs of infected animals. *J. Virol. Meth.* **106**(2); 197-207.

Barnes B.J., Moore P.A., Pitha P.M. (2001) Virus-specific activation of a novel interferon regulatory factor, IRF-5, results in the induction of distinct interferon α genes. *J.Biol. Chem.* **276**(26); 23382-90.

Brenna S.M.F., Syrjänen K.J. (2003) Regulation of cell cycles is of key importance in human papillomavirus (HPV)-associated cervical cancers. *Sao Paulo Med. J.* 121(3); 128-32.

Burk R.D. (1999) Hospital Practice: Human Papillomavirus and the Risk of Cervical Cancer. [<www.hosppract.com/issues/1999/11/burk.htm>.]

Cassel W.A., Garrett R.E. (1965) Newcastle Disease Virus as an antineoplastic agent. *Cancer* **18**; 863-8.

Cavanagh D., Mawditt K., Shaw K., Britton P., Naylor C. (1997) Towards the routine application of nucleic acid technology for avian disease diagnosis. *Acta. Vet. Hung.* **45**(3); 281-98.

Chen H., Ward M.H., Graubard B.I., Heineman E.F., Markin R.M., et al. (2002) Dietary patterns and adenocarcinoma of the esophagus and stomach. *Am. J. Clin. Nutr.* **75**(1); 5-7.

Chen L., Gorman J.J., McKimm-Breschkin J., Lawrence L.J., Tulloch P.A., Smith B.J. *et al.* (2001 (a)) The structure of the fusion glycoprotein of Newcastle disease virus suggests a novel paradigm for the molecular mechanism of membrane fusion. *Structure* **9**; 255-66.

Chen M.J., Chung-Faye G.A., Searle P.F., Young L.S., Kerr D.J. (2001 (b)) Gene therapy for colorectal cancer: therapeutic potential. *BioDrugs* **15**(6); 357-67.

Cobaleda C., Muñoz-Barroso I., Sagrera A., Villar E. (2001) Fusogenic activity of reconstituted newcastle disease virus envelopes: a role for the hemagglutinin-neuraminidase protein in the fusion process. *Int. J. Biochem. Cell Biol.* **1201**; 1-12.

Coleman N., Peeples M.E. (1993) The matrix protein of Newcastle disease virus localizes to the nucleus via a bipartite nuclear localization signal. *Virology* **195**(2); 196-607.

Crennell S., Takimoto T., Portner A., Taylor G. (2000) Crystal structure of the multifunctional paramyxovirus hemagglutinin-neuraminidase. *Nat. Struct. Biol.* **7**; 1068-1074.

Csatary L.K., Gergely P. (1990) Vaccine therapy of malignant tumors. *Orv. Hetil.* **131**(47); 2585-8.

Csatary L.K., Eckhardt S., Bukosza I., Czegledi F., Feyvesi C., Gergely P., et al. (1993) Attenuated Veterinary Virus Vaccine for the Treatment of Cancer. *Cancer Det. Prev.* **17**(6); 619-27.

Csatary L.K., Babács T. (1999 (a)) Use of Newcastle Disease Virus Vaccine (MTH-68/H) in a patient with high-grade glioblastoma. *JAMA Letters* **281**(17).

Csatary L.K., Moss R.W., Beuth J., Töröcsik B., Szeberenyi J., Bacaks T. (1999 (b)) Beneficial treatment of patients with advanced cancer using Newcastle disease virus vaccine (MTH-68/H). *Anticanc. Res.* **19**(1B); 635-8.

De Leeuw O., Peeters B. (1999) Complete nucleotide sequence of Newcastle disease virus: evidence of the existance of a new genus within the subfamily Paramyxovirinae. *J. Gen. Virol.* **80**; 131-6.

Denny L, Kuhn L., Pollack A., Wainwright H., Wright T.C. Jr. (2000) Evaluation of alternative methods of cervical cancer screening for resource-poor settings. *Cancer* **89**(4); 826-33.

do Horto dos Santos Oliveira L, Rodrigues Ede V, de Salles Lopes AP, Fernandez Ade P, Cavalcanti SM. (2003) *Sao Paulo Med. J.* 121(2); 67-71.

Du Plessis L., Dietzsch E., Van Gele M., Van Roy N., Van Helden P., Parker M.I., *et al* (1999) Mapping of novel regions of DNA gain and loss by comparative genomic hybridization in esophageal carcinoma in the Black and Colored populations of South Africa. *Canc. Res.* **59**; 1877-83.

Eiselein J.E., Biggs M.W., Walton J.R. (1978) Treatment of transplanted murine tumors with an oncolytic virus and cyclophosphamide. *Canc. Res.* **38** (11); 3573-9.

Ertel C., Millar N.S., Emmerson P.T., Schirrmacher V., von Hoegen P. (1993) Viral hemagglutinin augments peptide-specific cytotoxic T cell responses. *Eur. J. Immunol.* **23**(10); 2592-6.

Evan M.F., Cooper K. (2004) Human papillomavirus integration: detection by in situ hybridization and potential clinical application. *J. Pathol.* **202**(1); 1-4/

Fabian Z., Torocsik B., Kiss K., Csatary L.K., Bodey B., Tigyi J., *et al.* (2001) Induction of apoptosis by a Newcastle disease virus vaccine (MTH-68/H) in PC12 rat phaechromocytoma cells. *Anticanc. Res.* **21**(1A); 125-35.

Finzer P., Aguilar-Lemarroy A., Rösl F. (2002) The role of human papillomavirus oncoproteins E6 and E7 in apoptosis. *Can. Letters* **188**; 15-24.

Fonn S., Bloch B., Mabina M., Carpenter S., Cronje H., Maise C., et al. (2002) Prevalence of pre-cancerous lesions and cervical cancer in South Africa – a multicentre study. *S.A. Med. J.* **92**(2); 148-56.

Fueyo J., Gomez-Manzano C., Yung W.K.A., Kyritsis A.P. (1999) Targeting in gene therapy for gliomas. *Arch. Neurol.* **56**; 445-8.

Galanis E., Vile R., Russell S.J. (2001) Delivery systems intended for in vivo gene therapy of cancer: targeting and replication competent viral vectors. *Crit. Rev. Oncol. Hematol.* **38**; 177-192.

Gallili G.E., Ben-Nathan D. (1998) Newcastle Disease Vaccines. *Biotech. Adv.* **16**(2); 343-66.

Geh J.I. (2002) The use of chemoradiotherapy in esophageal cancer. *Eur. J.Canc.* **38**; 300-13.

Gelderblom W.C.A., Snyman S.D., Abel S., Lebepe-Mazur S., Smuts C.M., Van der Westhuizen L., et al. (1996) Hepatotoxicity and carcinogenicity of the fumonisins in rates: a review regarding mechanistic implications for establishing risk in humans. *Adv. Exp. Med. Biol.* **392**; 279-306.

Guimaraes D.P., Lu S.H., Snijders P., Wilmotte R., Herrero R., Lenoir G., *et al.* (2001) Absence of association between HPV DNA, TP53 codon 72 polymorphism, and risk of esophageal cancer in a high-risk area of China. *Canc. Lett.* **162**; 231-5.

Haas C., Schirrmacher V. (1996) Immunogenicity increase of autologous tumor cell vaccines by virus infection and attachment of bispecific antibodies. *Canc. Immunol. Immunother.* **43**(3); 190-4.

Haas C., Ertel C., Gerhards R., Schirrmacher V. (1998) Introduction of adhesive and costimulatory immune functions into tumor cells by infection with Newcastle disease virus. *Int. J. Oncol.* **13**(6); 1105-15.

Haas C., Herold-Mende C., Gerhards R., Schirrmacher V. (1999) An effective strategy of human tumor vaccine modification by coupling bispecific costimulatory molecules. *Canc. Gene Ther.* **6**(3); 254-62.

Hamburg S.I., Cassell G.H., Rabinovitch M. (1980) Relationship between enhanced macrophage phagocytic activity and the induction of interferon by Newcastle disease virus in mice. *J. Immunol.* **124**(3); 1360-4.

Hao D., DiFrancesco L.M., Brasher P.M., DeMetz C., Fulton D.S., DeAngelis L.M., Forsyth P.A. (1999) Is primary CNS lymphoma really becoming more common? A population-based study of incidence, clinicopathological features and outcomes in Alberta from 1975 to 1996. *Ann Oncol* **10**(1); 65-70.

Hopman A.H.N., Smedts F., Dignef W., Ummelen M., Sonke G., Mravunac M., Vooijs G.P., Speel E.M., Ramaekers F.C.S. (2004) Transition of high-grade cervical intraepithelial neoplasia to micro-invasive carcinoma is characterized by integration of HPV 16/18 and numerical chromosome abnormalities. *J. Pathol* **202**(1); 1-4.

Horn L.S., Raptis G., Fischer U. (2002) Familial cancer history in patients with carcinoma of the cervix uteri. *Eur. J. Obs. Gyn. Rep. Biol.* **101**; 54-7.

Iorio R.M., Glickman R.L., Sheehan J.P. (1992) Inhibition of fusion by neutralizing monoclonal antibodies to the haemagglitinin-neuraminidase glycoprotein of Newcastle disease virus. *J. Gen. Virol.* **73**; 1167-76.

Jain A. (1996) Preventing cervical cancer. *A.V.S.C. News* **34**(3); 2.

Jaskiewicz K., Marasas W.F., Van der Walt F.E. (1987) Esophageal and other main cancer patterns in four districts of Transkei, 1981-1984. *S.A. Med. J.* **72**(1); 27-30.

Kadish A.S. Doyle A.T., Steinhauer E.H., Ghossein N.A. (1981) Natural cytotoxicity and interferon production in human cancer: deficient natural killer activity and normal interferon production in patients with advanced disease. *J. Immunol.* **127**(5); 1817-22.

Kawaguchi H., Ohno S., Araki K., Miyazaki M., Saeki H., Watanabe M., *et al.* (2000) p53 polymorphism in Human Papillomavirus-associated esophageal cancer. *Canc. Res.* **60**; 2753-5.

Kirchner H.H., Anton P., Atzpodien J. (1995) Adjuvant treatment of locally advanced renal cancer with autologous virus-modified tumor vaccines. *World J. Urol.* **13**(3); 171-3.

Krishnamurthy S., Huang Z., Samal S.K. (2000) Recovery of a virulent strain of Newcastle disease virus from cloned cDNA: expression of a foreign gene results in growth retardation and attenuation. *Virology* **278**(1); 168-82.

Kuhn L., Denny L., Pollack A., Lorincz A., Richard R.M., Wright T.C. (2000) Human papillomavirus DNA testing for cervical cancer screening in low-resource settings. *J. Nat. Canc. Inst.* **92**(10); 818-25.

Lazarus C., Venter T.H. (1986) Carcinoma of the esophagus in Ciskei. *S.A. Med. J.* **69**(12); 747-8.

Levi F., Randimbison L., Luccini F., Te V.-C., La Vacchia C. (2001) Epidemiology of adenocarcinoma and squamous cell carcinoma of the oesophagus. *Eur. J. Canc. Prev.* **10**; 91-6.

Liang R., Coa D.J., Li J.Q., Chen J., Guo X., Zhuang F.F., Duan M.X. (2002) Newcastle disease outbreaks in western China were caused by genotypes VIIa and VIII. *Vet. Microbiol.* **87**(3); 193-203.

Locke D.P., Sellers H.S., Crawford J.M., Schultz-Cherry S., King D.J., Meinersmann R.J., Seal B.S. (2000) Newcastle disease virus phosphoprotein gene analysis and transcriptional editing in avian cells. *Virus Res.* **69**; 55-68.

Lomalisa P., Smith T., Guidozzi F. (2000) Human Immunodeficiency Virus infection and invasive cervical cancer in South Africa. *Gyn. Oncol.* **77**(3); 460-3.

Lorence R.M., Katubig B.B., Reichard K.W., Reyes H.M., Phuangsab A., Sassetti R.J. *et al.* (1994 (a)) Complete regression of human fibrosarcoma xenografts after local Newcastle disease virus therapy. *Canc. Res.* **54** (23); 6017-21.

Lorence R.M., Reichard K.W., Katubig B.B., Reyes H.M., Phuangsab A., Mitchell B.R., *et al.* (1994 (b)) Complete regression of human neuroblastoma xenografts in athymic mice after local Newcastle Disease Virus therapy. *J. Nat. Canc. Inst.* **86**; 1228-33.

Lorence R.M., Rood P.A., Kelley K.W. (1988) Newcastle disease virus as an antineoplastic agent: induction of tumor necrosis factor-alpha and augmentation of its cytotoxicity. *J. Nat. Canc. Inst.* **80**(16); 1305-12.

Martin P.M., Hill G.B. (1984) Cervical cancer in relation to tobacco and alcohol consumption in Lesotho, southern Africa. *Canc. Det. Prev.* **7**(2); 109-115.

Moodley M., Moodley J., Kleinschmidt I. (2001) Invasive cervical cancer and human immunodeficiency virus (HIV) infection: a South African perspective. *Int. J. Gyn. Canc. (Official Journal of the I.G.C.S.)* **11**(3); 194-7.

Morrison T.G. (2001) The three faces of Paramyxovirus attachment proteins. *Trends in Microbiol.* **9**(3); 103-5.

Moss R.W. (1994) MTH-68/N gets some respect. JNCI lauds Newcastle treatment; Hungarians to allow widescale use. *Canc. Chronicles* **#23**, Sept. 1994. At <wysiwyg://86/http://www.ralphmoss.com/html/newcastle/shtml> on 10/05/2002.

Muñoz-Barroso I., Cobaleda C., Zhadan G., Shnyrov V., Villar E. (1997) Dynamic properties of Newcastle disease virus envelope and their relations with viral hemagglutinin-neuraminidase membrane glycoprotein. *Biochim. Biophys. Acta* **1327**; 17-31.

Naidoo R., Tarin M., Reddi A., Chetty R. (1999) Allelic imbalance and microsatellite instability in chromosomes 2p, 3p, 5q and 18q in esophageal squamous carcinoma in populations from South Africa. *Diagn. Mol. Path.* **8**(3); 131-7.

Nair M.G. (2000) Quality of life in cancer of the cervix patients. *Int. Clin. Psychopharm.* **15**(3); S47-9.

Nakaya T., Cros J., Park M.S., Nakaya Y., Zheng H., Sagrera A., et al. (2001) Recombinant Newcastle Disease Virus as a vaccine vector. *J. Virol.* **75**(23); 11868-73.

Nemunaitis J. (1999) Oncolytic viruses. *Inv. New Drugs* **17**(4); 375-86.

Nemunaitis J. (2002) Live viruses in cancer treatment. *Oncology (Huntingt)* **16**(11);1483-92.

Norman K.L., Farassati F., Lee P.W.K. (2001) Oncolytic viruses and cancer therapy. *Cyt. Growth Fact. Rev.* **12**; 271-82.

Omar A.R., Ideris A., Ali A.M., Othman F., Yusoff K., Abdullah J.M., Wali H.S.M., Zawawi M., Meyyappan N. (2002) An overview on the development of Newcastle Disease virus as an anti-cancer therapy. *Malaysian J. Med. Sci.* **9**(2); 4-12.

Pecora A.L., Rizvi N., Cohen G.I., Meropol N.J., Sterman D., Marshall J.L. et al. (2002) Phase I trial of intravenous administration of PV701, an oncolytic virus, in patients with advanced solid cancers. *J. Clin. Oncol. (Official Journal of the A.S.C.O.)* **20**(9); 2251-66.

Peeples M.E., Wang C., Gupta K.C., Coleman N. (1992) Nuclear entry and nucleolar localization of the Newcastle disease virus (NDV) matrix protein occur early in infection and do not require other NDV proteins. *J. Virol.* **66**(5); 3263-9.

Pegoraro R.J., Rom L., Lanning P.A., Moodley M., Naiker S., Moodley J. (2002) P53 codon 72 polymorphism and human papillomavirus type in relation to cervical cancer in South African women. *Int. J. Gyn. Canc. (Official Journal of the I.G.C.S.)* **12**(4); 383-8.

Pennisi E. (1998) Training viruses to attack cancers. *Science* **282**; 1344-6.

Phuangsab A., Lorence R.M., Reichard K.W., Peeples M.E., Walter R.J. (2001) Newcastle disease virus therapy of human tumor xenografs: antitumor effects of local or systemic administration. *Canc. Lett.* **172**; 27-36.

Reed L.J., Muench H. (1938) A simple method of estimating fifty per cent endpoints. *Am J Hyg* **17**(3); 493-7.

Russel J., (2002) Review: RNA viruses as virotherapy agents. *Canc. Gene Ther.* **9**; 961-6.

San Román K., Villar E., Munoz-Barroso I. (1999) Acidic pH enhancement of the fusion of the Newcastle disease virus with cultured cells. *Virology* **260**(2).

Saville P. (1996) Newcastle Disease & Avian Influenza. *Animal Health Advisory Leaflet / South Pacific Commission* **8**, p 1-2.

Schirrmacher V., Ockert D., Hagmüller E., Saeger H.D. (1995) Newcastle disease virus infected intact autologous tumor cell vaccine for adjuvant active specific immunotherapy of resected colorectal carcinoma. *Eur. J. Canc.* **31**(975); S208.

Schirrmacher V., Haas C., Bonifer R., Ahlert T., Gerhards R., Ertel C. (1999) Human tumor cell modification by virus infection: an efficient and safe way to produce cancer vaccine with pleiotropic immune stimulatory properties when using Newcastle disease virus. *Gene Ther.* **6**(1); 63-73.

Schlag P., Manasterski M., Gerneth T., Hohenberger P., Dueck M., Herfarth C., *et al.* (1992) Avtive specific immunotherapy with Newcastle-disease-virus-modified autologous tumor cells following resection of liver metastases in colorectal cancer. First evaluation of clinical response of a phase II trial. *Canc. Immunol. Immunother.* **35**(5); 325-30.

Seal B.S., King D.J., Sellers H.S. (2000 (a)) The avian response to Newcastle disease virus. *Dev. Comp. Immunol.* **24**; 257-68.

Seal B.S., King D.J., Meinersmann R.J. (2000 (b)) Molecular evolution of the Newcastle disease virus martix protein gene and phylogenetic relationships among the paramyxoviridae. *Virus Res.* **66**; 1-11.

Seal B.S., Crawford J.M. Sellers H.S., Locke D.P., King D.J. (2002) Nucleotide sequence analysis of the Newcastle disease virus nucleocapsid protein gene and phylogenetic relationships among the Paramyxoviridae. *Virus Res.* **83**; 119-29.

Segal I. (2001) The gastro-esophageal reflux disease complex in sub-Saharan Africa. *Eur. J. Canc. Prev.* 10(3); 209-12.

Shoham J., Hirsch R., Zakay-Rones Z., Osband M.E., Brennert H.J. (1990) Augmentation of tumor cell immunogenicity by viruses – an approach to specific immunotherapy of cancer. *Nat. Immun. Cell Growth Regul.* **9**(3); 165-72.

Sinkovics J.G., Horvath J. (1995) Can virus therapy of human cancer be improved by apoptosis induction. *Med. Hypoth.* **44**(5); 359-68.

Sinkovics J.G., Horvath J.C. (2000) Newcastle disease virus (NDV): brief history of its oncolytic strains. *J. Clin. Virol.* **16**; 1-15.

Smagner B. (1999) The ever-recurring threat of emerging diseases. *Cornell Poultry pointers* **49**(2); 1-2.

Smith E.R., Chiocca E.R. (2000) Oncolytic viruses as novel anticancer agents: turning one scourge against another. *Exp. Op. Invest. Drugs* **9**(2); 311-327.

Stanziale S.F., Fong Y. (2003) Novel approaches to cancer therapy using oncolytic viruses. *Curr. Mol. Med.* **3**(1):61-71.

Steller M.A. (2002) Cervical cancer vaccines: progress and prospects. *J. Soc. Gynecol. Investig.* **9**; 254-64.

Stoeck M., Marland-Noske C., Manasterski M., Zawatzky R., Horn S., Möbus V., *et al.* (1993) In vitro expansion and analysis of T lymphocyte microcultures obtained from the vaccination sites of cancer patients undergoing active specific immunization with autologous Newcastle-disease-virus-modified tumor cells. *Canc. Immunol. Immunother.* **37**(4); 240-4.

Stoner G.D., Gupta A. (2001) Etiology and chemoprevention of esophageal squamous cell carcinoma. *Carcinogenesis* **2**(11); 1737-46.

Sur R.K., Levin C.V., Donde B., Sharma V., Miszczyk L., Nag S. (2002) Prospective randomized trial of HDR brachytherapy as a sole modality in palliation of advanced esophageal carcinoma – an international atomic energy agency study. *Int. J. Rad. Oncol. Biol. Phys.* **53**(1); 127-133.

Suzuki F., Pollard RB. (1982) Alterations of interferon production in a mouse model of thermal injury. *J. Immunol.* **129**(5); 1806-10.

Suzuki K., Curiel D.T. (2001) Viral therapy of cancer. [La terapia viral del cáncer.] *Rev. Inv. Clin.(Organo Del Hospital de Enfermedades de la Nutricion)* **53**(4); 346-56.

Syed A.M.N., Puthawala A.A., Abdelaziz N.N., El-Naggar M., Disaia P., Berman M., et al. (2002) Long-term results of low-dose-rate interstitial-intracavitary brachytherapy in the treatment of carcinoma of the cervix. *Int. J. Rad. Oncol. Biol. Phys.* **54**(1); 67-78.

Toneva V. (1977) Swine cell systems as interferon producers. IV. Comparative studies on the correlation between virulence of different Newcastle disease virus strains and their potency as interferon inducers. *Arch. Immunol. Therap. Exp.* **25**(5); 679-81.

Trigiante G., Huestis W.H. (2000) Selective virus-mediated intracellular delivery of membrane-impermeant compounds by means of plasma membrane vescicles. *Antiviral Res.* **45**; 211-21.

Turner P.C., Nikiema P., Wild C.P. (1999) Fumonisin contamination of food: progress in development of biomarkers to better assess human health risks. *Mut. Res.* **443**; 81-93.

Ueda Y., Enomoto T., Miyatake T., Ozaki K., Yoshizaki T., Kanao H., Ueno Y., Nakashima R., Shroyer K. R., Murata Y. (2003) Monoclonal expansion with integration of high-risk type human papillomaviruses is an initial step for cervical carcinogenesis: association of clonal status and human papillomavirus infection with clinical outcome in cervical intraepithelial neoplasia. *Lab. Invest.* **83**(1); 1517-27.

Ulane C.M., Rodriguez J.J., Parisien J., Horvath C.M. (2003) STAT3 ubiquitylation and degradation by mumps virus suppress cytokine and oncogene signaling. *J. Virol.* **77**(11); 6385-6393.

Walker A.R.P., Michelow P.M., Walker B.F. (2002) Cervix cancer in African women in Durban, South Africa. *Int. J. Gyn. Obs.* **79**; 45-6.

Washburn B., Schirrmacher V. (2002) Human tumor cell infection by Newcastle Disease Virus leads to upregulation of HLA and cell adhesion molecules and to induction of interferons, chemokines and finally apoptosis. *Int. J. Oncol.* **21**; 85-93.

Weaver B.K., Ando O., Kumar P., Reich N.C. (2001) Apoptosis is promoted by the dsRNA-activated factor (DRAF-1) during viral infection independent of the action of interferon or p53. *F.A.S.E.B. J.* **15**; 501-13.

Weaver E., Aron G.M. (1998) Synergistic anti-Newcastle disease virus activity of pokeweed antiviral protein, ribavirin and guanidine. *Can. J. Microbiol.* **44**(7); 702-705.

Wildner O. (2001) Oncolytic viruses as therapeutic agents. *Ann. Med.* **33**; 291-304.

Williamson A.-L., Marais D., Passmore J.-A., Rybicki E. (2002) Human papillomavirus (HPV) infection in Southern Africa: prevalence, immunity, and vaccine prospects. *I.U.B.M.B. Life* **53**(4-5); 253-8.

Witteveen P.O., Verhaar M.J., Jürgenliemk-Schulz, Van Eijkeren M.A. (2002) Update on the treatment of advanced cervical cancer. *Crit. Rev. Oncol. Hematol.* **43**; 245-6.

Young J.K., Hicks R.P., Wright G.E., Morrison T.G. (1997) Analysis of a peptide inhibitor of paramyxovirus (NDV) fusion, using biological assays, NMR and molecular modeling. *Virology* **238**(2); 291-304.

Young J.K., Hicks R.P., Wright G.E., Morrison T.G. (1998) The role of leucine residues in the structure and function of a leucine zipper peptide inhibitor of paramyxovirus (NDV) fusion. *Virology* **243**(1); 21-31.

Yu M., Wang E., Liu Y., Cao D., Jin N., Zhang C. W.-H., *et al.* (2002) Six-helix bundle assembly and characterization of heptad repeat regions from the F protein of Newcastle disease virus. *J. Gen. Virol.* **83**; 623-9.

Zeng J., Fournier P., Schirrmacher V. (*2001*) Stimulation of human natural interferon-α response via paramyxovirus hemagglutinin lectin-cell interaction. *J. Mol. Med.* **80**(7); 443-51.

Zorn U., Duensing S., Langkopf F., Anastassiou G., Kirchner H., Hadam M., *et al.* (1997) Active specific immunotherapy of renal cell carcinoma: cellular and humoral immune responses. *Canc. Biother. Radiopharm.* **13**(3); 157-65.

CHAPTER 5

5.1. SUMMARY

5.1.1. Abstract

Since the 1950's, several virus strains have been found to specifically infect and lyse human carcinoma cells, while not causing serious side effects in the patient. However, the available technology did not allow either sufficient characterization of, or research on these viruses until the development of molecular biology technology. We are now able to further explore this subject with renewed hope of "curing" cancer.

Newcastle Disease Virus (NDV) is a commonly occurring avian virus that is known not to infect normal human cells or cause side effects other than mild conjunctivitis and laryngitis in humans upon exposure to even the most virulent strains. Some NDV strains have been shown to be oncolytic in recent studies. Much research remains to be done on the molecular mechanisms, selectivity and biochemical apoptotic pathways involved in this oncolytic mechanism.

Two of the most commonly occurring cancers in South Africa are cervical and esophageal cancer.

The aim of this study was to assess the oncolytic properties of the two NDV strains *La Sota* and *Texas GB in vitro* (in cell culture) and *in vivo* (in an immune compromized mouse model).

In vitro results were promising: both strains were shown to be oncolytic, *Texas GB* more aggressively than *La Sota*. Both strains were shown to induce apoptosis and polycaryocyte formation, which leads to necrosis, in both cervical and esophageal cancer cell lines.

In vivo, it was shown that intratumoural administration of either virus strain had either a carcinostatic effect, or caused reduction in tumour volume in cervical cancer tumours in

immune compromized mice. In some cases the results were temporary and in other cases the treatment had a prolonged effect. This is probably due to leakage of the inoculation from the treatment site.
The results were not statistically significant due to the small number of mice used in the study.

These results warrant further evaluation of the oncolytic efficiency of the *La Sota* strain of NDV in immune competent mice, other cancer types and in clinical trials.

5.2. Opsomming

Sedert die 1950's is bewys dat sekere virusse menslike kankerselle infekteer en vernietig sonder om ernstige newe-effekte in die pasiënt te veroorsaak. Die tegnologie was egter nie van so 'n aard dat voldoende karakterisering van, of navorsing op hierdie virusse moontlik was nie, tot die ontwikkeling van molêkulere biologie. Ons kan nou hierdie onderwerp verder ondersoek met nuwe hoop vir die genesing van kanker.

Die Newcastle Siektevirus (NDV) is 'n algemene voëlvirus wat nie normale menslike selle infekteer nie en ook geen newe-effekte behalwe matige konjunktivitis en laringitis in mense veroorsaak nie, selfs nie met blootstelling aan die mees virulente stamme nie. Sommige NDV stamme is onlangs bewys om onkolities te wees. Daar is egter nog baie navorsing nodig om die molekulêre meganismes, selektiwiteit en biochemiese apoptose-weë wat betrokke is in hierdie onkolitiese meganisme te ontrafel.

Twee van die algemeenste kankertipes in Suid Afrika is servikale en esofagus kankers.

Die doel van hierdie studie was om die onkolitiese eienskappe van die twee NDV stamme *La Sota* en *Texas GB* te bepaal *in vitro* (in selkultuur) en *in vivo* (in 'n immuniteits-gebrekkige muis model).

Die *in vitro* resultate was belowend: albei stamme was onkolities, hoewel *Texas GB* meer aggressief was as *La Sota*. Albei stamme het apoptose en die vorming van polikariosiete, wat lei tot nekrose, geïnduseer in beide die servikale en esofageale kankersellyne.

In vivo is bewys dat intratumorale toediening van enige van die twee virusstamme of 'n karsinostatiese uitwerking het, of 'n afname in tumorvolume veroorsaak in servikale kanker tumore in immuniteits-gebrekkige muise. In sommige gevalle was die resultate tydelik en in ander gevalle van langer duur. Dit was waarskynlik na aanleiding van lekkasie van die inokulum van die tumor. Die resultate was nie statisties betekenisvol nie as gevolg van die klein getal muise wat in die studie gebruik is.

Hierdie resultate regverdig verdere evaluering van die onkolitiese effektiwiteit van die *La Sota* stam van NDV in immuniteits-kompetente muise, ander kankertipes en in kliniese toetse.