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# Environmental risk assessment of replication competent viral vectors in gene therapy trials

## Supplementary document:
Overview of replication competent viral vectors

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RIVM Report 601850002/2008

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**This report is a supplementary document to RIVM Report 601850001: 'Environmental risk assessment of replication competent viral vectors in gene therapy trials'.**

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# Abstract

**Environmental risk assessment of replication competent viral vectors in gene therapy trials**

The National Institute for Public Health and the Environment (RIVM) has developed a method to estimate the risks for man and the environment of the application of replication competent viral vectors in cancer therapy. Since such a method did not exist, this report will be a significant aid in the risk assessment of replication competent viruses and in guiding applications for a gene therapy license involving the use of these viruses through the regulatory process in the Netherlands.

Dutch scientists are planning to initiate clinical trials in which genetically modified replication competent viruses will be applied. These viruses are able to specifically replicate in cancer cells leading to their destruction.

Potential adverse effects of viral therapies are related to the exposure of man and the environment to the virus. In the Netherlands, exclusively clinical studies making use of replication deficient viruses have been permitted thus far. These 'crippled' viruses can only infect a limited amount of cells and are thereby able to repair the effects of a genetic defect, for instance in a patient with a metabolic disease. Using risk assessment it has been concluded that in most cases the environmental risks of this type of application are negligible.

Replication competent viruses, however, retain characteristics that make them able to multiply within a cancer patient and therefore a basic principle in the risk assessment of these viruses should be that there is a chance of spreading of the virus from the patient into the environment. The report gives points to consider for the environmental risk assessment of replication competent viruses taking into account the viral characteristics, the effects of the genetic modifications on the virus, the current clinical applications and future developments.

The report is expected to provide guidance to risk assessors and regulatory officers as well as to applicants for a gene therapy license.

Key words:
environmental risk assessment, virus, genetic modification, gene therapy, cancer

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# Rapport in het kort

**Leidraad voor de milieurisicobeoordeling van genetisch gemodificeerde replicatiecompetente virussen in gentherapiestudies**

Het RIVM heeft een methode uitgewerkt waarmee de risico's voor mens en milieu van replicatiecompetente virussen als kankertherapie kunnen worden beoordeeld. Zo'n methode bestond nog niet. De verwachting is dat de risicobeoordeling, en daarmee de vergunningverlening, van klinische studies die gebruikmaken van genetisch gemodificeerde replicerende virussen hierdoor kan worden bespoedigd.

Nederlandse onderzoekers zijn van plan om klinische studies te starten waarbij gebruik zal worden gemaakt van virussen die in staat zijn zich te vermenigvuldigen (replicatiecompetent). Dit zijn genetisch gemodificeerde virussen die zich in kankercellen kunnen vermenigvuldigen en ze op die manier kunnen vernietigen.

Mogelijke schadelijke effecten van gentherapieën met een virus zijn gekoppeld aan de mate waarin mens en milieu aan het virus worden blootgesteld. In Nederland zijn tot nu toe uitsluitend virussen die zich niet meer kunnen vermenigvuldigen als therapie toegepast. Deze ‘kreupele’ virussen infecteren een beperkt aantal cellen en kunnen bijvoorbeeld een genetisch defect in een patiënt met een stofwisselingsziekte opheffen. Uit de risicobeoordeling blijkt dat in de meeste gevallen de risico's hiervan voor mens en milieu verwaarloosbaar klein zijn.

Een belangrijk uitgangspunt in de risicobeoordeling van replicatiecompetente virussen is dat er een zekere kans is dat de toegepaste virussen zich vanuit de patiënt in het milieu verspreiden. Replicatiecompetente virussen hebben immers eigenschappen waardoor ze zich binnen een patiënt kunnen vermenigvuldigen. In de aanbevelingen voor de milieurisicoanalyse wordt rekening gehouden met deze eigenschappen, de eventuele effecten van de genetische modificaties op het virus, de huidige klinische toepassingen en toekomstige ontwikkelingen.

Het rapport biedt handvatten voor zowel risicobeoordelaars en beleidsmakers als voor aanvragers van een introductie in het milieu vergunning om gentherapiestudies uit te mogen voeren.

Trefwoorden:
milieurisicobeoordeling, virus, genetische modificatie, gentherapie, kanker

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# Contents

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# List of abbreviations

| | |
|---|---|
| ADP | Adenoviral death protein |
| AdV | Adenovirus |
| AZT | Azidothymidine |
| CAR | Coxsackie adenovirus receptor |
| CD | Cytosine deaminase |
| CEA | Carcino embryonic antigen |
| CIK | Cytokine induced killer |
| CMV | Cytomegalovirus (promoter) |
| CPA | Cyclophosphamide |
| CRAd | Conditionally replicating adenoviral vector |
| CVA | Coxsackievirus type A |
| DAF | Decay accelerating factor |
| DLT | Dose limiting toxicity |
| EGFR | Epidermal growth factor receptor |
| EM | Electron microscopy |
| Env | Envelope |
| ERA | Environmental risk assessment |
| EV | Echovirus |
| FC | Fluorocytosine |
| FDA | Food and drug administration |
| FIPV | Feline infectious peritonitis virus |
| fMHV | Felinized mouse hepatitis virus |
| GALV | Gibbon ape leukemia virus |
| (E)GFP | (Enhanced) Green fluorescent protein |
| GM-CSF | Granulocyte macrophage colony stimulating factor |
| HA | Hemagglutinin |
| HAdV | Human adenovirus |
| HCC | Hepatocellular carcinoma cells |
| HIF | Hypoxia inducible factor |
| HIV | Human immunodeficiency virus |
| HMEC | Human mammary epithelial cell |
| HSP | Heat shock protein |
| HSV | Herpes simplex virus |
| i.a. | Intra-arterial |
| ICAM | Intercellular adhesion molecule |
| IFN | Interferon |
| IGF | Insulin-like growth factor |
| IH | Immunohistochemistry |
| IL | Interleukin |
| i.l. | Intralesional |
| InflA | Influenza A |
| i.p. | Intraperitoneal |
| IRB | Institutional review board |
| IRES | Internal ribosomal entry site |

| | |
|---|---|
| ISH | In situ hybridization |
| i.t. | Intratumoural |
| i.v. | Intravenous |
| LacZ | β-galactosidase |
| LD50 | Median lethal dose for 50% of subjects |
| LTR | Long terminal repeat |
| MEF | Mouse embryonic fibroblast |
| MLV | Murine leukemia virus |
| MMP | Matrix metalloproteinase |
| MOI | Multiplicity of infection |
| MRI | Magnetic resonance imaging |
| MTD | Maximum tolerated dose |
| MU | Mumps virus |
| MV | Measles virus |
| MVM | Minute virus of mice |
| NA | Neuraminidase |
| NDV | Newcastle disease virus |
| NIH | National Institute of Health |
| NIS | Sodium iodide symporter |
| NP | Nucleoprotein |
| NYCBOH | New York City Board of Health |
| OBA | Office of Biotechnology Activities |
| PCR | Polymerase chain reaction |
| PEG | Polyethylene glycol |
| PFU | Plaque forming unit |
| PKR | Protein kinase R |
| PSA | Prostate-specific antigen |
| RAC | Recombinant DNA Advisory Committee |
| RCR | Replication competent retrovirus |
| RCVV | Replication competent viral vector |
| (c)RGD | (cyclic) Arginine-glycine-aspartic acid |
| RR | Ribonucleotide reductase |
| s.c. | Subcutaneous |
| scAb | Single-chain antibody |
| SCID | Severe combined immune deficiency |
| SIN | Sindbis virus |
| SVV | Seneca valley virus |
| TCID50 | 50% tissue culture infective dose |
| TK | Thymidine kinase |
| Tm | Temozolomide |
| UPRT | Uracil phosphoribosyltransferase |
| UTR | Untranslated region |
| VEGF | Vascular endothelial growth factor |
| VGF | Virus growth factor |
| vp | Viral particles |
| VSV | Vesicular stomatitus virus |
| VV | Vaccinia virus |
| WR | Western Reserve strain of Vaccinia virus |

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# Summary

This document is a supplement to the report ‘Environmental risk assessment of replication competent viral vectors in gene therapy trials’.

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# 1 Introduction

This document is accompanying the RIVM report ‘Environmental risk assessment of replication competent viral vectors in gene therapy trials’. This supplementary document provides a more complete overview of the types of replication competent viral vectors that are being applied in clinical and pre-clinical studies. The overview focuses on the types of modifications that are being generated in each of the applied viral backbones and on the available safety data. When appropriate, research developments and directions that may become of influence on the environmental risk assessment of certain viruses in the future are discussed. The information in this background document has essentially been summarized in Appendices A and B and in the tables presented in the RIVM report. This document provides an additional justification of the conclusions in the report and at the appropriate places the report refers to this document. More information about the rationale of this study and the conclusions of this overview can be found in the report itself.

# 2 Methods

The information on oncolytic viruses and their applications was primarily gathered by literature searches in the Pubmed database. A first inventory of replication competent viral vectors was made by using the search terms ‘oncolytic’ and ‘review’. There are a number of excellent reviews available that were used as a starting point (1-11). These reviews explain both the history of the use of oncolytic viruses and highlight the most important categories of viruses involved. Search terms based on these reviews that were applied later include ‘oncolytic’, ‘replication competent’ and ‘clinical trial’ in conjunction with the virus names shown in table III of the report and alternative names of these viruses. Additional information on ongoing clinical trials and clinical protocols involving replication competent viruses was gathered by studying the minutes of the RAC meetings since essentially all clinical protocols that include the use of GM viruses in the USA are submitted to the RAC (12). In the European Union the GTAC database is an important source of information showing an overview of trials held in the UK (13). The web pages of the limited number of biotech companies involved in the clinical development of oncolytic viruses were another source of online information (14-18).
The author has benefited very much from attending the Oncolytic Virus Meeting 2007 in Carefree, Arizona, USA. At this meeting, scientists involved in fundamental and clinical research from around the world presented and discussed their latest findings. The meeting was sponsored by all major European and North-American biotech companies involved in oncolytic virus research that for a large part presented an overview or an update of their (un-)published clinical data.
A special comment should be made about the developments in China. While the developments in China are moving fast, there is a lack of scientific transparency (19, 20). Papers are often not published in English and the attendance of international meetings is very scarce. There were for instance no representatives from the Chinese companies at the Oncolytic Virus meeting 2007. As far as China is concerned, the information in this report is therefore based on a limited number of papers published in English language journals, abstracts in English from papers published in Chinese journals, information available from general reviews and online information.

# 3 Overview of pre-clinical vectors

## 3.1 Vectors based on DNA viruses

The first viruses that were explored for oncolytic therapy or immunotherapy are DNA viruses, i.e. human adenovirus (HAdV-C), herpes simplex virus (HSV-1) and vaccinia virus (VV). General advantages of DNA viruses are that they can be easily genetically modified and are genetically stable. Especially HSV-1 and VV have a large capacity for genetic modification. Since these viruses have humans as their natural host they have been well characterized and the (vaccine) strains, on which the viral vectors are based, often have an established safety record in humans. Most of the current human population has pre-existing immunity against these viruses. While this may be good news from the standpoint of safety this may pose a problem for systemic administration (1). To circumvent this problem replication competent viral vectors (RCVVs) based on DNA viruses that have other species as their host are being applied. Myxoma virus is a poxvirus that causes a lethal disease termed myxomatosis in its specific host (the European rabbit) but that is nonpathogenic to other species. This and the lack of preexisting antibodies in the human population suggest that Myxoma could be an attractive agent for oncolytic therapy (21). Another animal DNA virus that is being explored is the rat parvovirus H-1 (22-24).
A drawback of the use of (modified) animal viruses (see also paragraph 3.2) are the specific environmental risks that may be associated with their use. Genetic modifications may for instance cause an altered pathogenicity of a virus in its natural host. Moreover, the spontaneous or engineered acquisition of an altered tropism may result in viruses crossing the host-range barrier and the establishment and persistence of a virus in the new host species, which may result in disease (zoonosis). The latter example is especially a concern in case of use of viruses not known to have been in contact with the human population previously (see Louz et al. (25)).

### 3.1.1 Adenovirus (Adenoviridae, Mastadenovirus)

The adenoviral (AdV) AdV-2 and AdV-5 serotypes, members of the serotype species AdV-C, are widely applied as a backbone for viral vectors with oncolytic properties. AdVs are double stranded DNA viruses with a linear genome of 36 kb which can be engineered to incorporate large enough stretches of DNA to permit the incorporation of foreign therapeutic genes. AdVs are relatively mild class 2 pathogens and humans are their primary and almost exclusive host. In general, AdV infections are asymptomatic but they can be associated with diseases of the respiratory, ocular and gastrointestinal system with AdV-C viruses primarily causing mild respiratory and alimentary tract infections in children. Although AdVs are able to integrate in the host cell genome no adverse consequences of this property are known; therefore the application of these viruses for gene therapy purposes does not impose the risk of causing insertional mutagenesis (26, 27). Given these properties, it is not surprising that AdV vectors are among the most commonly applied oncolytic vectors in both preclinical and clinical studies. However, AdV vectors also have some fundamental disadvantages. AdV administration may lead to hepatic toxicity (28). Wildtype AdV is not naturally specific for tumour cells and several tumour types are not efficiently transduced by AdVs. AdVs spread slowly and work poorly when administered intravenously, mostly due the pre-existing defence mechanisms present in most humans (approximately 80% of the human population is sero-positive for AdVs) (29). Importantly, there is a lack of fully permissive animal models to test the safety of AdV vectors. Mouse and rat tumours and normal tissue do not support efficient replication of human adenoviruses,

presumably because expression levels of the coxsackie adenovirus receptor (CAR) differ significantly between mice and humans. Therefore syngeneic immunocompetent models that are used to study viral replication and effectivity in presence of T-cell dependent immunity and neutralizing antibodies are not available. Most *in vivo* pre-clinical studies have made use of xenograft models in which tumour cells or tumour tissue are transplanted into nude mice. Cotton rats and Syrian hamsters (30-32) are semi-permissive models that are currently being applied to test safety and/or efficacy of AdV vectors.
The first adenoviral vectors were modified in such a way to render them specific for tumours. However, mainly for safety concerns, these vectors were also attenuated in their spreading and immune system avoiding properties (33). After initial establishment of the safety of these vectors for the patient (26), later generations with an increased efficacy are now being developed for application in the clinic. It is impossible to describe all AdV vectors separately, due to the vast amount of vectors that have been developed so far. Therefore in the following paragraphs strategies that are applied in the development of AdV vectors for oncolytic therapy are described, and examples of specific vectors are given.

### *Adenoviral vectors with deletions in viral genes leading to tumour selective replication*

The AdV genome has evolved a number of mechanisms involved in promoting viral replication and further spreading into the host. E1A is the first adenoviral gene that is transcribed after infection and is essential for viral replication; the E1A protein binds to pRb and other cell cycle checkpoint regulators, causing the release of E2F transcription factors that stimulate entry of arrested cells into the cell cycle. Furthermore E1A binds and trans-activates promoters of other early viral genes like E1B and E4; these proteins counteract intrinsic and extrinsic cellular apoptotic pathways that are activated by the actions of E1A, thereby preventing death of the cell before replication can occur (33, 34). The adenoviral E1B-19kD protein inhibits apoptotic stimuli of both the intrinsic (p53) and extrinsic (TNF/Trail-mediated) pathways and the viral-associated type I (VAI) and type II (VAII) RNAs block the interferon/PKR apoptosis pathway (34). The adenoviral E1B-55kD protein was recently shown to promote viral replication by yet another mechanism, i.e. stimulation of the export of late viral RNAs (35). E4 gene products regulate DNA replication, RNA transport and apoptosis in conjunction with the E1A and E1B gene products. For instance, E4orf6 is thought to play a role in regulating the nucleocytoplasmic shuttling of late viral messages, p53 degradation and shutoff of host cell protein synthesis together with E1B-55kD (34, 35).
Deletion of adenoviral genes can be applied to specifically target AdV vectors to tumour cells. Tumour cells are unrestricted in their growth because they accumulate mutations and other genetic defects that lead to increased proliferation and defective apoptosis. Many of these mutations occur in genes that have a role in the same cellular pathways as those targeted by the abovementioned AdV gene products and by proteins from many other viruses. Frequent mutations in tumours include inactivating mutations in the tumour suppressor genes p53 and pRb, and activating mutations in the Ras proto-oncogene, that targets the interferon/PKR apoptosis pathway. For yet unknown reasons, tumour cells are also altered in their mechanisms of RNA export. Therefore tumour cells (as opposed to normal cells) can, depending on the type of mutation occurring in those cells, complement for the loss of function of specific viral genes in viral vectors, leading to tumour-selective viral replication (33, 34).
Vectors containing deletions in specific adenoviral genes are designated type 1 conditionally replicating adenoviral vectors (CRAds). The first oncolytic vectors that have been extensively studied in clinical phase I-III trials are the E1B-55kD deleted vectors Onyx-015 (36) and H101 (37). Despite a limited efficacy in most trials, results were deemed promising and lead to the rapid development of more potent oncolytic AdV vectors. Since E1B-55kD is thought to have various functions involved in viral replication, deletion of the entire E1B-55kD gene may have contributed to its attenuated replication in some human cancer cell lines. Adenoviral mutants targeting specific E1B-55kD functions have been generated that show higher replication competency in tumour cell lines compared to Onyx-

015 (38). The E1B-19kD deleted vector Ad309 was shown to display enhanced anti-tumour potency and viral spread in preclinical *in vitro* and tumour xenograft models compared to wildtype AdV virus and Onyx-015 (34). Both AdΔ24 (39) and dl922-947 (40) contain a small deletion in the CR region of the E1A gene that abrogates binding to the pRb protein. In preclinical studies also these vectors show increased potency compared to Onyx-015 (40, 41). The dl1331 vector carries a deletion in the VAI RNA coding region and showed anti-tumour activity in a Ras dependent fashion both *in vitro* and in a xenograft model (42, 43).
Most of these first generation vectors (except dl1331) contain secondary deletions in the adenoviral E3 region that was initially thought to be non-essential for viral replication. In recent years it was shown that E3 region genes encode proteins with important anti-apoptotic and immuno-modulary functions. While these deletions may have increased the selectivity and the safety of the first generations of AdV vectors, they may have compromised the efficacy of AdV vectors (34, 44). For instance deletion of the E3-RID and E3-14.7 genes, which play an important role in preventing TNF-induced apoptosis, led to enhanced sensitivity to TNF and increased viral clearance in single E3B single and compound E3B/E1B-19kD deleted vectors compared to a single E1B-19kD deleted vector in preclinical models (34). Therefore in more recent AdV vectors the functions of certain E3 genes are retained or even enhanced. Examples are the KD1 and KD3 vectors that contain two small deletions in E1A that abrogate pRb binding plus the deletion of all E3 genes, except for the adenoviral death protein (ADP) gene (KD1) or the ADP and E3-12.5K genes (KD3). KD1 and KD3 show overexpression of the viral ADP protein leading to enhanced cell to cell spreading and higher cytopathic effects *in vitro* (compared to wildtype AdV and fully E3 deleted viruses) and *in vivo* (compared to fully E3 deleted viruses) (45). E1A-CR2 deleted vectors are able to replicate in cycling normal cells (40). Based on the initial data E1B-55kD deleted vectors were thought to be permissive for p53 deficient cells only. After more extensive analysis it was shown that E1B-55kD deleted vectors do replicate in certain tumours without p53 mutation and importantly also to a certain extent in normal cells (46). To improve the relative specificity of AdV vectors, vectors with additional mutations have been generated. The CB1 and Adl118 / AxdAdB-3 adenoviral vectors contain deletions in E1A-CR2 plus E1B-55kD and E1A-CR2 plus E1B-19kD, respectively (46-48). These vectors show similar anti-tumour efficacy compared to control vectors containing single deletions in tumour cell lines and *in vivo* xenograft models, with an improved specificity for tumour cells. CB1 was in contrast to AdΔ24 not able to replicate in dividing normal cells *in vitro* (47). Likewise, the AdVAdel vector that has deletions in both VAI RNA coding regions showed an increased relative specificity compared to dl1331 for Ras dependent tumours while retaining anti-tumour efficacy (43).

### *Adenoviral vectors with foreign regulatory sequences to promote tumour-specific expression*

An alternative strategy to specifically target AdVs to tumours is to drive expression of essential viral genes by ‘foreign’ regulatory sequences that are hyper-reactive in certain tumour cells, a strategy also known as ‘transcriptional targeting’. In a review from 2004 (33), 26 of these type 2 CRAds were counted and many more have been published since then. In most cases, expression of the E1A gene is placed under the control of a human promoter or other regulatory elements that are active in tumour cells. Promoters from the cellular genes telomerase reverse transcriptase (TERT) and E2F-1 were used to target E1A expression and viral replication to many types of tumours, making use of the fact that most tumour cells have elevated levels of TERT and E2F-1. Similarly, hypoxia inducible factor (HIF) responsive promoters were used because hypoxia occurs in most solid tumours. To target E1A expression to specific tumour types, regulatory elements of for instance the cellular genes PSA (specific for prostate tumours), AFP (liver tumours), Tyrosine enhancer (melanoma), DF3/Muc1 (breast cancer), and Tcf (colon cancer) have been applied (33).

Like type 1 CRAds, type 2 CRAds replicate preferentially but not exclusively in tumour cells. Small amounts of E1A protein are known to be sufficient to initiate 'leaky' adenoviral replication and in most cases a small amount of replication is observed *in vitro* in normal cells. Moreover, promoters may be activated *in vivo* in certain cell types or under conditions that are difficult to test *in vitro*.
To increase the specificity of replication, additional essential viral genes (i.e. E1B, E4) may be placed under control of the same or different tumour-specific regulatory elements as those applied for the E1A gene. For instance in the Ad.Flk-Endo vector (in which E1A and E1B gene expression are controlled by Flk-1 and endoglin regulatory elements, respectively) replication was 600 times reduced in control cells compared to dividing human endothelial cells[1], while for Ad-Flk-1 (E1A driven by Flk-1) replication was only 30 times reduced (49).
In two other CRAds designed for treatment of breast cancer, AdEHT2 and AdEHE2F, E1A expression was under control of minimal estrogen plus hypoxia responsive promoters and E4 expression under control of the TERT or E2F promoter, respectively. While for the AdEHE2F vector the E2F regulatory region contributed to the restricted replication in tumour cells, AdEHT2 did replicate in telomerase negative normal cells. These results clearly show that these modifications do not always result in a more restricted replication. While the use of minimal promoters may reduce the chance of replication in non-tumour tissues, deletion of negative regulatory elements may have the opposite result (50). This example shows that results with type 2 CRAds are difficult to predict and depend on the viral and cellular context.
Unlike the first generation vector CV706 (PSA promoter controlled E1A), the second generation vector CV764 (PSA promoter controlled E1A and hklk2 promoter controlled E1B) was found to be replication deficient in PSA negative cell lines (51). Although these vectors successfully inhibited tumour growth *in vitro*, they failed to show potent *in vivo* action. Inclusion of E3 sequences in a third vector (CV787; rat probasin promoter controlled E1A, PSA promoter controlled E1B) resulted in increased *in vitro* and *in vivo* efficacy, while retaining a greatly attenuated replication (more than 10.000 times compared to wildtype AdV) in PSA negative cells, again emphasizing the important role of E3 genes for retaining AdV oncolytic activity (52).
A different strategy to increase the tumour specificity is to combine transcriptional targeting with deletion of viral genes. For instance in the vector Onyx-411 the E1A-CR2 deletion is combined with an E2F-1 promoter driven E4 gene leading to reduced replication in normal cells, while retaining a tumour-killing efficacy similar to wildtype AdV (53). Moreover, a reduced hepatic toxicity was observed which was attributed to the reduced expression of E1A because of the transcriptional targeting of the E4 gene (54). By using heterologous promoters this strategy can be applied to target oncolytic vectors in either a pan-tumour fashion (e.g. Onyx-411) or to specific tumour types. An example is the KD1-SPB vector in which the SPB promoter limits E4 expression to lung cancer cells (55).

### *Translational targeting of adenoviral vectors*

Many tumour cells have a constitutively active Ras-MAP kinase pathway leading to tumour-selective stabilization of certain cellular mRNAs. Upregulation of Cox2 in Ras-transformed tumours is in part mediated through selective stabilization of the Cox2 messenger through a region in the 3'-UTR that is activated by the MAP kinase pathway. Ahmed et al. used this property to generate a tumour selective CRAd Ad-E1A-Cox in which the E1A gene is fused to the 3'-untranslated region (UTR) of the Cox2 gene. Ad-E1A-Cox was preferentially oncolytic *in vitro* in tumour cell lines with an activated MAP kinase pathway and was as efficient as wildtype AdV virus in an *in vivo* xenograft model (56).

[1] This example shows that AdV vectors are not exclusively generated for targeting tumor cells but also to target normal cells, in this case to counteract angiogenesis related to tumor growth.

***Adenoviral vectors armed with therapeutic transgenes***

The complexity of cancer necessitates that multiple treatment modalities (e.g. surgery, chemo- and radiotherapy) need to be combined for effective treatment. Therefore oncolytic viruses need to be complemented by additional agents to have long-term therapeutic outcomes. A strategy which can be applied is to arm replicating viruses with therapeutic transgenes to enhance the probability of tumour eradication through multiple modes of attack (26, 57, 58). In a recent publication Chinese researchers claim that they have generated several type 1 (E1B-55kD deleted) and type 2 (TERT promoter driven E1A) CRAds containing single insertions encoding therapeutic genes as diverse as tumour suppressor genes (e.g. p53, pRb), inhibitors of apoptosis (e.g. IAP), apoptosis inducing genes (e.g. Trail), immune regulatory genes (e.g. GM-CSF, IL12), angiogenesis inhibitory genes (e.g. VEGF) and suicide genes (e.g. TK, CD). Moreover they have shown that application of two different oncolytic vectors at the same time can have a synergistic therapeutic effect in animal models. Liu et al. indicate that they have already constructed type 1 and 2 CRAds containing two transgenic insertions working in the same pathway (e.g. hTERT-AFP-Trail/Smac, in which Smac increases the effects of Trail) (59). Although we have to await data to support all these claims, this publication gives a good idea of the wide spectrum of therapeutic inserts that already have been applied in replicating AdV vectors thus far.

A common transgenic insertion in AdV vectors in general has been the inclusion of suicide genes: enzymes that are able to convert non-toxic prodrugs into cytotoxic metabolites that in most cases are also able to spread to non-infected neighbouring cells. Examples of oncolytic vectors containing suicide genes are Ad5-TK(II)$^{RC}$ (30, 60) an E1B-55kD and E3 deleted vector containing the herpes simplex thymidine kinase gene (TK), AdV-hTert-CPG2, in which the E1A gene is driven by the TERT promoter and the E3-gp19kD gene is replaced by carboxypeptidase G2 (61) and Ad5-CD/TKrep, an E1B-attenuated, replication-competent AdV vector containing a cytosine deaminase (CD)/TK fusion gene (62, 63). Ad.OW34 is a variant of Ad5-TK(II)$^{RC}$ lacking the E1B-55kD deletion. This E3 deleted AdV vector has a CMV-TK insertion rendering it more potent in preclinical studies compared to Ad5-TK(II)$^{RC}$. While prodrug administration increases the anti-tumour efficacy of most of the above vectors, it decreased the anti-tumour efficacy of Ad.OW34. Since there are currently no antiadenoviral agents approved for clinical use suicide genes like TK may also be inserted as a failsafe mechanism should the adenovirus infection need to be terminated (30, 60, 64).

AdV oncolytic vectors have also been armed with immunostimulatory cytokines to increase their efficacy. Onyx-320 and Onyx-321 express TNF-α, a pro-inflammatory cytokine with a variety of antitumour activities, instead of the ADP or E3B gene (65, 66). In the vector Onyx-372 native viral promoters were used to express TNF-α and the co-immunostimulatory gene MCP-3 instead of the E3-gp19kD and ADP genes (58). Other examples of cytokine containing vectors are YKL-IL12 and YKL-IL12/B7, E1B-55kD deleted AdV vectors armed with CMV-driven IL12 inserted in the E1B region, plus or minus CMV-driven B7.1 (a T-cell stimulatory molecule) inserted in the E3 region, respectively (67).

In Adp53rc and Adp53W23S, the tumour suppressor gene p53 and a mutant form of p53 with increased stability were inserted via an internal ribosomal entry site (IRES) in the adenoviral fiber transcription unit of an E3 deleted AdV vector (68, 69). AdΔ24 vectors have also been armed with (mutant) p53 or with TIMP-3 (an angiogenesis inhibitor) using a SV40 or CMV promoter driven expression cassette inserted in the E3 region (70-72).

Recently siRNAs were included in oncolytic vectors in order to target specific genes (73). Internavec (for interfering RNA vector) is a novel version of Onyx-411 containing a siRNA directed against the oncogenic Kras gene. Internavec had a more potent *in vitro* and *in vivo* effect than Onyx-411 against tumours containing Kras-mutations, while maintaining the relative selectivity of Onyx-411 in non-malignant cells (53).

Marker and reporter genes like luciferase (74), Dsred2 (75) and the human sodium iodide symporter (hNIS) (76) have also been included in oncolytic vectors as they are useful for monitoring adenoviral replication, distribution and antitumour efficacy both in pre-clinical and clinical studies.

### *Adenoviral vectors with modifications of viral coat proteins*

AdV-C viruses use several receptor binding sites to gain access to the cell. Viral attachment is mediated by the knob of the fiber protein that binds to the cellular coxsackie and adenovirus receptor (CAR). Viral uptake into the cell is mediated by the RGD motif of the adenoviral penton protein that binds to cellular integrins. Moreover, lysine residues in the fiber shaft that bind to heparan sulfate glycosaminoglycans (HSG) are involved in viral binding and transduction.
Tumour cells show a great variety in their CAR receptor expression level which can limit the efficacy of AdV vectors (77, 78) . Moreover, AdV viruses are able to infect a broad range of cells which may limit the viral fraction that becomes available for target cell transduction *in vivo*, especially upon systemic administration. Also other factors may limit the clinical efficacy of AdV vectors. Upon systemic administration, especially the liver but also other organs function as an adenoviral sink. Kuppfer cells in the liver are known to play a major role in adenoviral clearance in a non-CAR mediated process. Pre-existing titers of neutralizing antibodies against AdVs are limiting the clinical efficacy of AdV vectors, especially in multiple dosing regimens. 'Transductional targeting' can be aimed at (a) enhancing the specific transduction of CAR-deficient tumour cells, (b) deleting the broad tropism of AdV-2/5 in normal epithelial cells, (c) preventing systemic clearance by neutralizing antibodies and (d) decreasing the toxicity of AdVs to the liver (79-82).
A number of strategies can be applied to redirect AdV viruses to tumour cells without ablating CAR binding. In the E1B and E1A-CR2 deleted vectors Adv-E1BdB-F/K20 and Ad5.pK7-D24 extra lysine residues were incorporated into the C-terminus of the fiber protein leading to increased activity against glioma and breast cancer, respectively (83, 84). In AdΔ24-RGD an additional RGD motif was incorporated in the HI loop of the fiber knob allowing direct attachment of the virus to cell-surface integrins. This modification allowed more effective oncolysis of cancers with Rb pathway abnormalities and low CAR expression *in vitro* and *in vivo* (85-88). Incorporation of the RGD motive in a type 2 CRAd, RGDCRADcox-2, resulted in an *in vivo* anti-tumour efficacy comparable to AdΔ24-RGD (89).
AdV vectors can be programmed genetically to be directed to different target cells by incorporation of sequences encoding adapter molecules. This strategy is an extension of the strategy in which AdVs are retargeted by systemic co-administration of dual-specific antibodies (79, 82). An example is AdΔ24-425S11, a CRAd that has a CMV driven expression cassette encoding a bispecific single-chain antibody directed towards the AdV fiber knob and the EGFR incorporated in its E3 region (90, 91). This vector was more potent compared to AdΔ24 in destroying neuroblastoma cells (that overexpress the EGFR and that have low expression levels of CAR) *in vitro* and *in vivo*.
To develop a targeted AdV vector that is systemically more effective the natural CAR tropism can be ablated by fiber (knob) replacement. An additional advantage of this strategy is the concomitant reduction in non-target organ transduction and sustained bloodstream persistence because the AdV-5 fiber (in a CAR independent manner) is responsible for viral uptake by, and toxicity to the liver (79, 82, 92).
In fiber chimeric vectors the knob domain of the AdV-5 fiber protein is replaced by the knob domain of for instance AdV-B serotype viruses (like AdV-3) that bind to a different cellular receptor (82). The fiber chimeric vector Ad5/3-Δ24, an AdV-5 CRAd pseudotyped with the AdV-3 fiber knob and therefore deficient in CAR binding, was superior to AdΔ24-RGD in killing ovarian cancer cells *in vitro* and *in vivo* (93, 94). The infectivity of fiber chimeric viruses can be further enhanced by incorporating

the RGD motif in the fiber protein allowing more effective binding to integrins that are overexpressed in many cancer types (95).
A strategy that has been recently developed for replication deficient AdVs and that has been proposed for future incorporation into CRAds is to replace the entire fiber protein with a fusion molecule comprising the virion-anchoring domain of the fiber and the oligomerization domain of the reovirus attachment protein S1 (96).
PEG or polymers can be conjugated to the AdV capsid to conceal AdV vectors from pre-existing antibodies and to decrease systemic clearance. The drawback of this method is that shielding is lost during viral replication. Recently a genetically based shielding method was developed. Large proteins like TK are incorporated in the virion capsid by fusion to the minor capsid protein pIX leading to reduced recognition by AdV antibodies. A shielded version of AdΔ24-RGD, AdΔ24S-RGD is being developed (81).

***Transcomplementing adenoviral vectors***

To increase the safety of use of AdV vectors several transcomplementing strategies have been developed (97, 98). An example is the co-administration of an E1-deleted non-replicating AdV vector expressing the TK gene (AV.C2.TK) and Ad5.dl1014, an E4-deleted/E4orf4-only expressing AdV in order to allow full replication competence in co-infected tumour cells (98). Binary systems also represent a flexible platform for screening of multiple gene products and the enhanced insert capacity of replication-deficient vectors compared to replication-competent vectors allows for the cloning of larger inserts. For instance co-infection of Ad Flk1-F, an replication deficient vector with a VEGF therapeutic transgene and dl922-947 resulted in repackaging of the replication deficient vector leading to tumour selective replication, increased transgene expression and increased *in vivo* antitumour activity in mice (99). Similarly expression of fusogenic glycoproteins, which promote cell fusion and increased dispersion of viruses throughout tumours, was increased by co-infection of a replication deficient AdV with replication competent helper virus (100).

***Multimodal adenoviral vectors***

Treatment of aggressive metastasized cancers requires the generation of AdV vectors that can be administered systemically, that are specifically infectious for tumour cells and that have profound tumour-specific cytotoxic effects. Several of the modifications mentioned in the previous sections have to be combined in order to construct AdV vectors with such qualities.
The CRAd Ad5/3cox2LΔ24 expresses a CR2 deleted E1A gene driven by the Cox-2 promoter and has an AdV-5/3 fiber chimeric protein. These modifications lead to increased selectivity and reduced toxicity compared to Ad5/3Δ24 and wildtype AdV, and an increased efficacy against ovarian cancers compared to wildtype AdV and Ad5/3Δ24. Although none of these CRAds replicated in primary human liver cells some replication was observed in dividing fibroblasts (101).
Ad.MCDIRESE1.71Hsp3 is another example of a vector combining several types of modifications. First, the vector contains the E1B-55kD deletion. Second, the E1A and CD genes (linked by an IRES) are driven by a tyrosinase enhancer element inserted in the E1 region, to make the replication of this vector and the induction of CD gene expression specific for melanoma cells. Third, this vector contains a CMV-Hsp70 expression cassette in the (deleted) E3 region allowing overexpression of Hsp70 that stimulates several innate immune responses in infected cells. Last, an RGD peptide is inserted in the fiber protein to increase the infection efficiency of this vector. This multimodal vector specifically replicated in melanoma cell lines (as opposed to Hela cells) and had an increased melanoma-specific cytotoxic effect in the presence of 5-fluorocytosine *in vivo* compared to a control vector without the Hsp70 insertion (102). The OV1195 vector is a third example of a vector containing several

modifications. E1A is driven by the E2F-1 promoter, the human GM-CSF gene is inserted instead of the E3-gp19kD gene and the vector contains a chimeric AdV-3/5 fiber with RGD insertion. The cytotoxicity and replication of this and other fiber chimeric viruses was attenuated in normal cells compared to wildtype AdV-5. Compared to an oncolytic virus with a normal AdV-5 fiber the chimeric viruses were more cytotoxic to head and neck tumours, but also to normal cells (102, 103).

***Future developments with AdV vectors***

The overview of multimodal vectors described in the previous paragraph shows that there is a tendency to include more modifications into AdV vectors to increase their efficacy while maintaining their relative safety. These vectors are still in the pre-clinical stage but may be expected to enter clinical trials in the future once the safety of vectors containing each of the individual modifications is proven. In this respect it is important to note that recently the first the tropism-modified AdV vector has entered a clinical trial in the USA.
AdV vectors based on serotype B are being investigated as an alternative for AdV-2/5 vectors because the immune prevalence for these viruses is lower and they bind to the CD46 receptor that is overexpressed on many tumour cells (81, 82). Insertion of measles virus MV-H/F proteins into AdV-5 is an attractive alternative strategy. These fusogenic glycoproteins determine the CD46 tropism and tumour selectivity of oncolytic MV (see paragraph 3.2.2) and moreover they induce syncytium formation and apoptosis of permissive tumour cells with considerable bystander effects (104, 105). Recently, the first non-human CRAd based on the canine AdV CAV-2 was generated. This vector was developed to be able to study the effects of immuno-suppression in a host system that is totally permissive for AdV virus. In the canine vector OC-CAVE1 the E1A gene was driven by the osteocalcin promoter, causing efficient replication and oncolysis of dog osteosarcoma cells *in vitro* and in a mouse xenograft model (106). This group has also generated fiber chimeric replication deficient AdV-5 vectors that contain the CAV-2 knob and a polylysin insertion to improve the infectivity of dog osteosarcoma cells and are planning to generate infectivity enhanced canine CRAds. AdV vectors with the canine knob were more effective and yielded more efficient gene transfer than vectors with the AdV-5 knob in canine cells and human cells. This may be linked to the fact that CAV-2 has the ability to transduce CAR-deficient cells suggesting that CAV-2 is using a second cellular receptor. This is potentially interesting for treatment of certain human cancers since the improvement of infectivity observed with AdV-5/3 fiber chimeric vectors in human epithelial-derived neoplasms, was not observed for mesenchymal neoplasms, like sarcomas (107).

### 3.1.2 Herpes simplex virus (Herpesviridae, Alphaherpesvirinae, Simplexvirus)

Herpes simplex virus, type 1 (HSV-1) is an enveloped double stranded DNA virus with a 152 kb genome encoding 84 known genes. Roughly half of the HSV-1 genes are essential for viral replication, and that the other half appears to have a function in blocking the host cell immune response. HSV-1 is a neutrophic virus that uses the retrograde axonal transport route to reach spinal ganglia. Persistent infections of neurons with HSV-1 occur that remain in latency until the occurrence of decreased immunity in the infected individual (7, 11). HSV-1 rarely produces life-threatening illness in healthy adults. Symptoms of HSV-1 infection may include epithelial lesions as well as disseminated disease and encephalitis.
HSV-1 attaches to the cell surface by interaction of viral envelope glycoproteins (such as gC and gB) with the glycosaminoglycan moieties of cellular heparin sulfates. Fusion of the viral envelope with the cell membrane involves a number of other glycoproteins of which glycoprotein gD binds to cell surface receptors that include nectins. Since HSV receptors are expressed in many cell types, HSV-1 has a very broad tropism. The HSV-1 replication cycle takes about 18-20 h. Immediate early genes start being

expressed after circularization of the HSV-1 DNA and are involved in transcriptional regulation and host-cell shut-off. Early genes, of which the expression is depending on the immediate early genes, are mainly involved in DNA replication. Structural genes are mostly encoded by the late viral genes (7, 11).
Several features of HSV-1 make it an attractive vector for oncolytic therapy; the fast replicative cycle (compared to AdV-5), the fact that it can transduce non-dividing and dividing cells, the high levels of transgene expression, the availability of models to test efficacy and safety and of anti-viral drugs (acyclovir). Moreover, HSV-1 has the ability to spread through cellular junctions allowing penetration of solid tumours. A potential risk of the use of HSV vectors is the generation of virulent forms containing therapeutic genes through homologous recombination in patients between the vector and wildtype HSV (7, 11). Genetically modified HSV vectors are based on different strains (laboratory strains or clinical isolates) of HSV-1. Most vectors are derived from the laboratory strains F, 17+ or KOS. The HSV-1 F strain is the most attenuated of these strains (108, 109).

***Conditionally replicating HSV-1 vectors with deletions of single viral genes***

One of the approaches to create tumour-selective HSV-1 virus is to delete gene functions that are critical for efficient viral replication in normal cells but that are dispensable in tumour cells. Current generations of conditionally replicating HSV-1 vectors have included mutations and/or deletions in one or more of the genes encoding thymidine kinase, DNA polymerase, uracil DNA glycosylase, ribonucleotide reductase (RR), and ICP34.5.
The TK gene has a function in synthesis of deoxyribonucleotides to facilitate viral DNA replication in cells with suboptimal precursor pools. TK deleted HSV vectors like dslptk induced tumour regression in several animal models but were not tumour selective in SCID mice, and have the disadvantage that they are not sensitive to acyclovir (11).
In the HSV-1 KOS strain derived hrR3 vector the UL39 gene is deleted. UL39 encodes the ICP6 protein which is the large subunit of the HSV-1 ribonucleotide reductase (RR), an enzyme necessary for DNA replication in non-dividing cells. The hrR3 vector replicated efficiently in tumour cells while replication in normal cells was attenuated. Moreover, hrR3 showed anti-tumour efficacy in animal models for brain, pancreas, colon and liver cancer (11, 110-112).
HSV1716 (derived from the HSV-1 strain 17) is deleted for both copies of the ICP34.5 gene, which gene product targets the protein kinase R (PKR) antiviral pathway by binding to a cellular phosphatase that dephosphorylates the PKR target eIF-2a leading to restored translation and virus production. The deletion of ICP34.5 attenuates HSV-1 neurovirulence. ICP34.5 deleted vectors (that also include the vectors R3616 (F strain) and R4009) are replication selective for dividing cells and showed anti-tumour efficacy in models for glioma, mesothelioma, melanoma, ovarian cancer and lung cancer. While in most studies HSV1716 was non-toxic (11), residual replication in and toxicity to normal cells has also been reported *in vitro* and *in vivo* (113, 114).
Intracerebral inoculation of the single gene deleted HSV vectors R3616, hrR3 and HSV1716 caused toxic effects and morbidity in immunodeficient and/or immunocompetent rodent models, sometimes at low doses ($10^3$ pfu for HSV1716 in nude mice) (115-117). Nevertheless, HSV1716 has been applied in several clinical studies.

***Conditionally replicating HSV-1 vectors with deletions of multiple viral genes (and in some cases extra insertions)***

G207 and MGH-1 are similar vectors based on the HSV-1 F strain containing deletions in the UL39 gene and both copies of ICP34.5. In G207 the UL39 gene is disrupted by insertion of the lacZ coding sequence. G207 was found to be non-toxic in various rodent models and did not induce toxicity after

high doses in non-human primates in contrast to wildtype HSV-1 strains. G207 demonstrated antitumour effects in immunocompetent and immunodeficient models and pre-existing immunity did not influence these results (11, 118-129). Several phase I and II trials of G207 for the treatment of malignant brain tumours have been initiated.
NV1020 is a replication-competent HSV-1 vector with an HSV-2 glycoprotein insertion that was genetically engineered to attenuate its pathogenic ability (130). The virulence of NV1020 is highly attenuated relative to the parental strain HSV-1 (F) due to deletion of one copy of the ICP34.5 gene, of 15 kb spanning the internally repeated region of the genome extending through UL55/56 (unknown function), and because of a 700-bp deletion that prevents expression of the UL24 neurovirulence factor. NV1020 is endogenous $TK^-$ but sensitive to anti-viral drugs since it expresses an exogenous copy of the HSV-1 TK gene driven by the viral α4 promoter. Although NV1020 has a higher proliferative rate than G207 *in vitro*, presumably due to the presence of one copy of the ICP34.5 gene, both vectors were effective in treating pancreatic and prostate tumour xenografts and orthotopic bladder cancer grafts *in vivo* (131-133). NV1020 replicates selectively in tumour cells and showed efficacy in various tumour models for e.g. liver, colorectal and head and neck cancer (127, 131, 132, 134, 135). NV1020 administration induced minor toxicities and local lesions in rodents and primates, but not disseminated disease (130, 136). NV1020 has been applied in clinical phase I trials in patients with colorectal carcinoma that has metastasized to the liver (see paragraph 4.2.3) (137).
NV1023 and NV1066 are derivatives of NV1020 expressing marker genes. In NV1023, compared to NV1020, the α47 gene is deleted preventing expression of UL23 (TK), the UL24 deletion has been repaired and the vector contains a copy of lacZ instead of the TK insertion behind the α4 promoter (138). NV1023 was effective in thyroid cancer and invasive carcinoma models (139, 140).
NV1066 contains the GFP gene driven by a CMV promoter. NV1066 (delivered intraperitoneally) was effective against human gastric cancer cell xenografts with almost no virus detectable by PCR and imaging in normal organs (141). NV1066 was amongst others also effective against models of neuroblastoma (142) and various combination therapies (radiation therapy, chemotherapy and estrogen) are able to potentiate the anti-tumour activity of NV1066 in specific pre-clinical cancer models (143-147).
G47Δ is a vector derived from G207 with an extra deletion in the ICP47 gene that normally inhibits the TAP protein, a cellular transporter involved in tumour-antigen presentation. Compared to G207, G47Δ was more potent in immunocompetent and immunodeficient animal models while remaining non-toxic (148, 149).

### *Conditionally replicating HSV-1 vectors with foreign regulatory elements*

The HSV-1 vector Myb34.5 contains deletions of both copies of ICP34.5 and a reinsertion of one copy of ICP34.5 in the ICP6 locus (causing disruption of this gene) under control of the tumour-specific B-myb promoter. This promoter is active in tumour cells with elevated levels of the E2F transcription factor. Compared to hrR3, Myb34.5 is more attenuated *in vitro* and *in vivo* in normal cells and less toxic in mice after intravenous administration while retaining anti-tumour efficacy against liver tumours (150, 151). Several other vectors with HSV-1 genes under the control of foreign regulatory elements combined with deletion of HSV-1 genes have been generated (11). An example is RQnestin34.5 that contains the foreign nestin promoter for selective targeting of virus to glioma tumour cells (152).

### *HSV-1 armed with therapeutic genes*

HSV-1 vectors have been armed with immunomodulatory genes, suicide genes, angiogenesis inhibitors and fusogenic proteins from other viruses (11).

Oncovex$^{GM-CSF}$ was constructed by deleting the genes encoding for ICP34.5 and ICP47 in a freshly isolated, more oncolytic, HSV-1 strain JS1 and inserting the gene encoding for granulocyte-macrophage colony stimulating factor (GM-CSF). Antitumour effects were observed *in vitro* and *in vivo* in mice in injected and non-injected tumours and these effects were more potent compared to control vectors without GM-CSF insertion (153). Oncovex$^{GM-CSF}$ has been subjected to several phase I clinical trials (154).
Replication competent HSV vectors deleted in ICP34.5 and instead containing two copies of the immunostimulatory cytokine IL4 or the immunosuppressive cytokine IL10 have been generated. While HSV-IL4 was effective in a glioma model in immunocompetent mice, HSV-IL10 was not effective (155). Another example of a cytokine expressing vector is NV1042, a derivative of NV1023 that expresses IL12 (a stimulator of T-cells and an inhibitor of angiogenesis) driven by the α4 promoter (138). NV1042 was more effective than its parental vector NV1023 in eradicating prostate, lung, colorectal cancer and squamous cell tumours in various mouse models (135, 138, 156, 157). Moreover, NV1042 was more effective compared to G207, G47Δ and NV1023 in treatment of metastatic breast cancer (158). A derivative of G47Δ double-armed with IL18 and B7-1 had significantly improved anti-tumour efficacy compared to G47Δ in two immunocompetent mouse tumour models for prostate cancer and neuroblastoma, by enhancement of T-cell mediated immune responses (159). Likewise, derivatives of G47Δ expressing the antiangiogenic factor platelet factor 4 or the dominant negative fibroblast growth factor receptor (an inhibitor of tumour cell migration) were more effective than the parental vector in neural tumour models (160, 161).
The vectors HSV1yCD and rRp450 respectively contain the CD and the cytochrome P450 gene instead of the disrupted RR gene. These vectors were more effective, in presence of prodrug, compared to their parental vector hrR3 in pre-clinical tumour models (162, 163).
Several HSV vectors with an incorporated cell membrane fusion capability have been generated. Fu-10, selected from G207 through random mutagenesis, had a significantly greater antitumour effect compared to the parental virus on xenografted lung metastatic breast cancer (164). Synco-1 and Synco-2, two ICP34.5 deleted HSV vectors that express the fusogenic gibbon ape leukemia virus (GALV) glycoprotein by means of the CMV promoter or the endogenous UL38p promoter, respectively, were more potent than the non-fusogenic control vector Baco-1 in killing cell cultures as well as human liver cancer xenografts (165). Synco-2D, a double fusogenic vector generated by random mutagenesis of the ICP34.5 deleted vector Baco-1 and insertion of GALV glycoprotein, was superior to all control vectors in controlling lung metastases of human prostate cancer xenografts and was effective against ovarian cancer xenografts and mammary tumours growing in immuno-competent mice (166-168).
Oncovex$^{GALV/CD}$ is a variant of Oncovex$^{GM-CSF}$ containing the GALV and CD genes instead of GM-CSF in the deleted ICP34.5 locus of the JS-1 oncolytic strain. When administration of this vector was combined with prodrug treatment more potent anti-tumour effects were observed *in vivo* in nude mice and immunocompetent rats compared to Oncovex$^{GM-CSF}$ or control vectors containing one of the inserts (169).

### *Transcomplementing HSV vectors*

Conditionally replicating HSV vectors can be applied in combination with defective HSV vectors that contain the HSV genes deleted in the conditionally replicating vector under the control of foreign regulatory elements that can be activated in tumour cells. This strategy targets the HSV virulence to specific tumour cells and enhances the therapeutic efficacy of conditionally replicating oncolytic HSV vectors. An example is the combined use of G207 in combination with a defective vector containing ICP34.5 (which is deleted in G207) driven by the musashi1 promoter in order to target ICP34.5 expression to glioma cells. This approach resulted in an increased anti-tumour activity compared to G207 alone in glioma models *in vitro* and *in vivo* (170). Likewise, therapeutic transgenes (e.g. IL12)

can be inserted in a replication defective HSV vector with a replication competent vector as a helper virus (171).

***Vectors based on HSV-2***

HSV-2 is an HSV variant involved in sexually transmitted disease. Attenuated vectors based on HSV-2 are currently being investigated. The HSV-2 ICP10 gene encodes serine/threonine protein kinase activity in its PK domain. This domain binds and phosphorylates the GTPase-activating protein Ras-GAP, leading to activation of the Ras/MEK/MAPK mitogenic pathway, and is required for efficient HSV-2 replication. FusOn-H2, a HSV-2 vector deleted in its PK domain, selectively replicates and lyses human tumour cells with an activated Ras signalling pathway. The virus had anti-tumour effects against pancreatic cancer xenografts (172).

***Future developments with HSV vectors***

Many of the modifications that have been described for adenoviruses are also being explored for HSV. Like adenoviruses, the HSV-1 vectors that are being constructed are becoming more complex, containing several modifications. In contrast to AdVs the development of tropism modified HSV is still in its infancy. Retargeting has been demonstrated to be feasible in replication deficient HSV-1 based vectors by abrogating binding to heparin sulfates through gB and fusing (truncated) gC to a ligand that binds to a specific receptor, and this may also be explored in the context of oncolytic vectors in the future (11). In literature, several other strategies for enhancing the anti-tumour efficacy of HSV vectors have been described. These include the use of more virulent clinical isolates of HSV-1 strains for genetic engineering and the isolation of more potent variants by serial passaging (11). In addition several strategies combining the use of replication competent HSV vectors with combination treatments are being developed.

### 3.1.3 Vaccinia virus (Poxviridae, Chordopoxvirinae, Orthopoxvirus)

Vaccinia virus (VV) is a member of the family of Poxviridae which are ~200 kb double stranded DNA viruses that remain in the cytoplasm and use virally encoded polymerases to replicate. VV may either be a laboratory survivor of an extinct virus or a laboratory derivative of a cowpox vaccine. VV was the first widely used vaccine and its use has resulted in the worldwide eradication of smallpox. VV is much less contagious compared to small-pox that is mainly transmitted via the respiratory tract. VV is regarded as a safe agent, although it induces a strong immune response and can be lethal in immunocompromised patients by causing encephalitis. Despite the presence of neutralizing antibodies, VV efficiently spreads in vaccinated persons making it possible to administer the virus over multiple injection cycles. VV has a wide host range and tropism and is able to infect most mammalian cell lines. VV particles are present in 4 stable forms that differ in abundance, localization and function. Intracellular mature virus (IMV) is the most abundant particle. At the trans-golgi network or early endosomes intracellular mature virus particles are wrapped by a double membrane to form intracellular enveloped particles that move to the cell surface. At the cell surface, cell-associated enveloped virus particles that are important in cell to cell spread, and extracellular enveloped virus particles that mediate long-term dissemination are formed (173).
Cellular entry, that is likely to involve cell surface binding to heparin and chondroitin sulfates, may be different for the VV forms because of differences in their lipoprotein envelope. Because of the cytoplasmic presence, insertional mutagenesis is no concern for VV. Since VV encodes its own replication machinery minimal interaction with host proteins is required allowing the virus to replicate in many different cell types and to avoid antiviral mechanisms. Half of the VV genome encodes early

genes, i.e. mostly enzymes involved in RNA synthesis and DNA replication that are transcribed before replication. VV has inverted terminal repeats that are required for DNA replication which starts 1–2 h after infection and that is followed by the expression of intermediate genes which drive expression of the late, mostly structural genes. Immature but infectious particles are generated 6h after infection which is much faster than AdV-5. Many of the approximately 200 genes contained in VV genome encode proteins that are involved in blocking apoptosis and immune responses against infected cells. Wildtype VV has a natural tropism towards tumours since following intravenous injection into tumour bearing animals, the highest amount of virus was recovered from the tumour, followed by the ovary, with little virus detected in other organs. It is thought that the leaky vasculature in most tumours and in ovarian follicles is responsible for this selectivity. This would allow more efficient entry of pox virus particles from the bloodstream. Wildtype VV has been used in human cancer trials and was shown to have antitumour efficacy against melanoma after intralesional injection with minimal side effects. Unlike adenoviral vectors, no hepatic toxicity has been observed with VV (174-177).

***Recombinant vaccinia with deletions in viral genes***

The systemic use of replicating VV in human cancer trials awaits the development of a safe, tumour-selective vaccinia virus for this purpose. Wildtype VV has been applied with variable outcome in humans as an intralesional vector with no reported toxicity (177). In preclinical studies, the Western Reserve VV strain (WR) has been used as a backbone for recombinant vectors because of its superior oncolytic properties compared to other VV strains. WR has a higher neurovirulence and replicates in brain, lung, spleen, ovary, and other organs in the mouse. When injected intradermally in rhesus monkeys WR lead to large necrotic ulcers without systemic spread. Strategies to turn this VV variant in an oncolytic agent with increased tumour-selectivity are currently being applied, focusing on the deletion of viral genes. The development of VV vectors with foreign regulatory elements or an altered tropism is unlikely since vaccinia replication is cytoplasmic and not much is known about entry of VV into cells (174).

The VV TK gene promotes viral replication in normal cells by increasing intracellular nucleotide concentration. Since tumour cells have an increased pool of intracellular nucleotides it was postulated that TK-negative VV would selectively replicate in tumour cells. Indeed, TK-deleted virus was found to be less pathogenic in mice than wildtype WR with preserved replication in tumour cells (178) and TK-deleted VV-luc, which expresses luciferase driven by a synthetic early/late promoter, replicated efficiently in a variety of tumour cell lines (179). In nude mice with subcutaneously implanted MC38 murine colon carcinoma cells, intratumoural, intraperitoneal and intravenous administration of VV-luc resulted in peak levels of luciferase activity in tumour tissue 4-6 days after delivery and this activity remained high up till the last measured time point (day 14). Levels in normal organs were at least 1000 times lower than in tumour tissue with highest levels in the ovaries (180). After administration of VV-luc in immunocompetent (intravenous, intraperitoneal or portal vein administration) and nude mice (intravenous administration) bearing hepatic metastases of MC38 cells a difference in luciferase activity of at least 100-fold was observed between tumour tissue and the ovaries, both in immunocompetent and nude mice. Luciferase activity was undetectable in normal tissues 10-12 days after injection in immunocompetent mice, but was present until at least day 38 in tumour tissue and in the liver, but not any other organ, in nude mice. The presence in liver was probably related to this specific model bearing hepatic MC38 metastases since no presence in liver was observed after VV-luc delivery in nude mice with intraperitoneally or subcutaneously implanted MC38 cells. This relative tumour selectivity of TK deleted VV has also been demonstrated in nude mice and rats bearing human melanoma or rat sarcoma tumour cells (174, 179, 181, 182). In rabbits containing liver metastases of rabbit VX-2 carcinoma cells systemic injection of a TK deleted VV-luc lead to dose dependent toxicity. In tumour tissue, reporter activity peaked at day 4 and became undetectable at day 12. The activity in

tumours was higher than in normal tissues with highest activity in the ovaries. Serum levels of VV-luc peaked at day 4 and antibodies were detected from day 6 onward. There was no difference found in viral biodistribution between tumour-bearing and control animals (183).
Besides TK also other genes have been deleted to generate a more tumour-selective VV.
The VGF (vaccinia growth factor) gene encodes a secreted protein that acts as a mitogen to prime surrounding cells for vaccinia infection. VGF is encoded twice by the VV genome, since it is present on both terminal repeats. A recombinant vaccinia containing β-galactosidase coupled to the late vaccinia P11 promoter instead of VGF sequences replicated less efficiently than wildtype virus in resting Swiss 3T3 cells and exhibited an attenuated phenotype following intracranial and intradermal inoculations into mice and rabbits, respectively (184).
VV-DD-GFP is a double mutated ($VGF^-/TK^-$) and GFP expressing recombinant VV that was generated to replicate more selectively in actively dividing cells (185). Similar amounts of virus were yielded by dividing murine NIH3T3 cells after infection with wildtype virus, single deleted $VGF^-$ and $TK^-$ viruses or VV-DD-GFP; in resting NIH3T3 cells however, the yield of VV-DD-GFP was significantly less compared to the other viruses supporting the above hypothesis. Eight days after i.p. injection in nude mice bearing MC38 tumours, VV-DD-GFP was recovered at significantly lower titers from brain, spleen and bone marrow compared to the other viruses. However, all viruses were equally infectious in tumours and in the ovary. Again, the viral biodistribution was similar in a non-tumour-bearing control group. In immuno-competent mice, a similar distribution pattern was observed but peak infectivity occurred earlier, and most organs were negative for all viruses by day 8. While i.p. injection of $10^8$ pfu of wildtype, VGF deleted or TK deleted virus in nude mice was uniformly lethal (mice dying respectively with a median of day 5, day 17 and day 29) all VV-DD-GFP injected mice survived. Ovarian necrosis, brain lesions and liver necrosis that were observed with the application of the other viruses was not observed in the case of VV-DD-GFP. In immuno-competent mice less toxicity was seen for all mutant groups compared to the wildtype virus. While $10^9$ pfu of VV-DD-GFP was lethal in nude mice (<100 days after treatment) this was not observed in the immuno-competent mice. Despite this viral toxicity a significant antitumour effect was seen in the VV-DD-GFP group compared to controls. As opposed to the wildtype WR strain VV-DD caused no long-term toxicity in primates following intradermal, intravenous or intralimb perfusion and no virus could be recovered from saliva, urine, faeces, tissues and serum opening the way for clinical trials (186). Recently it was shown that administration of cytokine-induced killer (CIK) cells preinfected with VV-DD-GFP results in increased regression of human ovarian cancer and murine breast cancer tumours in both immuno-deficient and immuno-competent mouse models showing the effectiveness of viral shielding and of combining an oncolytic virus with immunotherapy (187).
The VV vector vSP contains deletions of two genes designated Spi-1 and Spi-2 that are normally involved in blocking mitochondrial apoptosis, with the lacZ gene inserted in the Spi-2 locus. This virus was constructed to target more slowly growing solid tumours which may not be permissive for VV-DD-GFP that is selective for proliferating cells. While vSP and control virus replicated equally well in transformed human keratinocytes, human lung epithelial cells and mouse fibroblasts, replication of vSP was 10-100 times reduced in their normal non-transformed counterparts. While with virus with single deletions in the Spi-1 and Spi-2 genes no attenuation had previously been observed, vSP was less virulent *in vivo* compared to wildtype virus since an increased survival was observed in nude and immunocompetent mice. Viral distribution after i.p. administration was studied in nude mice with s.c. implanted murine colon carcinoma tumours. While equal amounts of control virus, VV-DD-GFP and vSP were present in tumour tissue, less recombinant virus could be recovered from normal organs, except for the brain. Anti-tumour activity was observed for vSP in both nude and immunocompetent mice with the virus inducing both apoptotic and necrotic cell death (188). Recently, the recombinant VV vSPT containing deletions in the TK, Spi-1 and Spi-2 was generated. While deletion of these three genes resulted in a further enhancement of the safety, the oncolytic potency was decreased in tumour bearing mice (189).

### *Oncolytic vaccinia armed with therapeutic genes*

Recombinant vaccinia is mostly generated by transfecting wildtype VV containing monkey cells with a plasmid that includes a VV promoter and the insert of choice flanked by VV sequences that drive recombination. Usually recombination as such results in the concomitant disruption of the TK gene. Early as well as late vaccinia promoters can be used in these constructs with late promoters driving stronger gene expression than early promoters. An example of a VV promoter that is widely applied is the P7.5 promoter that is active during both early and late phases of infection. Using this system stable insertion of DNA fragments up to 25 kb is possible.
Several TK-negative recombinant VVs expressing suicide genes have been generated. The advantage of this approach is twofold since depending on the timing of prodrug administration, suicide genes can be used to enhance the oncolytic response or to stop viral replication. When delivered systemically in a model of MC38 liver metastases no effect of VV-CD or VV-luc was observed. However in the presence of the prodrug, VV-CD induced antitumour effects and prolonged the survival in immunocompetent and nude mice. While in immunocompetent mice no toxicity was observed, moderate viral toxicity (weight loss, lethargy, and flu) was observed at a dosage of $10^6$ pfu in nude mice >6 weeks after vector administration without affecting the survival (181, 182).
Intraperitoneal administration of $10^6$ pfu of a TK-deleted VV expressing the suicide gene purine nucleoside phosphorylase (vPNP) in combination with the prodrug 6-methylpurine deoxyriboside prolonged the survival of athymic mice with hepatic MC38 metastases (190).
VV-CD and VV-luc had similar antitumour efficacy against MC38 tumours subcutaneously implanted in nude mice after i.p. administration with or without prodrug. Increased efficacy of VV-CD was observed after i.t. administration in presence of the prodrug. Significant viral pathogenicity of VV-CD in absence of prodrug was observed, in the form of weight loss, skin lesions and death at higher dose levels ($10^7$-$10^8$ pfu i.p. or $10^8$ pfu i.t.). The systemic pathogenicity was explained by the relatively high viral titers observed in the spleen, brain, and bone marrow. Minimal virus was recovered from the liver and no virus could be recovered from blood samples. A non-significant reduction of these viral titers and a decrease in virus-mediated death was seen in the group treated with prodrug. It was concluded that the addition of an enzyme/prodrug system to a replicating virus can improve the antitumour response and decrease the viral pathogenicity (180).
JX-594, a $TK^-$ VV vector expressing human GM-CSF, has been tested in preclinical models for intravenous delivery. Intravenous JX-594 was well tolerated and had significant efficacy against intrahepatic primary tumours in immunocompetent rat and rabbit models (191). JX-594 is being tested in clinical trials and two other vaccinia product candidates, JX-929 (VV-DD expressing CD) and JX-963 (VV-DD expressing GM-CSF) were scheduled to enter phase I clinical trials in 2007. JX-963 was as effective as wildtype VV and more effective compared to the AdV vector Onyx-015 in a wide spectrum of cancer cell lines and was systemically effective in primary carcinomas and metastasized cancers in immunocompetent mice and rabbits (15, 192). In JX-795, the B18R gene (an-anti IFN gene) was removed and the antiviral and anti-tumour cytokine IFN-β was cloned in the TK region, to limit replication in normal tissues and enhance the oncolytic potential. This vector was more selective and more efficacious compared to wildtype VV and a control vector without IFN insertion in immunocompetent mice bearing subcutaneous tumours (193).

### *Vaccinia tumour vaccines*

Poxvirus recombinants have been used as vaccines against infectious organisms, and more recently tumours. Many recombinant VV vectors have been generated that express tumour-specific antigens to elicit antigen-specific immune responses. Examples are VV-CEA and VV-PSA. These vectors encode antigens that are expressed in a variety of carcinomas and prostate cancer cells, respectively. These tumour vaccines have been extensively tested in clinical trials, are generally well tolerated and induce

cytotoxic T cell responses and systemic antibodies. Recombinant VV tumour vaccines have also been applied to deliver cytokines and costimulatory molecules, either as a stand-alone agent or in combination with other treatments. These immuno-stimulatory inserts recruit antigen-presenting and effector cells thereby eliciting a systemic tumour-specific immune response. Several vectors containing cytokines have been generated and are being tested in clinical trials. TRICOM, a recombinant VV expressing three co-stimulatory molecules (B7.1, ICAM-1, and LFA-3), was found to be more efficient at inducing antigen-specific T cell responses than vectors expressing less costimulatory molecules. In animal studies the antigen-presenting and immunostimmulatory strategies have been combined; VV-CEA and TRICOM induced strong activation of T cells leading to potent therapeutic antitumour immunity (194, 195). Clinical trials combining costimulatory molecules with tumour-associated antigens are ongoing.
Recombinant VV vectors containing IL4 and IL10 have been generated to circumvent viral clearance *in vivo*. While this approach may enhance direct viral-mediated immune reposes or oncolysis, there are safety concerns about viruses that are not recognized by the immune system (177).

***Future developments with VV vectors***

Recombinant VV is a promising vector, especially for systemic delivery. Since smallpox immunization was discontinued in 1978, increasingly more patients will not have preformed immunoreactivity against this vector. Nonetheless strategies for masking VV from neutralizing antibodies, like coating with liposomes or polyethylene glycol (PEG) are under investigation. Several vectors based on the highly oncolytic WR strain are currently being tested in clinical trials. Since vaccinia has a large capacity for foreign sequences, combinations of suicide genes, cytokine genes, tumour-associated antigens and costimulatory molecules may be included in recombinant vaccinia in the future (185).

### 3.1.4 Myxomavirus (Poxviridae, Chordopoxvirinae, Leporipoxvirus)

Myxoma virus is a rabbit-specific poxvirus that causes a lethal disease termed myxomatosis in the European rabbit but that is strictly nonpathogenic to other species. Myxoma has a large double-stranded DNA that allows for the potential insertion of large (25 kb) therapeutic inserts. This, combined with the lack of preexisting antibodies in the human population, suggest that Myxoma virus could be an attractive agent for oncolytic therapy. The oncolytic properties of myxoma may be dependent on defective interferon (IFN) signalling in human tumour cells. The vector vMyxgfp contains a GFP insert between the open reading frames M135R and M136R of the Lausanne strain genome. Most human glioma cell lines and primary gliomas were susceptible, although in a variable degree, to myxoma virus infection and replication. Intracerebral administration of myxoma virus was safe in immunocompetent and nude mice. Intratumoural administration of myxoma decreased tumour size and prolonged the survival in two mouse xenograft models for glioma. GFP expression was observed in residual tumour cells and not in neighbouring tissues. Replication of myxoma was long-lived since it was detected by plaque forming unit (pfu) assay up to 42 days post-injection, peaking at day 14. Myxoma virus infection however did not cause regression of glioma tumours in contra-lateral non-inoculated tumours (21).
Glioma cells are characterized by constitutively activate Akt signalling that is involved in cell survival. Recently, myxoma host range mutants containing specific mutations in host range genes like MT-5 (that is able to activate cellular Akt signalling) were selected. The host range mutants elicited similar oncolytic potential compared with wildtype myxoma in glioma cells but are attenuated in their natural host, possibly representing safer candidates for usage in human trials (196).
Future studies are aimed at arming myxoma virus vectors with therapeutic genes, facilitating the infection and improving the strategies for delivery (21).

### 3.1.5 Autonomous Parvovirus (Parvoviridae, Parvovirinae, Parvovirus)

Autonomous parvoviruses are small non-enveloped DNA viruses with a single-stranded genome of approximately 5kb. The rat parvovirus H-1 and its close relative the minute virus of mice (MVM) bind to ubiquitous sialic acid containing glycoproteins located at the surface of target cells. No pathogenicity or induction of tumours has been observed in adult animals and humans and there is little or no preexisting neutralizing antibody in the human population, which are clear advantages compared to for instance AdV or HSV. Autonomous parvoviruses contain two large open reading frames encoding for cytotoxicity-mediating non-structural (NS) polypeptides and viral capsid proteins that are driven by the left-arm P4 and right-arm P38 promoters, respectively. Parvovirus contains two viral hairpins that are necessary for replication and are thought to be involved in the selective killing of p53 mutant cells by triggering cellular pathways involved in apoptosis (22, 23). Normal cells were found to take up parvovirus particles as efficiently as their transformed derivatives. Stimulation of the P4 promoter in response to the cell cycle and/or activation of the P4 promoter by certain oncogenes (Ras, SV40 large T) are thought to be critical determinants in the selectivity of parvoviruses for tumour cells. Parvovirus replication in permissive cells can, depending on the cell type, result in apoptotic or necrotic cell death (22). Besides being directly cytotoxic to tumour cells, H-1 also has immunostimulatory effects; H-1 promotes the cross-presentation of tumour antigens by dendritic cells, resulting in activation of cytotoxic T-cells and production of pro-inflammatory cytokines (197). H-1 preferentially killed *in vitro*-transformed cell lines, tumour-derived human and rodent cell lines, and primary samples from human breast carcinomas, liver carcinomas and gliomas. Limited or no cytotoxicity was observed in non-transformed cells (22, 24). Repeated intracerebral injections of H-1 did not cause damage to cerebral and cerebellar tissues of immunocompetent adult rats and these animals remained fully asymptomatic (198). Intratumoural administration of H-1 in an intracranial glioma model in immunocompetent rats resulted in rapid tumour regression without any toxic or inflammatory side effects in the surrounding brain tissue (24). Clinical studies are currently being performed with wildtype H-1 parvovirus. Another parvovirus that is being explored for oncolytic purposes is canine parvovirus that is able to target human tumours overexpressing the transferrin receptor (199).
Although replication deficient parvoviral vectors encoding immuno-stimulatory molecules have been engineered, the packaging capacity of parvovirus is very limited preventing large inserts from being efficiently packaged into replicative recombinant particles. Mutant parvoviruses expressing more toxic forms of the cytotoxic parvovirus protein NS1 have been generated by mutagenesis (22, 200). Recently, replicating recombinant parvovirus has been generated that contains binding sites for Tcf family transcription factors inserted in the P4 promoter. This recombinant parvovirus was found to be selective for colon carcinomas with an activated Wnt signaling pathway. Compared to the parental virus, Tcf mutant viruses replicated at least 1000 times less efficiently in cells with an inactive Wnt signaling pathway. Replication and oncolytic effects were near the levels of wildtype parvovirus in some of the studied colon cancer cell lines. However in most tumour cell lines the recombinant parvovirus replicated poorly (201, 202).
Another strategy was to introduce immunostimulatory CpG motifs in the 3'-UTR of the parvoviral VP transcription unit. These ‘armed’ parvoviruses showed increased immunogenic effects in an *in vivo* rat immunization model compared to wildtype H-1 virus (203).

## 3.2 Vectors based on RNA Viruses

RNA viruses are classified into different groups depending on the nature of their genome and mode of replication. The genome of positive-sense RNA viruses can be directly translated into viral proteins by the ribosomes of the host cell. Novel (+) RNA genomes are synthesized via (-) strand intermediates. Negative-sense RNA viruses (and double stranded RNA viruses) first have their genome copied by RNA-dependent RNA polymerase which is contained within the viral particle. The resulting (+) RNA is translated into viral proteins and used as a template to produce novel (-) RNA genomes. Reoviruses have a double stranded RNA genome consisting of 10 segments. Initial transcription primarily results in synthesis of single stranded (+) RNAs that are used for translation and as a templates for (-) RNA transcription eventually resulting in the formation of novel dsRNA copies that are packaged into new virions. Retroviruses are single-stranded (+) RNA viruses that use DNA intermediates to replicate. Reverse transcriptase, a viral enzyme that is contained within the viral particle converts the viral RNA into a complementary (-) strand of DNA, which is subsequently used as a template resulting in a double stranded molecule of viral DNA, that integrates in the host cell DNA and directs the formation of new particles.
A main advantage of the therapeutic development of RNA viruses compared to DNA viruses is their rapid growth, which aids in their production (204). A fundamental disadvantage of RNA viruses in general is that they have very high mutation rates since they lack proofreading mechanisms to conduct repair of damaged genetic material during replication. Besides mutation, recombination (by template switching or copy choice mechanisms) and reassortment are mechanisms that are important in the evolution of specific RNA viruses and that should when appropriate be considered in an environmental risk assessment involving the use of a genetically modified viral variant. RNA viruses that are being developed for therapeutic purposes and that are known to recombine by template switching include picornaviruses, coronaviruses, sindbis virus and retroviruses (205-208). Although the rate of recombination in negative sense RNA viruses is low, presumably because of the properties of the ribonucleoprotein complex in these viruses, recent findings suggest that recombination may also play a role in the evolution of the negative stranded virus NDV (209). Reassortment especially plays an important role in the evolution of influenza virus (210, 211). A major concern with retroviruses is that they have been associated with insertional mutagenesis and oncogene activation (212).
Several RNA viruses including Newcastle disease virus (NDV), reovirus, vesicular stomatitis virus (VSV), mumps virus (MU), Coxsackievirus A21 (CVA21), echovirus 1 (EV1) and poliovirus are being applied (pre-)clinically for the treatment of cancer.

### 3.2.1 Vesicular stomatitus virus (Rhabdoviridae, Vesiculovirus)

Vesicular stomatitis virus (VSV), a member of the Rhabdovirus family, is an enveloped negative-sense RNA virus with an ~11 kb genome containing five subgenomic mRNAs that encode for five proteins; nucleoprotein, phosphoprotein, matrixprotein, glycoprotein and large polymerase protein. The natural hosts for VSV infection are cattle, horses, and swine but the virus has a broad tropism and is thought to spread between mammals via insect vectors. Except for rural areas most people do not have pre-existing antibodies to VSV. Infections in humans are mostly asymptomatic but can result in mild flu-like symptoms (204).
Since VSV efficiently replicates in rodent cells, model systems to test the efficacy of VSV are widely available. VSV is readily grown to high titers, has a short replicative cycle (1–2 hours in tumour cells) such that tumour destruction may have occurred before the initiation of potentially neutralizing antiviral immune responses in the host. VSV is very sensitive to the anti-viral action of interferon that is activated in normal cells during the formation of double stranded RNA during the VSV life cycle.

The tumour selectivity of VSV resides in defects in the interferon/PKR pathway that occur in most tumours and that abrogate the induction of cellular antiviral responses including inhibition of viral translation (213). VSV kills cells primarily by inducing apoptosis and is able to kill a large variety of tumour cell types (214-217). Notably, the wildtype Indiana strain of VSV replicated efficiently in a range of tumour cell lines even in the presence of doses of interferon that completely protected normal human primary cells (215). Human leukemia cells were estimated to be 1000 times more sensitive to VSV than peripheral progenitor cells in co-culture experiments (204, 218). Tumour types characterized by persistent virus infection, such as Burkitts lymphoma, T-cell leukemia and hepatocellular carcinoma are especially attractive candidates for VSV mediated oncolysis (217, 219). VSV was shown to delay tumour growth in athymic mice when administered intratumourally ($2x10^7$ pfu) and intravenously (3 times $2.5x10^7$ pfu every two days) and in immunocompetent mice when administered intratumourally (3 times $2.5x10^7$ pfu every three days). While no adverse effects were observed in immunocompetent mice, hindlimb paralysis (which is characteristic of VSV disease in immunodeficient mice) was observed after intravenous delivery in nude mice. Virus was detected (besides in the transplanted tumour tissue) in the brain of nude mice after intravenous but not after intratumoural administration (214). Administration of VSV in carrier cells may improve the therapeutic efficacy when compared with viral particle injection by circumventing the antiviral immunity (220). Intratumoural administration of VSV producing carrier cells inhibited growth of melanoma xenografts in nude mice that eventually succumbed because of VSV infection. The cytopathic effects observed in normal cells could be counteracted by interferon *in vitro* and in nude mice *in vivo* (215).

***VSV with mutations in viral genes***

AV3-GFP is a genetically modified VSV strain that contains the M51R mutation in the matrix (M) protein. The M protein blocks the function of the Rae1 protein that mediates nucleo-cytoplasmic transport of cellular mRNAs and that is involved in the interferon (IFN) antiviral response (221). AV3-GFP, and the naturally attenuated VSV strains AV1 and AV2, have a reduced *in vivo* toxicity compared to wildtype VSV because of an increased *in vivo* IFN response. Intraperitoneal administration of these strains effectively eradicated human carcinoma xenografts in nude mice without inducing symptoms of viral infection. Intravenous AV1 and AV2 produced durable cures in an aggressive, disseminated syngeneic model in immuno-competent mice (216). Repeated intravenous injections of VSV(M51R)-LacZ in a syngeneic mouse model for metastatic breast cancer prolonged the survival of tumour-bearing mice without apparent toxicity (222). *In vitro*, VSV(M51R)-GFP was toxic for human glioma cell lines but not to normal cells. Although intracranial administration of this virus was lethal at low dose by inducing neurotoxicity in nude mice, intravenous administration was well tolerated and resulted in prolonged survival in xenograft models of multifocal and invasive human gliomas. Virus replicated in the tumours and was present at day 7 after treatment but undetectable at day 15. No virus or toxic effects were detected in normal brain, lung, kidney, liver, or heart (223).

***VSV armed with marker or therapeutic genes***

Foreign sequences of up to 4.0 kb can be inserted in the VSV genome. The rVSV-GFP vector, a recombinant VSV containing GFP, showed severely attenuated replication in primary hepatocytes compared to hepatocellular carcinoma (HCC) cell lines correlating with absent GFP expression and absence of cythopathic effects. Intratumoural administration of rVSV-GFP or hepatic artery infusion of rVSV-lacZ (for multifocal disease) prolonged the survival of immunocompetent rats or mice after implantation of HCC cells or colon carcinoma cells in the liver. Viral replication was demonstrated in tumour tissue as opposed to normal surrounding liver tissue, and background levels of GFP expression and necrosis were observed in normal liver tissue. In these experiments virus became undetectable 3

days after administration in normal liver tissue and 7 days after administration in tumour tissue (224-227).
To improve the efficacy in the HCC model a novel fusogenic VSV vector rVSV-NDV/F(L289A) was generated. This vector contains the syncytia inducing mutant F protein (L289A) from NDV in the noncoding region of the VSV/G gene. Syncytia formation promotes viral spread and necrotic cell death by evoking cythopathic effects and triggering the immune response. This vector showed evenly efficient replication but superior oncolytic activity against HCC cells *in vitro* compared to wildtype VSV. Enhanced survival compared to wildtype VSV was demonstrated in a rat model for multifocal HCC after hepatic artery infusion(s), with no apparent toxic defects in normal liver tissue. Plaque assays confirmed that the virus was undetectable in the circulation and normal liver tissue at day 1 and day 2 following infusion. Neutralizing antibodies appeared 4 days after infusion and their presence correlated with viral clearance from the tumour (226, 228, 229).
Intra-arterial administration of rVSV-GFP above the maximum tolerated dose in immunocompetent rats was shown to induce neurotoxic effects that were linked to viral spread and replication, and hepatotoxicity defects due to an increase in proinflammatory cytokines. Prophylactic interferon administration decreased these effects (230).
VSV vectors carrying the herpesvirus thymidine kinase (TK) suicide cassette, the CD/UPRT (C:U) suicide fusion gene or the immunostimulatory cytokine interleukin 4 (IL4) gene have been generated by inserting these sequences between the VSV-G and VSV-L genes. These recombinant viruses containing IL4, TK, or GFP were attenuated compared to wildtype VSV (Indiana strain). *In vitro*, in tumour cell lines, these rVSV vectors replicated and induced apoptotic cell death as efficient as wildtype virus. Around 20% of normal human epithelial cells went into apoptosis by *in vitro* administration of wildtype or recombinant VSV vectors which could be prevented by adding IFN (231, 232). Intratumoural or intravenous injection of rVSV-IL4 or rVSV-TK (in the presence of prodrugs) increased the survival of immunocompetent mice with metastatic syngeneic breast and melanoma tumours compared to wildtype VSV or rVSV-GFP. Using plaques assays it was demonstrated that there was no residual virus in other tissues than the tumour (232). Intratumour administration of rVSV-C:U plus prodrug resulted in increased survival compared to rVSV-TK plus prodrug in syngeneic lymphoma and breast cancer models which was attributed to bystander activity. The cytoplasmic replication of the rVSV-C:U virus was not influenced by the administration of the prodrug *in vitro* (231).
The rVSV-IFN-β vector was generated to improve the safety of rVSV vectors. It was postulated that this vector would retain oncolytic activity in interferon-defective tumour cells and enhance the antitumour immune response, while activating the IFN pathway in surrounding normal cells leading to enforced protection against superfluous VSV. In normal mouse embryonic fibroblasts (MEFs) from mouse strains with different sensitivities to VSV, rVSV-IFN-β exhibited minimal replication and induced significantly decreased cell death compared to rVSV-GFP, while retaining oncolytic activity. rVSV-IFN-β and to a lesser extent rVSV-GFP were shown to be attenuated compared to wildtype VSV in the BALB/C mouse strain since intravenous or intranasal administration of these viruses resulted in a reduced virus-induced mortality. Intratumoural and intravenous rVSV-IFN-β were equally or more efficient compared to rVSV-GFP in increasing the survival in a syngeneic renal carcinoma model and a metastatic breast carcinoma model, respectively (233). Other ‘smart’ rVSV-viruses like rVSV-Rae1, that counteracts the action of the VSV M protein against mRNA transport or rVSV-PKR and rVSV-eIF2 that are able to control VSV translation are currently being developed (221).

***Transductionally targeted VSV***

The receptor for VSV-G is ubiquitously expressed and VSV infects most cell types. Since this is regarded as an obstacle for clinical use attempts to generate oncolytic VSV with a restricted host cell range have been made. VSV vectors have been designed that preferentially infect breast cancer cells expressing the Her2/neu receptor. In the first generation vector, the VSV-G gene was deleted and replaced with a hybrid gene containing modified surface glycoproteins from sindbis virus (with severely reduced native binding) fused to the Fc antibody-binding domain of *Staphylococcus aureus* protein and the GFP gene. Addition of Her2/Neu antibodies targeted this virus to Her2/neu overexpressing cells (234). In a second generation vector, rr-VSV, VSV-G was replaced by chimeric sindbis virus glycoproteins fused to single chain Her2/neu antibodies. Moreover, the genes for murine GM-CSF and EGFP were inserted in this vector. The rrVSV vector infected, replicated and killed cells expressing Her2/neu but retained a small degree of infectivity in cells not expressing Her2/neu indicating that not all of the native binding function had been eliminated. Both vector generations suffered from the inability to be grown in high titers. However a variant of the second generation vector showing increased stability and replication has been selected by serial passaging (235).
Replicating VSV vectors have also been generated for non-oncolytic purposes, i.e. for generating a live vaccine against HIV. A recombinant vesicular stomatitis virus VSVΔG-CC4 lacking VSV-G but expressing the HIV receptors CD4 and CXCR4 specifically infected, propagated in and killed T-cells previously infected with HIV-1 and was able to control HIV-1 infection in these cells over a prolonged culture period (236, 237).

***Future development with VSV vectors***

VSV is a promising vector that is systemically effective and that may be applied against a broad range of tumours. The efficacy in man however still has to be demonstrated, but clinical studies can be expected in the near future. Current preclinical strategies are mostly aimed at increasing the safety and efficacy of these vectors. Cell-based carrier platforms (i.e. the virus is administered within carrier cells) are being developed to increase the efficacy by circumventing pre-existing immunity or immunity evolving during repeated systemic administration of VSV (220).

### 3.2.2 Measles virus (Paramyxoviridae, Paramyxovirinae, Morbillivirus)

Measles virus (MV) is an enveloped negative sensed RNA virus with a 15 kb genome and is the causative agent of the (usually self-limiting) disease measles which is frequently acquired during childhood. The measles genome encodes six proteins, a nucleocapsid protein (N), a polymerase (L) and its cofactor (P), matrix (M) protein and two oligomeric glycoproteins, hemagglutinin (H) and fusion peptide, (F). The only natural hosts for measles virus are primates. Although many cell types are permissive for measles infection, replication occurs mainly in cells of the immune system. Cellular attachment and entry in these cell types is mediated by the envelope glycoproteins haemagglutinin (H) and fusion protein (F), respectively, that use CD150/SLAM as a receptor. The viral envelope glycoproteins of paramyxoviruses (and of type-C retroviruses and lentiviruses) have fusogenic properties, i.e. they induce extensive cell-cell fusion, also recruiting many uninfected cells into multinucleated syncytia, which ultimately go into apoptosis. The live attenuated Edmonston (Edm) strain has been widely used for vaccination and has a good safety record. The Edmonston vaccine strain mainly uses the ubiquitously expressed CD46 receptor to enter cells, as opposed to wildtype MV that enters more efficiently through the SLAM receptor. CD46 is expressed at higher levels in many tumours (leukemia, gastrointestinal, hepatocellular, colorectal, thyroidal, endometrial, cervical, ovarian, breast, renal and lung carcinomas and multiple myeloma samples) compared to non-transformed cells. The tumour-specificity and fusogenic efficacy of MV-Edm was recently shown to correlate with the

CD46 receptor density of tumour cells (104, 238). MV is inhibited by interferon by various mechanisms, including cleavage of viral mRNA, inhibition of virus translation and inhibition of cell growth. Next to the degree of surface expression of CD46, the tumour-specificity of MV may also partly rely on deficiencies in the interferon pathway present in many tumour cells (104, 238, 239). The biggest constraint of the use of measles virus for gene therapy is the fact that neutralizing antibodies exist to a large degree in the population although there is evidence that measles virus can infect cells despite their presence (240).

### *MV with marker and therapeutic inserts*

Foreign proteins can be inserted as an extra transcriptional unit upstream of the N protein of the MV genome with no known size constraint. Several of these vectors have been successfully applied in animal models.

The vector MV-lacZ induced regression of large established human B-cell lymphoma xenografts in SCID mice after intratumoural (also in presence of neutralizing antibodies) and intravenous administration. Replication competent virus was recovered from injected tumours for at least 20 days following injection, indicating that the tumour xenografts can sustain viral replication *in vivo* (241).

MV-GFP efficiently killed cultured myeloma cell lines or primary human myeloma cells while syncytium formation remained minimal in control peripheral blood lymphocyte and patient non-plasma cell cultures. No cytopathic effect was seen in prolonged cultures (up to 10 days after infection) of peripheral blood lymphocytes or in colony cultures of normal human haematopoietic progenitor cells (242). Intratumour or intravenous administration of MV-GFP prolonged the survival of nude mice with implanted myeloma xenografts (243) and T-cell lymphoma xenografts (exclusively after intratumoural administration) (244).

MV-CEA contains the gene encoding the extracellular domain of human carcinoembryonic antigen (CEA) upstream of the N protein. CEA is an inert soluble marker peptide that can be used to monitor virus propagation. MV-CEA killed ovarian cancer cell lines (that have high levels of CD46) *in vitro*, with minimal cytopathic effects in non-transformed cells that line the peritoneal cavity. At an MOI of 10 about 90% of non-transformed cells remained viable as opposed to 20% of tumour cells. MV-CEA increased the survival of nude mice with subcutaneous and orthotopic xenograft transplants of ovarian cancer cells after intratumoural and IP injection respectively. Viral replication was demonstrated by increase in CEA levels, by mRNA in situ hybridization on residual tumours and isolation of live virus from these tumours using plaque assays. In residual tumours, there appeared to be a dynamic equilibrium between formation of new tumour cells and death of infected tumour cells (245). MV-CEA efficiently killed glioma cell lines *in vitro* and prolonged the survival of subcutaneous and intracranial nude mice xenograft models after intravenous and intracranial administration respectively. The rise and fall of serum CEA levels paralleled growth and regression of subcutaneously implanted tumours with levels peaking at day 10 and declining to baseline levels at day 24 post-administration. In line with earlier studies in primates with MV-Edm, no toxic effects or an increase in serum CEA levels were observed after intracranial administration of MV-CEA in CD46 transgenic mice (246). MV-CEA also had potent antitumour activity against breast cancer cell lines and breast cancer xenografts after intravenous administration (247).

Biodistribution experiments with MV-GFP after intraperitoneal administration in measles-susceptible CD46 transgenic mice showed that the virus was mainly present in macrophages in the peritoneal cavity, and outside the peritoneal cavity in lymph nodes and spleen. Other organs were negative. By day 5 after virus administration, fluorescent GFP-positive macrophages were no longer detectable in these mice, presumably because the viral infection was cleared by the immune system (248).

An engineered MV with increased oncolytic potency was designed since xenografted myeloma tumours persisted *in vivo* despite repeated MV-Edm injections. MV-NIS contains the thyroidal iodide

symporter downstream of the H gene. NIS is able to trap radioiodine and enhances the therapeutic potential of the virus by bystander killing. Moreover, expression of NIS allows non-invasive *in vivo* monitoring of the distribution and expression of the virus *in vivo*. Intravenous MV-NIS plus radiotherapy showed an enhanced effect on myeloma xenografts in SCID mice compared to virus alone, also in cells completely resistant to MV-Edm. Changes in iodide uptake indicated a peak in NIS expression 9 days after injection (249). MV-NIS had minimal cytopathic effects in normal fibroblast and peripheral blood lymphocytes cells compared to ovarian cancer cell lines. The vector MV-NIS-CEA that contains both the NIS and CEA inserts had poor replication kinetics and the maximal titer was 10-fold less than either of the parental viruses. Dual intraperitoneal therapy with MV-CEA and MV-NIS in mice with intraperitoneally implanted ovarian cancer xenografts was superior to treatment with either virus alone (250).
Besides the insertions described above measles virus has also been armed with IL12 (251), GM-CSF (252) and hepatitis-B surface antigen (253).

***Tropism modified MV***

Application of MV-Edm for cancer therapy will require systemic administration of the virus because of the disseminated nature of many cancers. In order to prevent collateral damage to normal tissues and to prevent binding of MV to its natural receptors on B-cells, T-cells, dendritic cells and macrophages that may cause immunosuppression, strategies to change the tropism of measles have been conducted.
The tropism of measles virus can be modified by adding C-terminal extensions to the H-protein. A recombinant virus expressing a hybrid protein consisting of the epidermal growth factor (EGF) linked to the C-terminus of the MV-Edm H protein showed similar replication as the wildtype virus and efficiently entered CD46-negative rodent cells expressing the human EGF receptor, causing syncytium formation and cell death (254).
Also single-chain antibodies (scAb) specific for the tumour-associated antigen CEA have been used to redirect the tropism of MV-Edm to cells that overexpress CEA, while maintaining CD46 tropism in CD46 positive cells (255). MVHαCD20 that displays anti-CD20 (a lymphoid marker) showed similar growth kinetics as the parental strain in CD46+ cells and was stable over 11 passages *in vitro*. MVHαCD20 was able to bind to CD20 overexpressing hamster cells and induced syncytia formation while only background syncytium formation was seen with the parental strain. Although the viruses showed similar replication kinetics *in vitro* on human $CD20^+$ and $CD20^-$ cells, growth of $CD20^+$ human fibrosarcoma tumours in nude mice was retarded more by intraperitoneal administration of MVHαCD20 than by control virus. The viruses had equivalent effects on the $CD20^-$ tumours (256).
Very similar results were obtained with MV-αCD38 (encoding a myeloma antigen). This virus infected and killed CD38 overexpressing hamster cells *in vitro* and *in vivo* as opposed to MV-Edm. However, human myeloma cell lines and a CD38 overexpressing fibrosarcoma cell line were equally susceptible *in vitro* to MV-αCD38 and Mv-Edm with minimal cell death in peripheral blood lymphocytes (248). A different strategy was used with MVT7 that contains the high affinity T-cell receptor that was able to fuse murine cells expressing an MHC-peptide complex (257). In these studies, an engineered factor Xa protease recognition site in the hybrid H proteins made it possible to cleave off the displayed domain and abrogate virus entry.
Fully retargeted GFP expressing recombinant measles viruses were generated by displaying C-terminal antibodies on mutant H-proteins in which CD46 and SLAM binding is almost completely ablated. These viruses also contain a His-tag fused to the single chain antibodies to accommodate the propagation of the virus in Vero-α-his cells. Ablated viruses containing α-CD38 or α-EGFR scAb retained some residual infectivity on normal monkey vero cells without inducing cytopathic effects. The viruses efficiently infected hamster cells or human tumour cells and demonstrated specific anti-tumour activity against $EGFR^+$ ovarian cancer xenografts and $CD38^+$ lymphoma cell line xenografts

after intratumoural and intravenous administration, respectively. Compared to viruses containing only the mutated H-protein these viruses showed reduced infectivity and replication in 'non-permissive' human cells *in vitro* and *in vivo* suggesting that the scAb insertion is responsible for the further reduction of residual CD46 tropism. Viruses rescued from residual tumours did not display a reversal to CD46 tropism (258, 259). Oncolytic measles virus strains retargeted to a mutated EGFRvIII receptor had comparable therapeutic efficacy compared to MV-GFP against EGFRvIII-expressing glioma lines and intracranial xenografts. Absence of nucleocapsid protein expression and complete protection of normal fibroblasts and astrocytes was observed after infection with the retargeted strain as opposed to MV-GFP at high viral doses improving the therapeutic index (260).
MV depends on the ubiquitous intracellular protease furin to process and activate its envelope fusion (F) protein. Recombinant MV-GFP with an altered F-protein that is activated by tumour-secreted matrix metalloproteinases (MMP) specifically fused MMP-expressing cells as opposed to Vero cells and had similar oncolytic activity as MV-GFP after intratumoural injection in a mouse s.c. xenograft model with $MMP^+$ fibrosarcoma cells. This virus was apathogenic when given intracerebrally to CD46 expressing / IFN knockout mice (261). This alteration may be combined with other modifications adding an extra level of selectivity.

***Future developments with MV vectors***

MV-CEA virus is currently being tested in a phase I trial for i.p. delivery into patients with recurrent ovarian cancer. MV-NIS virus will be tested for intravenous administration into patients with relapsed multiple myeloma (239). These current clinical strains may be 'over-attenuated' because of mutations in the V-protein that is involved in antagonizing the interferon response. Currently, novel MV and canine distemper virus strains are being engineered that maintain moderate replication in normal host cells (262). Canine distemper virus is the canine Morbillivirus equivalent of MV that may be applied for parallel pre-clinical studies in permissive ferrets. Morbillivirus therapy has also been proposed for treatment of canine lymphoma, since an attenuated CDV expressing GFP was able to infect and kill neoplastic dog lymphocytes. Thus, oncolytic viruses may also be used in veterinary practice in the future (263).

### 3.2.3 Mumps virus (Paramyxoviridae, Paramyxovirinae, Rubulavirus)

In clinical studies performed in Japan in the 1970s, tissue culture attenuated mumps virus was reported to cause responses in breast and cervical cancer patients. However, these studies lacked appropriate controls and subsequent studies were disappointing (1, 2). Like measles virus, mumps virus expresses hemagglutinin and fusion proteins that are able to induce extensive fusion of infected cells. Jeryl-Lynn mumps live attenuated vaccine was compared with Moraten measles and MV-CEA in a murine model of intraperitoneal human ovarian cancer. *In vitro*, Jeryl-Lynn mumps virus was slower than MV-CEA but faster compared to Moraten measles virus in causing cell fusion, but comparable levels of cell death were observed 6 days post infection. *In vivo* all three viruses displayed equivalent antitumour potency (264). The application of recombinant mumps virus in (pre-)clinical studies has not been reported thus far.

### 3.2.4 Paramyxovirus SV5 (Paramyxoviridae, Paramyxovirinae, Rubulavirus)

The paramyxovirus SV5 has been isolated from human, canine, porcine and non-human primate populations and has been implicated in upper respiratory tract disease in dogs and multiple sclerosis and chronic fatigue in humans (265). The vector rSV5-P/V-F is based on the CPI strain of SV5 that contains mutations in the P/V gene that is involved in counteracting the interferon response (266) and

furthermore the vector contains a glycine to alanine mutation in the fusion protein to enhance cell-cell fusion. The CPI strain and rSV5-P/V-F have both been tested for their ability to kill prostate tumour cells *in vitro* and in *vivo* in nude mice (267).

### 3.2.5 Newcastle disease virus (Paramyxoviridae, Paramyxovirinae, Rubulavirus)

Newcastle disease virus is an avian paramyxovirus that has chicken as its natural host. Like other RNA viruses, NDV replicates rapidly with progeny virions being first detectable *in vitro* within 3 hours after infection. Sialic acid, the natural receptor for NDV is found on a diverse number of cells including human cancer cells of neurectodermal, mesenchymal and epithelial origin. NDV is non-pathogenic to humans; exposure to NDV was reported to result only in occasional mild conjunctivitis (268, 269). NDV lentogenic (non-pathogenic to poultry) and mesogenic (pathogenic to poultry) vaccine strains appear to be genetically stable and human-to-human transmission has not been observed (270). Also in athymic mice and other animal models NDV does not induce disease even at high doses.
Like VSV and other negative strand RNA viruses, NDV is thought to be selectively cytolytic for tumour cells as a result of defects in the interferon pathway common among diverse tumour types. Although the NDV strains can have immunostimmulatory effects, the primary cytolytic effect is most probably the induction of apoptosis (271).
Initial results using the mesogenic 73-T strain have shown that NDV is directly oncolytic to murine tumour cells. Very large doses of 73-T were nonpathogenic in a variety of animal models, even when directly inoculated into the CNS (272). The 73-T strain was able to replicate in several human tumour cells to the same extent as in chicken cells. In normal fibroblasts also some replication occurred without inducing cytopathic effects. Intratumour injection in subcutaneous xenografts of human fibrosarcomas, cervical, lung, prostate, breast and colon carcinomas and neuroblastomas resulted in most cases in almost complete tumour regression in athymic mice without any symptoms of viral infection. *In vivo* replication occurred after injection in tumours but not after injection into muscle. Intraperitoneal injection also resulted in neuroblastoma tumour regression in nude mice with better results after multiple injections compared to single treatment (268, 269, 273, 274).
PV701 is a naturally attenuated mesogenic strain of Newcastle disease virus that has been tested in pre-clinical and clinical trials. PV701 showed oncolytic activity *in vitro* in tumour cells while being 1000 times less toxic toward normal human cells. There is a 1000 fold difference in the intravenous dose resulting in 50% tumour regression in athymic mice and the median lethal dose in athymic mice (270).
NDV strains that have been or that are currently tested in clinical trials are non-recombinant strains. However, several groups have recently developed recombinant NDV strains for oncolytic or (cancer) immunotherapy purposes. Transgenes that have been inserted include marker genes (275, 276), pro-drug converting enzymes (277), IL2 (278), GM-CSF (279), therapeutic antibodies (277) and a modified fusogenic glycoprotein (278).

### 3.2.6 Influenza A virus (Orthomyxoviridae, Influenzavirus A)

Influenza A virus is a segmented negative sense RNA virus encoding 11 proteins: HA (hemagglutinin), NA (neuraminidase), NP (nucleoprotein), M1, M2, NS1, NS2(NEP), PA, PB1, PB1-F2 and PB2. The tropism of influenza A viruses is mainly determined by the HA protein that binds to sialic acid sugars on the cell surface and the NA protein that is involved in viral release. Of both proteins several different subtypes exist and the combination of these proteins is used to classify different influenza A serotypes. Influenza A is an avian pathogen, but is also a common pathogen in humans and other mammals, and may cause severe disease in these hosts; the evolution of high-pathogenic (like the H5N1 subtype) from low-pathogenic strains is believed to involve both mutation and the reassortment of genome segments between influenza viruses (210, 211).

The cellular protein kinase PKR is activated by interferon and plays a major role in the antiviral response. Many tumours have activating mutations in the Ras oncogene and these are thought to counteract PKR. Influenza A virus is able to repress PKR activation and the resulting inhibition of viral replication via the virulence factor and non-structural protein NS1. Recombinant oncolytic influenza strains have been generated by genetically modifying NS1 function in the influenza A/Puerto Rico/8/34 (PR8) strain (H1N1). A recombinant influenza PR8 virus delNS1, in which NS1 is deleted, replicated efficiently in host systems defective in IFN production or signaling, and in otherwise non-permissive cells after transfection with activated Ras. The recombinant virus delNS1 replicated selectively in Ras transfected melanoma cells compared to non-transfected cells and had *in vivo* antitumour activity against subcutaneously implanted xenografts in nude mice after intratumoural administration. Normal cells did not support the growth of delNS1 *in vitro*. Despite a lack of viral replication in non-Ras transfected melanoma cells cytopathic effects were observed *in vitro*. Immunostaining two days after infection showed some evidence for viral replication in Ras-transformed cells with faint staining visible in adjacent muscle tissue. Subcutaneous application of influenza delNS1 was completely apathogenic for SCID mice (280, 281).
NS1-99, a recombinant influenza A virus in which only the N-terminal 99 amino acids of NS1 are deleted, is less attenuated compared to delNS1. Both mutants killed and efficiently replicated in tumour cell lines resistant to interferon. Interferon protected against the cytopathic effects of the attenuated viruses as opposed to wildtype virus. In mice bearing a subcutaneous IFN-resistant melanoma tumour the beneficial effect of the NS1-99 virus was improved compared to the delNS1 virus. Recombinant influenza viruses like delNS1 may (like for instance reovirus, VSV and NDV) be applied in the future against tumours with a defective antiviral interferon/PKR response (282).

### 3.2.7 Enteroviruses (Picornaviridae, Enterovirus)

Enteroviruses are mild class 2 pathogens that mainly cause asymptomatic or mild upper respiratory tract infections. Enteroviruses have a single stranded positive sense RNA genome. A number of enteroviruses have been shown to have naturally occurring selectivity for cancer cell lines. CoxsackievirusA21 (CVA21) is a human common cold virus that does not induce dramatic disease after deliberate administration in humans. There is a low prevalence of neutralizing antibody against CVA21 in the human population. CVA21 has been shown to selectively lyse and destroy melanoma, multiple myeloma, breast cancer and prostate cancer cells both *in vitro* and *in vivo*. Slowly growing melanoma tumours were more permissive to CVA21 lysis than rapidly growing tumours. Melanoma cells and some other cancer types show relatively high surface expression of the intercellular adhesion molecule-1 (ICAM-1) and decay accelerating factor (DAF) that mediate CVA21 binding and viral entry. Bioselected variants of CVA21 (CVA21-DAFv) may be interesting for targeting of tumours that overexpress DAF (283-288). Phase I clinical trials with CVA21 in melanoma patients are underway. It is important to note that Kelly et al. have reported severe myositis, paralysis and death in exclusively tumour-bearing mice following CVA21 therapy, suggesting that this was the result of viremia following intratumoural replication. Micro-RNA mediated targeting is being explored as a mechanism to control myositis associated with CVA21 therapy, as CVA21 is not well suited for insertion of large transgenes. Certain cellular micro-RNAs are able to mediate tissue-specific inhibition of viral replication presumably by repressing translation of viral RNAs. The introduction of miRNA target sites for muscle-specific miRNAs into CVA21 specifically abolished CVA21 replication in muscle cells (289).
Echovirus 1 (EV1) has a natural tropism for human ovarian cancer cell lines that show high expression of the integrin α2ß1. EV1 is therefore being investigated for the treatment of ovarian cancer. EV1 has been administered to human α2ß1 expressing transgenic mice or nontransgenic mice without clinical symptoms and in the absence of neoplastic cells EV1 is rapidly and effectively cleared from the

circulation. Preliminary serologic surveys show that protective levels of serum antibody to EV1 are about 6.0%. The virus was shown to have oncolytic activity against *in vitro* cultured ovarian cancer cells and in mouse xenograft models *in vivo*. Cultured immortalized normal ovarian cells and mononuclear blood cells were relatively resistant to infection although some replication and cytolysis occurred. Tumours may also be targeted by sequential treatment with two different enterovirus serotypes, e.g. EV1 and EV8 that are both tropic for integrin α2ß1 overexpressing cells or by the combination of EV1 and CVA21, since ovarian cancer cells and melanoma cells both have increased expression of ICAM and α2ß1 (290).
The oncolytic capacity of Echovirus 5 is being tested in colon cancer cell-lines (291).
Bovine enterovirus is an animal virus which is non pathogenic for the natural host and humans and that was shown to be able to replicate and lyse human cancer cells in culture (292).

### 3.2.8 Poliovirus (Picornaviridae, Enterovirus)

Poliovirus is a nonenveloped positive-stranded RNA virus belonging to the Picornaviridae and is the causative agent of paralytic poliomyelitis. Poliovirus infections mostly remain asymptomatic, with 1-2% of cases resulting in neurologic complications. Most people have been vaccinated against poliovirus but available evidence suggests that neutralizing antibodies do not exist in the CNS of vaccinated individuals (293).The motor neuron tropism of polioviruses is determined by the expression of the CD155 receptor and structural features of the poliovirus IRES (294).
In the Sabin vaccine strains and PV1-RIPO, an intergeneric recombinant poliovirus type 1 bearing the internal ribosome entry site elements from a common cold human rhinovirus, neurovirulence is attenuated. PV1-RIPO was shown to grow poorly in tissue culture cell lines of neuronal origin and was avirulent in a poliovirus permissive mouse model (CD155 overexpressing mice), and non-neuropathogenic to non-human primates (295). PV1-RIPO eliminated malignant glioma cells *in vitro* and in a mouse xenograft model after intratumoural but not systemic administration. Intratumoural replication was demonstrated and it was shown that the virus isolated from the xenograft tumours was stable and grew poorly in non-permissive cells neuroblastoma cells. PVS-RIPO, a derivative of PV1-RIPO containing the coding region of the Sabin strain was also shown to be tropic for malignant glioma cells and breast cancer cells overexpressing CD155 and efficiently replicated and lysed these cells. PVS-RIPO prolonged the survival in athymic rat xenograft models for glioma, breast cancer and intracranial disseminated breast cancer. No adverse toxic effects in the CNS or other organs were observed after intrathecal administration in CD155 overexpressing mice, and athymic rats and in the xenograft models (293, 296, 297). A133Gmono-crePV is a novel attenuated poliovirus containing an indigenous replication element (cre) in the 5' UTR and the A133G host-range mutation to confer replication in neuroblastoma cells. This virus destroyed subcutaneously implanted neuroblastoma tumours and newly introduced tumour cells in CD155 transgenic mice, despite presence of pre-existing antibodies, and with no signs of paralysis observed (298).
A protocol involving the use of PVS-RIPO has been submitted to the RAC. A safety concern for poliovirus is that the virus could revert to a neurovirulent genotype. Stability should therefore be carefully monitored in human trials (293, 295).

### 3.2.9 Seneca valley virus (Picornaviridae, Senecavirus)

Seneca valley virus (SVV-001) is a recently discovered picornavirus isolated from cultured cells. It is believed that the virus was introduced via fetal bovine serum and that swine may be the natural host. Pre-existing antibodies were observed in 1 of 70 human samples tested. Various cell-lines expressing neuroendocrine markers supported SVV-001 replication, leading to cell lysis. Several cell lines from other species including mouse, cow and pig also supported replication. The tested normal primary

human cells were resistant to lysis. Serial passaging in three non-permissive cell lines did not lead to a change in tropism suggesting that the genome is stable. Intravenous doses of up to $1x10^{14}$ vp per kg were well tolerated in immunocompetent mice (although a temporal decrease in white blood cell count was observed) and a single intravenous dose of $1x10^{8}$ vp per kg resulted in durable responses in mouse xenograft models (299, 300). SVV-001 is being tested in clinical trials.

### 3.2.10 Coronavirus (Coronaviridae, Coronavirus)

Coronaviruses are positive-stranded enveloped RNA viruses with a 30 kb genome that have a fast replication cycle of 6-9h and that have the specific ability to induce large syncytia between infected and noninfected neighbouring cells amplifying the cytotoxicity. The tropism of coronaviruses is determined by the spike glycoprotein of the envelope that is responsible for viral entry and syncytia formation by binding to a cellular receptor. Coronaviruses exhibit high mutation rates and are prone to recombination. Coronaviruses were initially thought to exhibit a strict species restricted tropism and to be nonpathogenic to human cells. However since the emergence of SARS, coronaviruses are considered as emerging pathogens and SARS coronavirus is believed to have a zoonotic origin. SARS coronavirus most likely evolved through direct transmission from palm civets to humans, possibly followed by adaptation of the spike protein to humans (208).
FIPV (feline infectious peritonitis virus) and fMHV, a recombinant felinized mouse hepatitis virus carrying a chimeric spike of which the ectodomain is from FIPV, infect and fuse feline cells through the feline aminopeptidase N (fAPN) molecule. Attempts to modify the tropism of these viruses by incorporating tumour binding into different parts of the spike protein were not successful, as no viable viruses could be rescued. Bispecific antibodies recognizing the fAPN molecule or the EGF receptor (EGFR) were used as an alternative to direct the FIPV / fMHV viruses to human EGFR overexpressing tumour cells. Likewise MHV was targeted with antibodies recognizing the EGFR fused to the soluble domain of the MHV receptor, the murine carcinoembryonic antigen cell adhesion protein 1a (mCEACAM1a). The targeting antibodies mediated specific infection of EGFR-expressing human cancer cells followed by replication and rapid cell killing (301, 302). Foreign genes like GFP and luciferase can be efficiently expressed from the MHV genome by using of viral transcription regulatory sequences, without significantly affecting the *in vitro* growth properties of the virus. Extensions of the MHV genome with 10% of its natural size are accepted. The stability of coronaviral vectors appears to be dependent on the nature of the heterologous gene, on the particular coronavirus vector used, and on the particular site of gene insertion (303). To reduce the risks associated with viral mutations attenuated coronaviruses lacking specific but non-essential virulence genes or scrambled coronaviruses that are less likely to recombine with wildtype viruses are being applied (302, 303). MHV viruses expressing bispecific antibodies instead of the genes 2a and HE were recently generated. The bispecific antibodies are composed of the spike-binding domain of mCEACAM1a fused to a His tag targeting peptide. The recombinant MHVs could establish a receptor-specific infection with His expressing human cells, resulting in extensive cell-cell fusion and efficient killing of the target cells (304).

### 3.2.11 Sindbis virus (Togaviridae, Alphavirus)

Sindbis virus (SIN) is an enveloped, single-stranded, positive-sense RNA virus that is transmitted to birds and mammals by mosquito bites. The SIN genome is translated into four nonstructural proteins that comprise the viral transcriptase-replicase complex. The complementary, full-length, negative-sense RNA is a template for the synthesis of full-length genomic positive-sense RNA and for the production of a subgenomic RNA that is translated into the viral structural proteins via the internal 26S promoter sequence (305). In humans, SIN infection can induce mild symptoms including fever, rash, and pain in the joints. SIN virus entry in mammalian cells is mediated by the 67 kDa high affinity laminin receptor

that is overexpressed in various human cancers. SIN induces apoptosis in infected mammalian cells. SIN (AR339 strain) showed no replication and induced minimal cytopathic effects in normal keratinocytes compared to reovirus (Dearing strain) and was more oncolytic in cervical and ovarian cancer cell lines. SIN induced cytopathic effects and replicated efficiently in primary cultured human fibroblasts and mouse fibroblasts *in vitro*. SIN was demonstrated to have antitumour activity against s.c. cervical tumour xenografts (after intratumoural and intravenous administration) and peritoneal ovarian cancer tumours (after i.p. administration) in nude mice. Mice in which tumours disappeared remained healthy for 6 months.
In recombinant SIN vectors the GFP or VP7 (of bluetongue virus) transgene was linked to the gene encoding the 2A protease of foot-and-mouth disease virus and inserted in frame between the capsid and E3 genes of SIN virus. Intramolecular cleavages mediated by the capsid and 2A proteases facilitate the release of the 2A fusion proteins from the viral structural polyprotein. These vectors show more stable transgene expression compared to alphavirus-based expression vectors that use a duplicated 26S promoter to drive expression of the foreign genes but are not attenuated compared to the parental strain, and they induce 100% mortality in CD-1 newborn mice. Bivalent vectors expressing both GFP and VP7 were less stable and caused low mortality rates in newborn CD-1 mice but induced a similar serological response in adult CD-1 mice compared to univalent vectors (305). The biodistribution of *GFP-SIN* was studied after intravenous injection in mice implanted with s.c. cervical cancer xenografts. Tumour-specific GFP expression was observed from 2 days after injection, and persisted for more than 10 days. GFP expression in various organs (tumour, brain, heart, lung, liver, kidney, and intestine) was reported to be low at 72h post-injection. SIN is stable in the bloodstream and therefore proposed to be more suitable for i.v. administration than for instance retrovirus or adenovirus (306).

### 3.2.12 Reovirus (Reoviridae, Orthoreovirus)

Human reovirus is a ubiquitous, nonenveloped virus containing 10 segments of double-stranded RNA as its genome, which code for non-structural proteins involved in replication and pathogenesis, and structural proteins that make up the capsid. Most adults are seropositive for reovirus and infections with reovirus do not cause severe clinical manifestations in humans. Reovirus infections in humans are mainly restricted to the upper respiratory and gastrointestinal tracts. Human reovirus uses ubiquitously expressed terminally sialylated cellular glycoproteins and junction adhesion molecule as cellular receptors and therefore reovirus binds to most mammalian cells (307, 308).
Reovirus serotype 3 (Dearing strain) is selectively cytolytic in tumour cells because of defects in antiviral pathways that result in downregulation of the double-stranded RNA-activated protein kinase PKR that inhibits translation of viral mRNA in untransformed cells. Sensitive cells include tumour cells in which the Ras oncoprotein is activated by mutations or by altered upstream signaling involving receptor tyrosine kinases. Reovirus has direct oncolytic effects on tumour cells, inducing cell cycle arrest and apoptosis and does not require the expression of foreign genes.
Reovirus has been tested with success in various preclinical mouse tumour models including glioma, medulloblastoma breast cancer, pancreatic cancer, ovarian cancer, bladder cancer, colon cancer and lymphoma. Control cells derived from normal tissue and haematopoietic progenitors were mostly resistant to virus-induced cell killing (307-310). Compared to SIN, reovirus was more cythopathic and did show replication in normal keratinocytes (306).
Reovirus was able to kill most glioma cell lines and fresh human glioma specimens. Intratumour administration of reovirus suppressed the growth of human glioma cells implanted subcutaneously or intracranially in SCID and nude mice and improved the survival of immunocompetent mice with implanted murine fibroblast tumour cells. Antibody staining indicated no replication in muscle underneath the tumour or in other tissues. Intratumour administration of reovirus also significantly suppressed the growth of both s.c. and intracranially implanted racine glioma cells in

immunocompetent rats. Growth of contralateral tumours was not suppressed correlating with a significantly lower viral load. Reovirus mRNA and protein were present in ipsilateral tumours up to 48 hours after viral inoculation but were barely detectable in contralateral tumours or ipsilateral tumours 96 hours after infection. Reduced amounts of virus reaching the contralateral tumours may account for the inability to kill these remote tumours. Reovirus was found in the serum 2 hours following inoculation but not at later time points (311-313). Reovirus has also been administered intravenously to treat remote breast cancer tumours in an immune competent setting. Intravenous therapy with reovirus not only inhibited metastatic tumour growth but also led to a significant improvement in survival. Combining intravenous reovirus treatment with immune suppression resulted in further reduction in tumour size and a considerable prolongation in survival, compared with viral therapy alone. Combined therapy was also effective in overcoming preexisting immunity to reovirus and to induce metastatic tumour regression. (314).
Intracranial reovirus infection can be lethal in immunodeficient mice. Inoculation of reovirus into the brain of nonhuman primates however did not produce significant toxicities at clinical doses except at the injection site. Evidence of viral shedding was found in urine and other excreta (313). Non-recombinant reovirus is being tested in several clinical trials both as monotherapy and in combination with other treatments.
Genetic manipulation of reovirus is difficult due to the double stranded RNA genome and constraints on the size of the insertion. Although modification of reovirus using reverse genetics is possible, genetic manipulation of reovirus for oncolytic purposes still has to be explored (307). Although reoviruses are not associated with severe disease in healthy individuals, pathogenic responses in immunocompromized individuals can not be excluded. The S1 gene segment plays an important role in reoviral virulence. S1 attenuated reovirus is a bioselected variant with a reduced infectivity of normal tissues in the immunodeficient host but with intact oncolytic activity (315).

### 3.2.13 Murine leukemia virus (Retroviridae, Gammaretrovirus)

Replication deficient retroviruses have been widely applied in the clinic to deliver genes to target cells. Recently, also the first clinical trial making use of a replication deficient lentiviral vector was approved. However, inadequate levels of gene transfer were observed *in vivo* with replication deficient retrovirus for treatment of solid tumours like glioma. To increase the efficiency for the treatment of solid tumours replication competent MLV based retroviral vectors (RCR) that can replicate and spread after initial delivery, are under pre-clinical development.
The transmission route of MLV is through mucosal contact or via direct inoculation in bloodstream and MLV survives poorly *ex-vivo*. MLV has a built-in level of specificity for tumour cells since it can only infect cells that are actively dividing. However RCR can infect dividing normal cells like normal fibroblasts and HMECs *in vitro*. This may be advantageous for *in vivo* therapy as these cells when present in the vicinity of tumours may contribute to tumour growth and invasion. Extratumoural spread was not detected after intratumoural injection of RCR in animal models suggesting that spread is restricted to the immunosuppressive tumour environment (316, 317). A pro of RCR compared to other viruses is the ease of manipulation and production. Moreover, since RCR vectors do not have inherent cytolytic properties they may not elicit a persistent inflammatory response. For the main proposed purpose of these vectors, oncolysis, suicide genes have to be included in RCR vectors. Safety is a concern for retroviral vectors since rare cases of oncogenesis after treatment with retroviral vectors have occurred. However insertional mutagenesis has only been shown to occur in treatment regimens in which stem cells were *ex vivo* transduced with retrovirus. No pathology was observed in primates after systemic administration of wildtype MLV and the virus is rapidly cleared from the bloodstream by the immune system in primates. Furthermore there are retrovirus inhibitors available like azidothymidine (AZT), and suicide genes like CD could also be incorporated to increase the safety.

***RCR vectors with therapeutic inserts***

A main problem with replicating retroviruses is their instability; inserts can be lost within three or fewer passages and it was found that the size of the insert should not exceed over 1.3 kb, or contain recombinant sequences. Experiments to improve the stability have been conducted with MLV based vectors. ZAPd-GFP, an MLV-based RCR containing vector with an IRES-GFP transgene cassette inserted between the ecotropic envelope and the 3'-UTR was found to efficiently transduce cultured cells *in vitro* and propagate over multiple infection cycles *in vitro* and *in vivo* in solid tumours in mice. The virus was found to replicate in normal cells within tumours but no replication outside of the tumour was observed (317). Although ZAPd-GFP and AZE-GFP (that contains the amphotropic envelope from 4070A) were relatively stable, during later infection cycles recombinant transgene deleted viruses became dominant (318). ACE-GFP and ACE-CD are vectors of a later generation that contain a CMV promoter instead of the U3 region of the 5'-UTR to increase transcription of the RCR genome during the first cycle and an IRES-transgene cassette inserted between the amphotropic envelope and the 3'-UTR. Some recombination was observed with ACE-CD (316, 319, 320). ACE-CD efficiently killed glioma cells in culture, but ACE vectors do slightly replicate in control cells *in vitro* (318, 321). *In vivo*, ACE-CD increasingly prolonged (depending on the number of 5-FC cycles) the survival of athymic mice (that have an absent immune response) implanted with intracranial gliomas. A transduction efficiency of up to 100% was observed while with replication defective vectors efficiencies up to 10% have been reported. Although extratumoural spread could not be formally excluded, the CD transgene could only be detected by PCR in tumour cells. IH staining indicated that anti-MLV signal was confined to tumour-tissue and disseminated tumour tissue suggesting that stable integration of the replicating vector resulted in persistent infection. No significant staining in peritumoural tissue was observed. Thus, RCR vectors are able to transmit a transgene throughout an entire tumour mass *in vivo* without spread to extratumoural organs. Specific RCR spread and efficacy without severe inflammatory effects was also observed in an immunocompetent rat model for glioma (322). ACE-CD can be used to kill escapee tumour cells for instance after surgery at a desired time point by administration of the prodrug (316, 320).

Solly ***et al.*** generated replicating retroviruses with the 4070A envelope and GFP inserted behind an IRES downstream of the envelope (Env) gene. Efficient spread of this vector was shown *in vitro* and in subcutaneously implanted murine and rat tumour cells in nude and immuno-competent mice after intratumoural injection of virus producing cells. The biodistribution of replicative vectors was investigated after intravenous injection in immuno-deficient and immuno-competent mice, with and without AZT treatment. In nude mice, proviral genomes could be detected in bone marrow and spleen by PCR two weeks after administration, only in the absence of AZT. No virus was detected in any tissue analyzed from immuno-competent mice, emphasizing the potency of anti-MLV immune responses (323).

Recently, secretory therapeutic transgenes have been stably integrated in MLV RCR vectors with a 4070A amphotropic envelope by fusing them with the N-terminus of the envelope protein via a furin cleavage site. During production of the Env protein in cells the therapeutic protein is cleaved off and secreted. EGF, GM-CSF, the chemokine CXCL-10 and single chain antibodies binding laminin and T-cells (7A5) have been incorporated via this strategy and these vectors were infectious for various cancer cells, normal fibroblasts and HMECs, causing secretion of biologically active protein from infected cells. Importantly, these vectors were stable for 12 passages in culture (324). The vectors IP10-A and IP10-V express interferon inducible protein (IP10), an angiostatic and immuno-stimulatory protein. IP10-V contains the gibbon ape leukemia virus (GALV) envelope and IP10-A the 4070A MLV envelope directing the tropism of the vector to human cells or human and murine cells, respectively. IP-10 production from RCR transduced cells was maintained for at least three months in culture and tumour cells transduced with these vectors showed similar *in vitro* growth kinetics as the parental cell line. The *in vivo* growth of IP10-G transduced human fibrosarcoma cells subcutaneously implanted in

SCID mice, IP10-A transduced murine lung carcinoma cells subcutaneously implanted in immunocompetent mice and IP10-A transduced melanoma after intravenous injection was reduced compared to non-transduced cells or cells transduced with wildtype MLV (325).

### *Transcriptional targeting*

Transcription of retroviral genes is mainly determined by elements in the U3 region of the LTR. Transcriptionally targeted RCR vectors like ACE-GFP-At have been generated by using a hybrid LTR that contains promoter sequences of the rat probasin gene fused to the U3 region TATA box to target replication to prostate cells (319). The CTP4-TF and E2-TF vectors contain the synthetic CTP4 promoter or the chimeric EII-Pa1AT promoter to target RCR to cells deregulated in the beta-catenin pathway or liver derived cells, respectively. Increased specificity for specific cell lines was observed compared to the parental vector ACE-GFP *in vitro* showing that transcriptional targeting increases specificity. A small amount of replication persisted in non-permissive cell lines (e.g. cells not deregulated in the beta-catenin pathway in case of CTP4-TF). Vectors were stable in culture but started to lose their GFP transgene but not the promoter sequence after multiple infection cycles. It is assumed that complete transduction of solid tumours would occur before the virus becoming unstable (321).

### *Transductional targeting*

The ecotropic envelope of MLV is not able to mediate infection of human cells. The amphotropic 4070A MLV envelope however can infect cells of many species since it interacts with a sodium-dependent phosphate transporter which is present in many tissues. Tandem repeats from the IgG binding domain from *Staphylococcus aureus* were inserted into the proline rich region of the envelope to enable retargeting of RCR vectors with anti-HER2 antibodies to breast cancer cells. Both the ecotropic (ZV-ZZ-GFP) and the amphotropic construct (AZE-ZZ-GFP) showed increased binding of human Her-2 overexpressing breast cancer cells in the presence of antibody, but increased infectivity was only observed in presence of the wildtype envelope, because the ability of the targeted envelope to mediate virus-cell membrane fusion and entry is thought to be attenuated. The constructs were very instable (326). In the future the application of more stable amphotropic retroviruses with modified envelopes displaying a single-chain antibody at the N-terminus directed against overexpressed surface proteins in cancer cells may be a useful strategy for modifying the RCR tropism (324, 327).

### *Transcomplementing vectors*

Semi replication competent systems have been developed recently that make use of transcomplementing retroviral vectors; a gag-pol vector in which the Env sequence is replaced by a GFP transgene and a second Env vector that contains the amphotropic envelope and an IRES-transgene cassette. An advantage of this system is the fact that more and larger therapeutic transgenes can be inserted. *In vitro*, a transgene propagated better with this system than a dual system based on a replication deficient vector with replication competent helper virus, and the method was as efficient as a RCR vector. In contrast with the application of wildtype virus or an RCR vector, leukemia development after bone marrow transduction/transplantation was not observed with this system (328). In a similar approach, the TK insert was included in the gag-pol vector and the VSV-G envelope that can also mediate cytotoxic cell killing, in a second vector. These vectors were highly stable. Although compared to RCR vectors decreased *in vivo* propagation was observed, increased survival was observed in *in vivo* tumour models (329).

***Future developments with RCR***

The application of replication competent retroviruses in clinical trials in the near future appears to be unlikely given the problems associated with their use. These include stability problems, the tropism for normal dividing cells and especially the genotoxic effects (leukemia development in immunocompromized persons).

# 4 Overview of replication competent viral vectors in clinical trials

From the mid-1800s there have been several reports of tumour regression coinciding with natural virus infections, but it was not until 1949 that the first serious clinical trial with an oncolytic virus was performed. In this study serum or tissue extracts containing hepatitis virus were administered to Hodgkin disease patients. During the 50's-70's various other wildtype viruses, including adenovirus, Egypt 101/West Nile Virus and mumps virus were tested in clinical trials. The limited success and the apparently unethical circumstances under which some of these trials were performed were held responsible for many researchers abandoning the field in the 70's and 80's (2). The possibilities of genetic engineering and the availability of better pre-clinical models resulted in a renewed interest in the 90's leading to several clinical trials with both genetically engineered (e.g. adeno, herpes and poxvirus) and non-modified viruses (e.g. specific strains of NDV and reovirus).
This chapter gives an overview of the clinical trials that have been performed in the second era of oncolytic viruses (90's-to present) or that are currently in progress. Where available a short overview of pre-clinical data that have lead to the approval of the clinical studies is given. Besides engineered viruses also published trials with non-engineered viruses are included since it is in most cases likely (see previous chapter) that genetically modified variants of these viruses will be used in the future. Specifications of the applied vectors and (pre-clinical) data concerning safety, efficacy, biodistribution and shedding are, when available, included.
To generate a comprehensive overview, published trials (gathered via Pubmed) and trials presented or announced at the 2007 Oncolytic virus conference (Carefree, AZ) are included. Web pages of companies involved in oncolytic research (14-18) were visited to get an overview of the progress of non-NIH funded trials. Moreover, several public clinical trial databases concerning clinical trials in the USA and Europe were searched (13, 330-332). Especially the OBA/RAC database (330) (an overview of trial protocols submitted for recommendation to the RAC) gives a good indication of the current status of clinical trial protocols in the USA. It should be noted that RAC approval is not necessary for initiation of a trial. First of all, RAC approval is voluntary for non-NIH funded trials. The initiation of a trial can take place after approval of the Institutional Review Board (IRB) at the clinical site and the approval of an investigational new drug application by the FDA (confidential). The sites of Clinicaltrials.gov (332) and NCI/RAID (that facilitates translation of novel therapies to the clinic (331)) provide additional information about whether a trial protocol is still waiting for regulatory approval or is open for enrolment of patients.

## 4.1 Adenoviral vectors

### 4.1.1 E1B deleted vectors; Onyx-015, H101, Ad5CD/TKrep and Ad/L523S

***Onyx-015: pre-clinical data***

In most of the published clinical trials with oncolytic vectors, Onyx-015 and a number of highly related vectors were used. Onyx-015 is an HAdV-2/5 chimera containing a deletion between nucleotides 2496 and 3323 in the adenoviral E1B region and a stop codon at position 2022 (the third codon of the protein), eliminating E1B protein expression. Furthermore the E3B genes 10.4, 14.5 and 14.7 are deleted in this vector. While these combined deletions lead to severely attenuated, but not completely abolished replication in normal cells, efficient replication is supported by many tumour cell lines leading to cytolysis (35, 333). Before its use in clinical trials, the efficacy of Onyx-015 was tested in pre-clinical models. When Onyx-015 was injected in s.c. implanted human cervical carcinoma xenografts in nude mice, the virus was able to spread to contralateral (non-injected) established tumours since three weeks after injection 4/5 tumours had a positive hexon stain. In contrast to established tumours, growth of newly implanted tumours was inhibited. Intravenous injection of $5x10^9$ pfu in nude mice with implanted xenografts resulted in 50% lethality. Livers from nude and immuno-competent mice that received $5x10^9$ pfu exhibited severe hepatic necrosis. When nude mice with subcutaneous tumours were injected intravenously with $10^9$ pfu of Onyx-015, most of the virus (90%) was detected in liver tissue 3-6 h after injection. However, after 24h, the titer in the liver had decreased 1000-fold, and virus became undetectable by 72h. In tumours a 150-fold increase in virus titer between 3 and 72h after injection was observed. Spread of virus was shown on tissue sections from tumours but no evidence of viral replication was detected within the livers at any time point. Intravenous administration of Onyx-015 also decreased tumour growth and increased survival in cervix and (metastatic) colon carcinoma models (334).

***Onyx-015: clinical trials***

Clinical trials with Onyx-015 were performed in the USA, the UK and Egypt. In a staged clinical research approach, systemic exposure to Onyx-015 was sequentially increased from intratumoural injection via intraperitoneal injection to intra-arterial and intravenous administration. No maximally tolerated dose could be established and toxicities were in most cases mild. $2x10^8$-$2x10^{13}$ viral particles were administered under various regimens for treatment of several types of tumours. Treatment with Onyx-015 was also found to be safe for the patient in combination with chemotherapy (10, 335, 336).

***Intratumoural injection: Onyx-015***

In a phase I study, 4/22 patients with recurrent head and neck cancer showed evidence of viral replication by DNA ISH on tumour biopsy samples at day 8 or day 22 after receiving $10^7$-$10^{11}$ pfu of Onyx-015. Normal skin and mesenchymal tissue were negative. Blood DNA samples were uniformly negative at day 0, 3, 8, 15, 22, and 29 post-treatment by PCR. In addition no virus could be detected at these time points after inoculation of HEK293 cells with cultures of swabs from the oropharynx and the injection site, followed by a direct fluorescence hexon protein assay. Only grade 1 and 2 toxicities were observed of which fever was observed most frequently. In one patient grade 2 tracheal obstruction was reported. In patients that received treatment for multiple cycles no added toxicity was observed. Although no objective responses were observed because of general disease progression, MRI of the injected tumours suggested necrosis in 5/22 patients (337).

In a phase II study, Nemunaitis et al. treated patients with advanced head and neck cancer with intra-tumoural Onyx-015 ($1x10^{10}$ pfu=$2x10^{11}$ vp for 5 days in the first week or twice daily for 10 days during the first two weeks). A partial to complete response (>50% tumour destruction) was observed in 14% of patients. Although mostly low grade toxicity was observed (e.g. fever), 33 grade 3 and 5 grade 4 adverse events were observed of which some were possibly related to Onyx-015 injection. Peri-tumoural tissue did not appear to be affected after Onyx-015 injection. Biopsies revealed presence of Onyx-015 in 7/11 patients by DNA ISH with 5 of 7 samples being positive on days 1-3 post-treatment and 2 of 4 samples being positive on days 7-10 post-treatment. Normal skin and mesenchymal tissue within the tumour samples was negative.14-17 days post-treatment all 10 samples tested were negative. In the peripheral blood, Onyx-015 could be detected by PCR on viral DNA in 12/29 (41%) samples at day 6-7 of the first treatment cycle (24h after the last injection), while 9% and 0% were positive at day 15 and day 22, respectively. In the second and third cycles, 6 of 21 and 2 of 8 patients were positive 24 hours after the last injection, respectively and all samples were negative by day 15 (338, 339).
In a phase II trial 3/5 tumour biopsies on day 5 and 1/2 biopsies on day 15 stained positive by DNA ISH after local administration of Onyx-015 in head/neck cancer patients ($10^{10}$ pfu each day for five days) in combination with chemotherapy. No indication of replication was observed in normal surrounding tissue. Grade 3 and grade 4 toxicities were observed in 46% and 14% of patients, respectively. No grade 3 or 4 hepatic dysfunction was observed. For some of the grade 3 and 4 toxicities involvement of Onyx-015 could not be excluded. The objective response rate (53%) was better than in trials using only chemotherapy (340).
In a phase I trial, no evidence for viral replication was observed after injection of Onyx-015 into pancreatic tumours ($10^{8}$-$10^{11}$ pfu in three week cycles). In 10/11 patients virus was detected in the blood by PCR 15 minutes after injection. Bloodsamples taken 1, 5 or 15 days post-injection (tested by quantitative PCR) and tumour biopsy samples taken 22 days postinjection (tested by ISH) were negative. One dose-limiting toxicity (DLT) was observed; a patient died from liver function abnormalities and the involvement of Onyx-015, although unlikely, could not be ruled out. 4% of patients showed a grade 4 toxicity (leucopenia) and 43% grade 3 toxicities that could be related to Onyx-015 treatment were observed. Although no objective responses were reported, 6 of 22 patients had a minor response (35-45% tumour shrinkage) (341).
In a phase I/II trial, intratumoural injections ($2x10^{10}$-$2x10^{11}$ vp at day 1, 5, 8, 36, 43, 50 and 57) were combined with chemotherapy during the last 4 injections. Treatment with Onyx-015 was well tolerated. One grade 3 fever and one grade 3 increase in thromboplastin time were observed. Other grade 3 and 4 toxicities could be attributed to the injection technique or chemotherapy. No objective responses were seen at day 35 but 2/21 patients (10%) showed a partial response after the combination treatment. No adenoviral DNA could be detected by ISH on biopsy samples or by PCR on blood (342).
Following intratumoural injection of Onyx-015 in advanced sarcoma combined with chemotherapy (phase I-II trial) viral genomes were detected one day before the last injection in the blood of 5/6 patients receiving $10^{9}$-$10^{10}$ pfu for five days in a one month cycle. In one patient, additional sampling was performed 3, 5 and 7 days post-injection. Virus was detected with a slight increase from day 5 to 7 post-injection, indicating viral replication. 2/4 patients showed viral DNA in the sarcoma biopsies taken at day 5 by ISH, with negative surrounding tissue. No DLT was encountered and no high grade toxicity related to Onyx-015 treatment was observed. Grade 3 toxicities related to the treatment combination were anemia in two patients and diarrhoea in one patient. 1 of the 6 patients showed a partial response (343).
Morley *et al.* administered $10^{10}$ pfu of Onyx-015 in patients with primary oral carcinoma or in normal mucosa surrounding the tumour. The treatment was well tolerated and no side effects attributed to Onyx-015 were reported. 10/15 tumour samples and 3/15 normal samples (taken 24h, 3 days or 14 days post-delivery) were positive respectively, by IH on hexon protein or DNA ISH. In 3 of 15 cases virus

was detected in saline injected hemi-tumours suggesting viral translocation. Direct injection in the normal tissue resulted in high levels of apoptosis 24-72 h after injection (344).
Injection of Onyx-015 ($10^7$-$10^{10}$ pfu) into normal brain tissue adjacent to freshly excised glioma was well tolerated. 10 of 24 patients showed one or more adverse events. None of the observed adverse effects were judged to be due to Onyx-015. No objective responses were observed. No data about replication were reported in this trial (345).

### *Intratumoural injection: H101*

Several clinical trials with H101, an oncolytic virus highly related to Onyx-015 (H101 contains a slightly larger E3 gene deletion), were performed in China. Sunway Biotech has conducted preclinical phase I/II/III trials for H101 and this company is also planning to perform phase III trials with Onyx-015 (17). Intratumour injection of H101 was found to be safe when administered intratumourally ($5.0x10^7$-$1.5x10^{12}$ vp for five consecutive days each cycle) in a phase I trial. No grade 3 or 4 toxicity was reported. Main side effects were injection side pain and fever. No adenoviral DNA was detected by PCR in plasma, urine and swabs from the oropharynx and the injection site. Blood was positive by PCR in all 15 patients but the day of sampling is unknown (6, 346). In a phase II trial intra-tumour injection of $5x10^{11}$ vp H101 for 5 consecutive days every three weeks was well tolerated in combination with chemotherapy in patients with various advanced types of solid tumours. 14/46 patients (30.4%) showed a complete or partial response, a better result than with chemotherapy alone. Treatment related toxicity included fever, leucopenia and liver dysfunction but no statements about the relationship with H101 were done. 30 minutes after injection plasma was positive in 4/11 cases. Evidence for viral replication was observed in 2/3 tumour biopsy samples taken at the end of treatment on day 22 or 44 by IH (37). Also in a phase III trial intratumoural injection of H101 ($5.0x10^{11}$-$1.5x10^{12}$ vp) plus chemotherapy for treatment of squamous cancer of the head and neck or esophagus was found to be relatively safe. A response rate of 79% was observed for H101 plus chemotherapy compared to 40% for chemotherapy alone. H101 was approved for treatment of head and neck cancer by the Chinese State Food and Drug Administration in November 2005 (20, 347).

### *Intratumoural injection: Ad5-CD/TKrep*

The Ad5-CD/TKrep vector contains a cytosine deaminase (CD)/herpes simplex virus thymidine kinase (HSV-1 TK) fusion suicide gene, but is otherwise very similar to Onyx-015. Delivery of this vector to tumour cells renders them sensitive to the agents FC and GCV, and makes the cells more sensitive to radiation. In a phase I trial performed in the USA this vector was injected intratumourally ($10^{10}$-$10^{12}$ vp) in patients with prostate tumours followed by prodrug treatment starting two days later. A partial response (PSA decrease for more than 50% for 4 weeks) was observed in 3/16 patients; 7 of 16 patients showed a decrease of 25% or more in serum PSA levels. Two patients were negative for adenocarcinoma during a 1 year follow-up period. 94% of adverse events were grade 1 or 2. No DLT was observed and there was no correlation between the viral dose and the incidence of an adverse event. Shedding in the blood and urine was evaluated up till one year after injection. In 12/16 patients (i.e. in all patients except those from the lowest dose group) viral DNA was detected in the blood peaking between day 2 and 4. In some patients a second peak was observed and in two patients high levels of the virus persisted until at least day 16 and 37. Virus could be detected in the blood of patients up to day 76 postinjection and 4/12 tumour biopsies stained positive for the transgene CD/TK two weeks post-injection. However, no infectious adenovirus was detected in the blood or urine at any time point. In a second phase I trial performed in the USA, Ad5-CD/TKrep ($10^{12}$ vp) was combined with prodrug treatment and radiotherapy. Again serious adverse events were rare, transient and mostly unrelated to the virus treatment. All patients showed a significant decline in serum PSA levels. 4 of 6

patients were negative for adenocarcinoma at the 1 year time point. Prostate biopsies were positive for CD expression (IH) in 3/3, 1/3 and 0/3 cases, 2 weeks, 3 weeks and 4 weeks post-treatment, respectively. While viral DNA was detected in the blood for up to 45 days no infectious adenovirus was detected at any time point (348, 349).
Ad5-γCD/mutTK$_{SR39}$rep-ADP is a second generation vector containing an improved version of CD/TK and an insertion of the adenoviral death protein (ADP) gene that increases the rate of vector spread. This vector was tested in a phase I trial in 9 patients with prostate cancer (intraprostate injection of $10^{12}$ vp on day 1, or on day 1 and day 22, each followed by prodrug therapy and radiotherapy). Compared to the earlier trials with Ad5-CD/TKrep, no enhanced toxicity was observed. 5 grade 3 or higher toxicities were observed. All patients showed significant declines in PSA level and 7/8 patients were negative for adenocarcinoma in their last biopsies. Although viral DNA was detectable in serum up to day 118 post-treatment, no infectious adenovirus was recovered. Urine and stool were not monitored in this trial (350).
In a recent update it was reported that 46 subjects have now received adenovirus-mediated suicide gene therapy in 4 trials. Toxicities were low with 93% of the adverse effects being grade 2. For several parameters a significant improved outcome was observed in these trials. Phase III trials in prostate cancer patients will commence in 2007 (351). Phase I trials applying adenovirus-mediated suicide gene therapy in patients with penile cancer and pancreatic adenocarcinoma have been reviewed by the RAC (330).

***Intra-peritoneal administration***

In a phase I trial Vasey et al. treated patients with ovarian cancer with i.p. injections of Onyx-015 ($1x10^9$-$1x10^{11}$ pfu) for 5 days every three weeks. 3/16 patients displayed grade 3 viremic (flu-like) toxicities and in one patient a DLT was reached (grade 3 pain and diarrhoea). On day 5 and day 15, Onyx-015 was detected by PCR in the intraperitoneal cavity in 7/8 and 5/8 patients, respectively. In one patient, Onyx-015 was detected for up to 354 days after the fourth treatment cycle. Onyx-015 was not detected by PCR in the peripheral blood 15 min-24 hours post-injection. There was no conclusive evidence for viral replication since levels on day 15 were lower than on day 5. No objective response was observed (352).
In a phase II trial Onyx-015 ($6x10^9$-$3x10^{10}$ pfu) was administered in patients with hepatobiliary tumours by i.p. or intratumoural injection. Treatment was well tolerated and treatment related toxicities were mostly grade 1 or 2. Two cases of grade 3 hepatic toxicity were observed. Grade 4 toxicities were unrelated to Onyx-015 administration. 1 of 16 patients showed a partial response of the injected lesion and 8 of 16 patients had a 50% decline in serum tumour markers. Bile (2/2 samples) and ascites (4/4 samples) were positive by PCR 1-5 days and 1-9 days post-injection, respectively. Although live virus was detected in ascites on day 1-6 postinjection, urine samples obtained from 12 patients between 1 and 14 days after injection remained negative by CPE assay suggesting effective systemic clearance. The positivity of bile was regarded as a safety concern warranting future analysis of stool samples (353). In a patient with a hepatobiliary tumour, virus was detected in tumour and normal surrounding tissue 37 hours and 7 days after intralesional administration of Onyx-015 ($10^{10}$ vp) by in situ reverse transcription PCR that detects transcription of late viral genes and that therefore appears to be a better measure for viral replication than ISH, IH or PCR. No evidence of necrosis or apoptosis was observed (353-355).

***Intra-arterial and intravenous administration***

In a combined phase I/II study performed in Egypt, patients with liver cancer received either intra-tumoural, intra-arterial or intravenous injection with Onyx-015 ($3x10^9$-$3x10^{11}$ pfu) under different regimens. In the phase II trial patients received Onyx-015 ($3x10^{11}$ pfu) intra-arterially for three consecutive days plus chemotherapy. Viral particles were detected 14 days after virus administration by electron microscopy in liver biopsies but no other biodistribution data were presented. No serious adverse effects were reported. In 4 of 7 patients in the phase II trial levels of the tumour serum marker CEA decreased after treatment. Patients were allowed to go home 2 hrs after administration or were kept overnight to guard bleeding from the liver capsule puncture site. Viral presence in liver tumour cells was associated with a pre-apoptotic phase and necrosis (356). In a phase II UK trial patients with hepatocellular carcinoma metastatic to the lungs received Onyx-015 intravenously at day 1 ($3x10^{11}$ pfu) followed by intratumoural Onyx-015 at days 2, 15, 16, 29 and 30. Only grade 1 or 2 toxicity (fever), and no rise in liver enzymes was observed. 1 of 10 patients showed a partial response. Virus was detected by EM in tumour tissue but not in the blood by PCR 4 hours after injection (357).
In another phase I trial Onyx-015 was given intravenously (weekly dosis of $2x10^{10}$-$2x10^{13}$ vp in a 21 day cycle) to patients with carcinoma metastatic to the lung. Most serious grade 3 and 4 events were unrelated to Onyx-015. No progressive toxicities were observed and no DLT was established. Viral genomes could be detected minimally 90 minutes after infusion in 9/9 patients. In all patients receiving a high dose level (>$2x10^{12}$ vp) Onyx-015 was detected in plasma 6 hours post-infusion after the first and second treatment cycle. Overall, 17/37 (46%) and 8/21 (38%) plasma samples had detectable levels of Onyx-015 7 days after the first or second cycle of treatment, respectively. An increase in viral genomes was detected between 6h and 48h post infusion in 3/3 patients receiving at least $2x10^{12}$ vp, indicating viral replication. A biopsy sample of one of these patients showed evidence of intra-tumour replication by IH and PCR. Surrounding pulmonary tissue was negative. Autopsy of this patient, 134 days after the last infusion, provided no evidence for replication in any organ including tumour tissue. No objective responses were observed (358). In a pilot trial, intravenous Onyx-015 ($2x10^{12}$ or $2x10^{11}$ vp given weekly for 6 weeks) was combined with chemotherapy or with treatment with the growth factor IL2. Treatment was well tolerated and the only toxicity that could be attributed to Onyx-015 was a transient fever. No objective response was observed. In three patients uptake of the vector in malignant tissue was demonstrated by PCR (2 patients) or EM (1 patient). In the EM sample no virus was observed in adjacent normal tissue. 48h post infusion, 3 out of 10 blood samples were PCR positive (359).
Patients with metastatic gastrointestinal carcinomas to the liver were treated in a phase I trial with intra-arterial Onyx-015 ($2x10^8$-$2x10^{12}$ vp) for two cycles on day 1 and day 8, and subsequent cycles on day 22, 50 and 78 included chemotherapy. No grade 4 toxicities were reported. No DLT was established and grade 3 toxicities possibly related to Onyx-015 included chills (3%) and leukopenia (6%). No significant liver toxicity was observed. Onyx-015 did not appear to increase the side-effects of chemotherapy. Input virus was rapidly cleared from the blood 6h after the first infusion, and no virus was detected 24h or 48h post-infusion in two high dose patients. However, Onyx-015 was detected by PCR on blood DNA from of all high dose patients (>$2x10^{11}$ vp) 4 days after infusion suggesting viral replication. Liver biopsies taken at day 4 were mostly necrotic and therefore no viral replication could be demonstrated. 1 of 11 patients had a partial response. In a phase II trial patients received $1x10^{11}$ pfu (~$2x10^{12}$ vp) of Onyx-015 plus chemotherapy in a similar treatment regimen. Mostly grade 1 or 2 events were observed; grade 3 events probably related to virus treatment included an increase in liver hyperbilirubinemia from grade 1/2 to (transiently) grade 3/4 in two patients. A reversible grade 4 systemic inflammatory response with altered cytokine profiles was seen in one patient after the fourth cycle. Virus was rapidly cleared from the blood within 6h after injection. In 4/6 and 1/1 patients virus was detected in the blood 3 and 4 days after infusion, respectively. From data from 1 patient it was estimated that $10^{11}$ genomes were produced and shed in the blood over the 72h replication cycle.

Furthermore, a second cycle of replication and shedding was observed in treatment cycle 4. 7/27 patients showed a minor or partial response. It was estimated that less than 0.1% of tumour cells was initially infected (28, 360-362).
In another trial patients with metastatic colorectal cancer received Onyx-015 intravenously ($2x10^{12}$ vp at day 1 and 15 of a 28 day cycle). 4/18 patients showed a minor decrease in serum CEA levels. One patient displayed grade 3 flu-like symptoms. After 6 and 24 hours respectively 100% and 50% of 14 blood samples were positive for Onyx-015 by PCR on viral DNA. At 72 hours this percentage had decreased to 36%. In three patients, levels at 48 hours were higher than at 6 hours indicating ongoing viral replication. In a patient that died 56 hours post-injection because of tumour progression, the vast amount of virus was present in the spleen and in normal liver cells. Little virus was found in liver and mesenterial tumour cells (suggesting limited replication in tumour tissue), lung, heart and small intestine by PCR on DNA and IH. No virus was detected in pancreas, kidney and brain (363).
In a phase I trial of intravenously administered Onyx-015 (up to $1x10^{12}$ vp weekly for 4 weeks) in combination with enbrel (an anti-TNF therapeutic agent) no significant adverse effects could be attributed to the therapy. 4/9 patients had stable disease. 2/3 patients had detectable viral DNA 3 or 8 days post-administration and levels were considerably higher during cycle 1 (plus enbrel) compared to cycle 2 (no enbrel) suggesting that enbrel reduced the (TNF-induced) systemic clearance of Onyx-015 (364).

***Mouthwash***

In a phase I trial Onyx-015 was administered by mouthwash ($10^{10}$ pfu weekly for twelve weeks or for 5 days ever 4 weeks, or 2 cycles $10^{11}$ pfu for 5 days and then 5 times weekly) to 19 patients with dysplastic lesions of the oral epithelium. Treatment was well tolerated and no grade 3 or 4 toxicities were observed. 37% of the patients showed transient histological improvement. 2 of 3 tissue biopsies showed Onyx-015 by ISH on day 8. Only one patient showed a slight two-fold increase in AdV-5 serum antibodies, suggesting minimal systemic exposure (365).

***Tumour vaccination***

Ad/L523S is an E1B deleted adenoviral vector expressing the L523S lung cancer antigen. In a phase I trial performed in the USA 1, 20 or 400 $x10^{9}$ vp were administered intra-muscularly on day 28 and 56 to 13 patients previously vaccinated with a plasmid expressing L523S. No significant toxic effects were identified (366).

### 4.1.2 Type II prostate-specific CRAds; CV706 and CV787

CV706 was constructed by removing the E3 region of the adenoviral genome and inserting PSA promoter and enhancer elements upstream of the E1A region. This resulted in selective toxicity of CV706 for PSA-producing prostate cancer cells. Single intratumoural injection of CV706 in human prostate cancer xenografts resulted in regression of these tumours and a decrease in PSA levels (367).
In a phase I trial performed in the USA CV706 was injected directly into prostate tumours ($1x10^{11}$-$1x10^{13}$ vp). All observed grade 3 toxicities were reversible and unrelated to the treatment. No hepatic toxicity was observed. In 13/20 patients PSA levels decreased more than 30% and 4/20 patients showed a partial response (>50% decrease in PSA level). In post-treatment biopsies taken at day 4, 22 and at 3 months adenovirus was detected by light microscopy or EM and this was confirmed by IH. Viral hexon staining was exclusively observed in prostate epithelial cells. Virus could be detected in all patients shortly after administration. The initial half life of virus in the circulation was approximately 23 minutes and circulating virus was near baseline 12-24h after administration. Evidence for viral

replication was observed by PCR on blood samples: a second peak of detectable CV706 occurred in 13/16 patients 2-8 days after treatment. By day 15, all blood samples were negative. Urine of 11/19 and 2/19 patients was positive for live virus (plaque formation), 2 and 8 days post-treatment respectively. By day 15 and 29, all urine samples were negative. No wildtype recombinant virus was detected since no plaques were formed on cells permissive to wildtype AdV but not to CV706 (368).
CV787 is a second-generation vector with more specificity than CV706 for PSA-producing cells. This virus was constructed by inserting the PSA promoter and enhancer elements upstream of the E1B region of the virus and the rat prostate probasin regulatory elements upstream of the E1A region, resulting in the preferential replication of CV787 in PSA-producing cells. The E3 region of the genome was reintroduced into the vector in order to achieve greater cell killing. Several trials with this virus were performed in the USA. Initially this virus was tested by i.t. administration in prostate carcinomas at a dose of $10^{12}$-$10^{13}$ vp. These doses were reported to be well tolerated (336). In a phase I/II trial 23 patients received a single intravenous injection of CV787 at various doses ($1x10^{10}$-$6x10^{12}$ vp). Grade 3 adverse events (fever, fatigue) were rare and temporal. Grade 1 and 2 liver toxicities were observed at the highest dose level used. Five patients showed a decrease (less than 50%) in serum PSA levels. Circulating genomes were found 90 minutes post-injection in all patients, at day 8 in 13/23 patients and at day 29 in 3/23 patients. Secondary peaks indicative for viral replication occurred in 70% of patients between day 2 and day 8. Urine remained negative up to 29 days post-treatment (plaque assay). Saliva samples were positive for live virus in three patients (2/5 and 1/18 receiving $>10^{12}$ or $10^{12}$ viral particles, respectively) on day 4 or day 8, but all saliva samples were negative on day 15 and 29 (369). A clinical protocol in which CV787 is administered intraperitoneally to patients has been submitted to the RAC (330)

### 4.1.3 Adenoviral vectors in future and ongoing clinical trials

Ad5-CD/TK*rep*-hNIS is a second generation adenovirus-mediated suicide gene therapy vector that is presently being applied in a phase I trial in prostate cancer patients. After i.t. delivery of a single dose of $10^{12}$ vp, 25% of the prostate showed NIS gene expression by SPECT imaging (351). H103 is an E1B deleted AdV-5 vector encoding the heat shock protein HSP70. A phase I trial in China has been completed (17). A phase I clinical trial for intravesicular treatment of bladder cancer has been initiated with CG0070, an armed oncolytic adenovirus that has been engineered to include the therapeutic gene GM-CSF. In CG0070, E1A expression is driven by the E2F promoter and GM-CSF is inserted in the E3 region instead of gp19kD (330, 370). A phase I trial with the tropism modified vector AdΔ24-RGD in ovarian cancer patients (i.p.) has been initiated in the USA. A protocol involving the use of this vector in glioma patients (i.t.) has been reviewed by the RAC (330, 331). VRX-007 lacks all E3 genes except 12.5K and has a reinsertion of the ADP gene in the E3 deletion such that the ADP major late mRNA is formed abundantly to promote release and spreading. This vector showed similar replication as AdV-5 in lung epithelium (one week after i.v. or i.t. injection) in Syrian hamsters and low toxicity / pathogenicity. VRX-007 was reviewed by the RAC for use in a phase I/II trial (i.t. delivery) in patients with various solid tumours but has yet not been approved by the FDA awaiting toxicology (330, 370). Another phase I clinical protocol submitted to the RAC makes use of the type 2 CRAd OBP301, in which E1A and E1B expression are driven by human telomerase reverse transcriptase (TERT) promoter elements for i.t. treatment of various cancers (330). In Korea a clinical trial has started in 2007 with DWP418, an AdV-5 vector that expresses a relaxin insert for better penetration of solid tumours. The RAC has also reviewed a protocol for the use of Ad4-ΔE3-Hiv-env and Ad4-ΔE3-Hiv-gagpro for use as AIDS vaccines after the demonstration that these vectors were as safe as wildtype AdV-4 in cotton rats (330, 371).

## 4.2 HSV vectors

### 4.2.1 HSV1716

HSV1716 is derived from the HSV-1 strain 17, but is deleted for both copies of the gene encoding γ34.5, reducing its virulence (HSV can cause fatal encephalitis in humans) and causing the selective replication of this vector in rapidly dividing cells. Preclinically, HSV1716 was shown to replicate as efficiently as wildtype HSV-17 in actively dividing, but not in terminally differentiated cells (372). HSV1716 is able to replicate in and kill human melanoma and glioblastoma cells *in vitro* (373, 374). Although replication is attenuated compared to wildtype virus, HSV1716 does replicate in cultures from normal keratinocytes, melanocytes and rat brain cells (113, 375). The LD50 of HSV1716 in BALB/C mice is $7x10^6$ pfu. In comparison, the LD50 for wildtype HSV is $10^2$ pfu (376). Intratumour injection of HSV1716 was effective against experimental brain tumours in nude mice (377, 378) and against intracranial implanted melanoma in immunocompetent mice. In this last study viral replication was restricted to melanoma and not detected in surrounding brain tissue (373). While in one study infection of the brain of BALB/C mice lead to a finite and self-limiting response (379), in a second study HSV1716 replicated in ependymal cells leading to loss of ependymal lining (114). HSV1716 was shown to replicate in neurons and to be significantly toxic in nude mice suggesting that immunocompromised patients treated with this vector may possibly also suffer from significant viral pathogenesis outside the tumour (116). HSV1716 was also shown to replicate in human skin xenografts (380).

Various trials have been performed in the UK with HSV1716. In a phase I trial HSV1716 was injected at $10^3$ pfu (1-4 times) in metastatic melanoma nodules. Treatment was well tolerated. No virus shedding or reactivation of endogenous HSV was observed in all 5 patients that were seropositive before injection. In 1 patient a response (flattening of two nodules) was observed. 3 patients showed signs of necrosis in excised nodules with no damage in adjacent normal tissue. Nodules were excised 8 or 14 days after the last injection and in 5/5 patients specific HSV protein staining was observed in melanoma cells and not in adjacent connective tissue or normal melanocytes (375).

Intratumoural administration of $10^5$ pfu HSV1716 in 21 patients with recurrent glioma was safe and no toxicity was reported. No dose escalation was performed since the investigators reasoned that the final dose could be several orders higher than the input dose. There was no induction of encephalitis. Adverse clinical symptoms or reactivation of latent HSV were also not observed. Of 9 patients treated, four were alive and well 14-24 months after HSV1716 administration. In 2/12 patients infectious HSV1716 was recovered at a higher titer than the input dose from tumour biopsies taken 4-9 days after injection. No virus was recovered from a paired distal tumour sample in one of the positive patients. Using a PCR for HSV DNA, it was found that 24 hours post-injection 10 of 12 patients were positive at the injection site, 4/10 in paired distal tumours and 1/12 in the blood. The positivity in this one patient rapidly cleared. In a post-mortem sample taken 251 days after HSV injection $5x10^3$ HSV genomes were recovered from tumour centre samples but not from peri-tumour and more distal brain samples. HSV antigen was detected in two patients at the injection site using IH. Evidence for replication was found in patients that were HSV sero-positive or sero-negative before treatment. A number of the patients with glioma have lived longer than would be normally expected but no conclusive data about survival were reported. Tumour tissue from one patient treated 2.5 years earlier with HSV1716 was negative for virus (381-383). In another phase I UK trial the same dose of HSV1716 was injected in the rim of resected tumours. Again no toxicity directly related to treatment was reported. In 4 of 12 patients (of which three were HSV seropositive before treatment) HSV was detected in serum after injection (1 at day 24 and 1 at three months, other time points were not reported) but unfortunately the PCR can not distinguish between wildtype HSV and HSV1716 (384). A phase I trial in which HSV1716 is used for treatment of sarcoma and neuroblastoma has been approved by the RAC (330).

### 4.2.2 G207

G207 is a modified HSV-1 virus that has a 1 kb deletion in both copies of the major neurovirulence gene γ34.5. Furthermore the UL39 gene that encodes for the ICP6 protein is disrupted by insertion of the lacZ coding sequence. ICP6 is the large subunit of the HSV-1 enzyme ribonucleotide reductase, which is necessary for viral replication in non-dividing cells.
G207 is non-toxic in HSV permissive mice and primates at high doses. In immunocompetent mice, G207 ($10^7$ pfu intracerebroventricular or $3x10^7$ pfu intrahepatic) caused no apparent side-effects. After inoculation in the brain no infectious G207 could be detected 4 days after injection but G207 DNA was still present 6 months after inoculation. No reactivation of latent HSV was observed after application of G207 in animals previously infected with wildtype HSV-1. This last issue was addressed because of the previous finding that avirulent herpes strains are able to generate lethal recombinants *in vivo* (109, 385). In owl monkeys, that are highly susceptible to HSV infection, single or repeated intracerebral inoculation of G207 (up to $10^9$ pfu) did not cause symptoms or detectable changes in the brain with a follow-up time of in some cases more than three years (128). Shedding and biodistribution of G207 ($3x10^7$ pfu) were evaluated after intracerebral inoculation in owl monkeys. Neither infectious virus nor viral DNA were detected in tear, saliva, or vaginal secretion samples up to 1 month (and in one case up two 2 years) post-inoculation. G207 DNA was only detected in the brain, and from brain or other organs no infectious virus could be recovered. Histopathology revealed that brain tissue, including the sites of inoculation, were normal ascertaining the safety of this application (386).
Intraprostate injection of G207 ($10^7$ pfu) in mice and owl monkeys did not induce signs of disease, pathology or morbidity. In monkeys no shedding or spread of infectious virus to other organs was observed using plaque assays. PCR analysis on DNA samples indicated limited spread of virus to neighbouring organs (penis, spleen and lymph nodes) in 1/4 monkeys (129).
Anti-tumour activity of G207 against a variety of tumours has been demonstrated, including malignant brain tumours, gastric cancer, prostate cancer, bladder cancer, breast cancer, pancreatic cancer, ovary cancer and head and neck cancer (11, 118-126).
Phase I and II trials with G207 for the treatment of malignant brain tumours have been initiated in the USA. In a phase I dose escalation trial G207 was intratumourally injected ($10^6$-$3x10^9$ pfu) in 21 glioma patients. No toxicity was observed. In 2/6 patients positivity for lacZ (PCR) was shown in biopsies taken 56 or 157 days post-inoculation. No shedding was observed: saliva or blood cultures taken 4 days to 1 year after inoculation were negative for recombinant HSV. No data about replication were reported. In some patients evidence suggestive of anti-tumour activity was observed (387). Long-term follow-up trials and new trials in which G207 is combined with chemotherapy for glioma treatment are underway (330).

### 4.2.3 NV1020

NV1020/R7020 is a replication-competent HSV-1 vector with an HSV-2 glycoprotein insertion that was genetically engineered to attenuate its pathogenic ability. The virulence of NV1020 is highly attenuated relative to the parental strain HSV-1 (F) due to the deletion of 15 kb spanning multiple genes; ICP0, ICP4, UL56, one copy of γ34.5, and a 700-bp deletion that prevents expression of the UL24 gene. NV1020 is sensitive to anti-viral drugs since it expresses an exogenous copy of the HSV-1 TK gene under the control of the viral α4 promoter. NV1020 kills colorectal cancer cell lines, *in vitro* and in mouse models (127, 137, 388). NV1020 replicates poorly in normal mouse liver tissue, and produces significant toxicity in the liver only at high doses (389). When injected intracranially in mice NV1020 was 7000-fold less neurovirulent than wildtype HSV. In owl monkeys injections of up to $1x10^9$ pfu into the liver or hepatic artery resulted in transient elevations of liver enzymes and local lesions but not in disseminated disease. Virus was isolated from tears, saliva or vaginal secretions for

up to 30 days depending on the route of administration (intra-ocular, intravenous or intravaginal)(130, 136).
NV1020 has been delivered as a vaccine candidate in humans. Subcutaneous delivery of up to $1x10^5$ pfu did not cause any serious adverse events (137).
NV1020 has been used in clinical phase I trials in the USA in patients with colorectal carcinoma metastasized to the liver. A single dose of $3x10^6$-$1x10^8$ pfu was infused via the hepatic artery in 12 patients. Mild transient adverse events related to treatment were observed (headache, pyrexia and rigors were most common). Three serious adverse events possibly related to the treatment were observed; leukocytosis, diarrhoea and a rise in liver enzymes. No DLT was observed and no MTD was established. In two patients reductions in tumour size (20-39%) were noted 28 days after treatment. Samples of saliva, urine, conjunctival and vaginal secretions were tested by PCR and culture assays throughout the course of the study. 1-60 minutes after infusion no virus was recovered from the hepatic vein by PCR in 7/12 patients receiving treatment at the lower dose levels (up to $3x10^7$ pfu) indicating viral clearance. Five patients receiving higher doses ($3x10^7$ - $1x10^8$ pfu) were positive by PCR, but from only one patient live virus was recovered. One patient was positive by PCR in a saliva sample taken at day 3 and two serum samples taken at days 4 and 6. No live virus was recovered from these samples and no symptoms of herpes infection were observed (137). In a phase I/II trial intra-arterial infusions of NV1020 (up to $1x10^8$ pfu every 4 weeks) were combined with standard chemotherapy. Several patients showed tumour regression and in one patient a significant response was observed. No DLT or MTD was reached (330, 390).

### 4.2.4 Oncovex$^{GM\text{-}CSF}$

Oncovex$^{GM\text{-}CSF}$ is based on the HSV-1 JS1 strain (a strain with increased oncolytic capacity compared to other HSV strains) and was made tumour-selective by deleting the genes encoding ICP34.5 (reducing the neurovirulence) and ICP47 in the HSV-1 genome and inserting the gene encoding for GM-CSF. Each of the last 2 modifications increased the oncolytic effect against tumour xenografts in nude mice compared to control vectors. Moreover, treatment with Oncovex$^{GM\text{-}CSF}$ protected against further tumour challenge in mice (153). In a phase I trial performed in the UK, Oncovex$^{GM\text{-}CSF}$ was administered intratumourally ($10^6$-$10^8$ pfu) to cutaneous metastases of various tumour types. Injection sites were protected with an occlusive dressing. Patients were discharged if they felt well and the 24h swab of the dressing and the injected lesion were negative. The virus was well tolerated with low grade systemic flu-like symptoms and local inflammation being the most observed side-effects. This last effect was more profound in patients that were HSV sero-negative before treatment. The DLT in this group was $10^7$ pfu at first treatment. In sero-positive patients (including initially sero-negative patients that became sero-positive during treatment) $10^8$ pfu was well tolerated also in a multiple dosing regimen, and no DLT was observed. The GM-CSF levels were below detection in the serum of all patients. PCR on tumour samples indicated increased GM-CSF expression in high-dose compared to low-dose patients. In the group receiving a single treatment, virus DNA was detected by PCR in blood and urine from 2/13 patients between 8 hours and 1 week post-injection. In 3 of these patients virus was detected at the tumour surface by plaque assay for up to two weeks. In 8 of 17 patients in the multiple treatment group, DNA was detected 1-8 hours after injection exclusively in the blood. In one patient of this group virus was detected at the tumour surface. Live virus was not detected by swabs at other sites (inflamed areas and lip sores appearing in one patient). In 6/26 patients a minor response (flattening of the tumour) was observed. In 14/19 tumour biopsies taken 3 weeks after the last injection necrosis correlating with HSV staining was observed. HSV staining was rarely seen in non-necrotic tumour tissue and even more rarely in normal tissue. Normal tissue showed no evidence of necrosis. Several phase I/II trials are planned in the USA with melanoma, head an neck cancer and pancreatic cancer patients (154, 330, 391).

### 4.2.5 HSV vectors in future and ongoing clinical trials

HSV-CYP2B1 (rRp450), a ribonucleotide reductase (RR) deleted HSV-1 mutant expressing the rat prodrug converting enzyme CYP2B1 has been reviewed by the RAC for use in phase I trials in patients with sarcoma / neuroblastoma (i.t. delivery) and liver tumours (i.a. delivery), respectively. Herpes simplex HF10, a naturally selected mutant herpes simplex virus, has been tested in clinical studies in Japan involving patients with recurrent breast cancer and pancreatic cancer. Intratumoural administration in breast cancer patients was well tolerated with no adverse effects and tumour regressions (30-100%) were reported (392).

## 4.3 Vaccinia vectors

### 4.3.1 Oncolytic vaccinia vectors

A wildtype low pathogenic live vaccinia virus vaccine (Dryvax; Wyeth strain) has been administered after revaccination, intravesicullary ($10^6$-$10^8$ pfu, 3 times) in 4 patients with bladder cancer. 24 hours after administration evidence of inflammation and viral infection was observed by IH on biopsies, both in tumour and normal tissue. No toxicity was observed and 3 of 4 patients remained disease free (393).
VV-IL2 (NYCBOH strain) has been administered intratumourally in 6 patients with mesothelioma ($10^7$ pfu weekly for twelve weeks). Culture and PCR on sputum, urine and blood samples indicated that virus was not excreted. Patients and contacts of patients did not show symptoms of VV infection or an increase in VV antibodies. VV-IL2 mRNA was detected in 4/6 tumour biopsies; expression was highest 1-3 days after injection and detected for up to three weeks. T-cell infiltrates were detected in 50% of biopsies. From one tumour biopsy live virus was recovered. No toxicity or response was observed in this study (394).
JX-594 is a TK deleted recombinant VV based on the New York City Board of Health strain (a low pathogenic strain from which the Wyeth strain was derived) containing GM-CSF and β-galactosidase inserts. Besides the inherent lytic and immune-stimulating nature of VV, GM-CSF further enhances the immune response by stimulating maturation of granulocytes and macrophages and increasing MHC antigen expression. 7 patients with incurable melanoma were revaccinated with wildtype vaccinia and received from day 4 after vaccination, intratumoural injections of JX-594 ($10^4$-$8x10^7$ pfu 3 times per week for up to 6 weeks). Injection sites were protected with an occlusive dressing. Revaccination led to major local reactions. Following injection with JX-594 systemic mild flu-like symptoms were observed. No change in hepatic, renal or haematological function was observed. Dose related inflammation of the injection sites with necrosis and infiltration of T-cells and macrophages occurred after injection. There were no cases of interpersonal transmission of wildtype or recombinant virus but it was not reported how this was established. A humoral antibody response against β-galactosidase developed after 3-6 weeks indicating *in vivo* replication. 3/3 patients were positive for GM-CSF PCR on tumour biopsies taken 18 hours after the first injection and after later injections (in week 5 or week 31). No change in serum GM-CSF levels was observed in 5 patients throughout treatment. Three patients showed temporal regression of tumours, 1 patient had a partial response and 1 patient a complete response. In 4 of 5 patients non-injected tumours also regressed (395).
JX-594 is currently in a phase I/II trial in patients with refractory liver tumours (i.t. delivery) that is being conducted in South-Korea. In this trial no serious adverse events were reported. All patients had mild flu-like symptoms and second waves of viremia were observed in 8/10 patients (15, 396). JX-594 is also in a phase II trial in melanoma patients. Moreover, the RAC has reviewed clinical protocols for use of JX-594 in lymphoma patients (by means of transduced tumour-infiltrating cytokine induced killer carrier cells) and in patients with solid tumours (intravenous administration) (330, 397).

Two vaccinia product candidates, JX-929 and JX-963, were scheduled to enter phase I clinical trials in 2007. JX-963 and JX-929 have been engineered from the vaccinia WR strain that is less attenuated compared to the NYCBOH strain used for JX-594 and which contains two deletions of viral genes that restrict replication to tumour cells with large nucleotide pools and/or cells with an activated Ras pathway. Furthermore JX-963 and JX-929 contain a GM-CSF and CD encoding transgene, respectively (15). The RAC has also reviewed a phase I clinical protocol in which a double deleted vaccinia virus expressing CD and the somatostatin receptor (vvDD-CD/SMR) will be applied in patients with superficially injectable tumours (330).

### 4.3.2 Vaccinia tumour vaccines

Recombinant live tumour vaccines are designed to produce either a tumour-specific T-cell response against tumour-specific antigens (e.g. CEA, MUC-1, PSA) and/or to enhance T-cell responses by insertion of co-stimulatory molecules (e.g. TRICOM, see below). All recombinant vaccinia viruses are based on the Wyeth NYCBOH strain that has the lowest incidence of clinical complications. Approximately 45 protocols making use of increasingly more complex recombinant vaccine tumour vaccines have been received by the RAC in the USA (330). None of these have been selected for RAC public review indicating that no novel scientific, ethical or safety issues were associated with their use. Besides in the USA, several clinical trials with recombinant vaccinia tumour vaccines have been performed in the UK. Only a selection of the published trials in this field is given, to show the developments in this field of research.
Recombinant vaccinia virus expressing PSA (RV-PSA) was administered intradermally to prostate cancer patients in different trials ($2.65x10^5$-$2.65x10^8$ pfu, three doses given at 4 week intervals plus or minus GM-CSF). Three grade 3 toxicities were seen in one trial (fatigue, fever, myalgia), but otherwise all toxicities were of a lower grade. T-cell responses against PSA were demonstrated and in one of the trials 14/33 men had stable disease for 6 months (398, 399). RV-CEA has been administered to 32 carcinoma patients ($10^7$-$10^8$ pfu, two doses intradermally, s.c or by scarification at 4 or 8 week intervals). Toxicities included local inflammation and low grade fever or fatigue. Seven patients developed CEA specific antibodies (400, 401). RV-B7.1 was given by monthly intralesional vaccination ($4.26 \times 10^7$-$4.26 \times 10^8$ pfu, two times). Only low-grade fever, myalgias, fatigue and vitiligo were reported. Patients showed an increase in T-cells specific to melanoma antigen. One patient showed a partial response and two patients had stable disease (402). The limited efficacy in these trials warranted the exploration of augmentation strategies like the prime-boost strategy. RV-CEA (one injection $10^7$ pfu intradermally) in combination with injections of avipox-CEA and GM-CSF/IL2 was well tolerated mainly inducing mild skin reactions and no other significant toxicity. In 8/11 patients CEA-specific T-cell responses were observed but no anti-tumour response was seen (403).
TRICOM is a recombinant VV expressing three co-stimulatory molecules (B7.1, ICAM-1, and LFA-3). In a phase I trial TRICOM was administered in patients with metastatic melanoma intralesionally ($5.1x10^6$-$5.1x10^8$ pfu, 3 times per month plus or minus IL2). Only low grade toxicities were observed (injection site pain, fatigue). All patients developed antibody and T-cell responses against the virus. 5/13 patients showed a partial response in the injected lesions. In 2/13 patients a partial response was observed in non-injected lesions (404).
VV-CEA-TRICOM has been administered as a tumour vaccine ($1.2x10^6$-$1.2\ x10^8$ pfu) by s.c. administration (in a combination treatment schedule with a recombinant fowlpox vector containing the same inserts, and plus or minus GM-CSF treatment) in patients with CEA expressing carcinomas. Mild toxicities (local inflammation, flu-like symptoms) were observed. Of the 58 patients treated one had a complete response and 11 had a decrease in or stable serum CEA levels. Enhanced CEA-specific T-cell responses were observed in 10/13 patients monitored (405). No replication or shedding data were presented in any report. In one report it was stated that infection of tumour cells by recombinant

vaccinia viruses results in a lytic process 7 hours after exposure, and that virus is generally cleared from patients within 5-7 days, especially following repeated exposure (402). Currently phase III trials in pancreatic cancer patients are being undertaken with PANVAC-V (a recombinant vaccinia virus expressing TRICOM, CEA and MUC-1) in a prime-boost regimen with fowlpox virus expressing the same transgenes and in combination with GM-CSF treatment. In a phase I trial this treatment was well tolerated and led to increased survival (406).
In the UK several trials have been performed with a live recombinant vaccinia strain encoding the human papilloma virus (HPV) E6 and E7 oncoproteins (TA-HPV) as a vaccine for treatment of ano-genital neoplasias. In total 119 patients were vaccinated using percutaneous scarification of the upper arm. Live virus could only be recovered from the vaccination site and the scab and dressings in immediate contact with the vaccination site, but not from the outside of the dressing. No virus was recovered from the throat or genital swabs, suggesting no systemic spreading. Patients were not isolated and only patients in household contact with at risk individuals were excluded from the study. The final scab (4 weeks after vaccination) was found to be positive for live virus in 5/12 patients in one of these trials. Treatment with TA-HPV was well tolerated and resulted in a limited number of clinical responses. TA-HPV has also been used in a prime boost regimen with TA-CIN, a recombinant HPV16 L2E6E7 fusion protein. There were no serious adverse events and clinical responses were seen in 5/29 women (17%) which is an efficacy comparable to previous studies with TA-HPV as a stand-alone agent (407-409).
Recombinant vaccinia and recombinant fowlpox vectors expressing the testis antigen NY-ESO-1 have been tested in Germany in a phase I trial in patients with various tumour types. These vaccinations were well tolerated and induced NY-ESO-specific immune responses (410). A trial with a vaccinia strain containing sequences encoding for MUC-1 (a carcinoma antigen) and IL2 (a growth factor for T-cells) has been performed in the UK and a phase II trial with PANVAC-VF in patients with colo-rectal cancer is currently being conducted.

## 4.4 Trials with other recombinant viruses

### 4.4.1 Measles virus

Recombinant MV vectors are in three phase I trials at the Mayo clinic in the USA. MV-NIS, a recombinant measles virus expressing the humane sodium iodine symporter, is being applied intravenously in presence or absence of cyclophosphamide with subsequent $I^{123}$ imaging in patients with multiple myeloma (330, 411). MV-CEA is being tested in patients with recurrent glioma (i.t.) and ovarian cancer (i.p.) (330, 412, 413). 3/15 ovarian cancer patients have shown a partial response in the last trial. Viral doses of $10^3$-$10^7$ TCID50 were well tolerated. Viral genomes were detected in peripheral blood mononuclear cells but not in the urine or sputum (414).

### 4.4.2 VSV

The FDA has approved a clinical trial with live-attenuated recombinant VSV expressing HIV env and gag genes for use as an AIDS vaccine vector (415).

### 4.4.3 Poliovirus

A clinical protocol in which glioma patients are treated i.t. with a recombinant poliovirus (PVS-RIPO) was reviewed by the RAC in 2005 (12) and is currently under FDA review. PVS-RIPO contains the IRES of human rhinovirus type 2 (HRV2) in a poliovirus type 1 (Sabin) background resulting in a reduced neurovirulence in pre-clinical models.

## 4.5 Trials with wildtype viruses

### 4.5.1 NDV

PV701 is a replication-competent naturally attenuated mesogenic strain of Newcastle disease virus. In preclinical testing, PV701 demonstrated efficacy against a wide array of tumour cell types and induced a high percentage of complete regressions in a variety of solid tumour models. In phase I trials PV701 has been administered intravenously in patients with advanced solid cancers. In the first trial, 79 patients were treated under multiple regimens for multiple cycles, receiving up to 6 doses of PV701 in the first (two) weeks of a 28 day cycle (1 time $12x10^9$ pfu and 5 times $120x10^9$ pfu) or 3 doses in a 21 day cycle (first injection $12x10^9$ pfu and subsequently 2 times $144x10^9$ pfu). Several grade 3 and 4 events occurred during the first cycle. Adverse events decreased with subsequent dosing. The MTD was $120x10^9$ pfu after the first dose of $12x10^9$ pfu. 1% of 821 sputum samples and 15% of 899 urine samples from 67 patients tested positive for infectious virus. No sputum samples were positive at day 14 of the first cycle or beyond. Three weeks after the last dose of the first cycle all tested urine samples were negative. During cycles 2 to 6, 5% of urine samples were positive for PV701. While during the first cycle the urine of 54% of the patients was positive this was 0% from cycle 7 onward. A tumour biopsy taken two weeks after the eighth cycle showed evidence of viral presence in tumour tissue by EM. Nine of 62 patients showed a partial or minor response (tumour regression) (270). In a second trial, doses were escalated from $1x10^9$ to $120x10^9$ pfu during each 21 day cycle of six doses. Grade 1 and 2 flu-like symptoms were the most common adverse event and grade 3 transient leukopenia was observed in ~30% of patients. Infectious virus was recovered from urine in 19% and 7% of the samples from cycle 1 and cycle 2, respectively. Cycle 3 samples and beyond were negative indicating that there was no persistent shedding. In one of 16 patients tumour regression was observed (416). In a recent trial it was shown that toxicities were lower upon slower infusion of virus in a two-step desensitization protocol (417).

NDV-HUJ is a newly isolated lentogenic strain of NDV that has been reported to have a selective cytopathogenicity for human and animal cancer cell lines and that primarily induces apoptosis. Normal fibroblasts were not affected by this virus *in vitro*. NDV-HUJ was tested in phase I trials in patients with glioma in Israel. Intravenous administration (11-55 billion particles) induced only grade 1 and 2 adverse events and no MTD was established. One of 14 patients had a clinical response. Infectious NDV particles were recovered from 5/5 patients from blood, urine and saliva during the first dosage cycle and in 9/10 blood samples obtained 9 days after the last dosage (from the previous cycle). One tumour biopsy sample was positive by IH (271).

MTH-68/H, a live attenuated mesogenic oncolytic strain of the Newcastle disease virus has been administered to 14 patients with untreatable glioma intravenously (and in previous studies by inhalation) at doses up to $2.5x10^8$ pfu for up to five times a week. No adverse effects were observed. Treatment resulted in survival rates in 4 patients of 5-9 years as opposed to the expected six months to one year. Partial responses (tumour regression) were observed in some of the patients. Continuous administration of MTH-68/H in combination with the antineoplastic agent valproic acid led to temporal

tumour regression in a twelve year old boy with glioma and virus particles were detected in tumour cells (418-420). A phase II trial with this virus was started in 2006 (421).

### 4.5.2 Reovirus

Reolysin (Dearing strain) is being tested in several trials in patients with glioma, solid tumours and skin cancer in the UK and Canada (16). Intratumoural delivery of reovirus (two doses of $10^8$-$10^{10}$ TCID50) in combination with radiation therapy in patients with various tumours was well tolerated with no DLT. No evidence for viral shedding (in blood, urine, stool, sputum) was observed at any stage. 3/18 patients have shown partial responses (16, 422). Stereotactic administration ($1x10^7$-$1x10^9$ TCID50) of reovirus in glioma patients was well tolerated. No grade 3/4 responses and MTD were reached and no objective responses were observed. Phase II studies are planned (16, 423, 424). In a phase I trial in patients with subcutaneous tumours i.t. reovirus ($10^{10}$ pfu) caused no adverse events. 61% of the patients demonstrated a (variable) response (32% - complete tumour regression), in some cases in remote tumours. In a phase II trial 4 of 6 patients showed apoptosis of tumour tissue following intraprostate injection of reovirus. No toxicity was observed and replication was restricted to tumour tissue (16, 307). Intravenous reovirus administration in patients with solid tumours was well tolerated with mostly grade 1-2 toxicities and transient grade 3 toxicities. No MTD was reached (maximum dose was $3x10^{10}$ TCID50 for 5 days or $3x10^{11}$ TCID50 for 3 days). No shedding was observed in blood, urine, stool or sputum by reverse transcriptase PCR immediately or one week after administration. 4/33 patients showed partial responses and tumour necrosis associated with viral replication was observed in one patient. 7/33 patients showed signs of stable disease. Phase II studies are being planned (16, 425). In a phase I trial of systemic reovirus (maximum dose $3x10^{10}$ TCID50 daily) performed in the USA stable disease was observed in 8/18 patients with advanced tumours. One patient with progressive breast cancer showed 28.5% shrinkage of tumour volume. Toxicities were generally mild (grade 1 and 2) (16). Characterization of the immune responses during a phase I trial of intravenous reovirus in patients with advanced tumours (up to $3x10^{10}$ TCID50 on 5 consecutive days of a 4 week cycle) demonstrated that even heavily pre-treated patients have a considerable immune response, including increases in neutralizing anti-reoviral antibodies. The data indicate that future studies should be biased at rapid repeated administrations before mounting of the adaptive immune response and at modulating the immune response by administration of immunomodulating substances like CPA (426).
Various other trials using reovirus either as monotherapy or in combination with chemo-, radio- or immunotherapy are ongoing or are being filed (16).

### 4.5.3 Other wildtype viruses

Several other non-recombinant oncolytic viruses are currently in, or will soon enter clinical trials. Two picornaviruses have entered phase I clinical trials: CVA21 is in a phase I trial in glioma patients in Australia (427) and Seneca valley virus (SVV-01), a neurotropic apparently non-pathogenic virus, is being tested in a phase I trial in patients with solid tumours with neuroendocrine features in the USA (428). In this trial, after i.v. infusion of SVV-01, in one of the patients, a 10,000 fold amplification of the input dose was observed with evidence for viral replication in tumour but not surrounding tissue. Little or no replication was observed in other patients. No DLT has been observed (429). A phase I/II trial with Parvovirus H1 in glioma patients in Germany has been announced.

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