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REVIEW

Non-human viruses developed as therapeutic agent for use in humans

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SUMMARY

Viruses usually infect a restricted set of host species, and only in rare cases does productive infection occur outside the natural host range. Infection of a new host species can manifest as a distinct disease. In this respect, the use of non-human viruses in clinical therapy may be a cause for concern. It could provide the opportunity for the viruses to adapt to the new host and be transferred to the recipient's relatives or medical caretakers, or even to the normal host species. Such environmental impact is evidently undesirable. To forecast future clinical use of non-human viruses, a literature study was performed to identify the viruses that are being considered for application as therapeutic agents for use in humans. Twenty-seven non-human virus species were identified that are in (pre)clinical development, mainly as oncolytic agents. For risk management, it is essential that the potential environmental consequences are assessed before initiating clinical use, even if the virus is not formally classified as a genetically modified organism. To aid such assessment, each of these viruses was classified in one of five *relative environmental risk* categories, ranging from "Negligible" to "Very High". Canary pox virus and the *Autographa californica* baculovirus were assigned a "Negligible" classification, and Seneca Valley virus, murine leukemia virus, and Maraba virus to the "High" category. A complicating factor in the classification is the scarcity of publicly available information on key aspects of virus biology in some species. In such cases the relative environmental risk score was increased as a precaution. Copyright © 2011 John Wiley & Sons, Ltd.

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INTRODUCTION

Already in the 19th century, the observation was made that cancer patients who contracted an infectious disease occasionally went into brief periods of clinical remission. It was recognized that contraction of influenza sometimes produced beneficial effects in leukemic patients. These early observations predate the formal demonstration that influenza was, in fact, caused by a virus. In the 1950s clinical studies were initiated in which a range of human viruses was administered to cancer patients, including HBV, West Nile virus, adenovirus, and mumps virus. The clinical experience with this approach has been discussed in several excellent and extensive historic reviews [1–3].

Although some anecdotal evidence of antitumor efficacy was obtained, the side effects were severe. In an effort to reduce the side effects and to circumvent the inhibitory effects brought about by neutralizing immunity, several non-human viruses entered the stage, including Newcastle disease virus and vesicular stomatitis virus. However, after the advent of new cytostatic drugs and the problematic attenuation of some candidate viruses, the oncolytic virus approach was largely abandoned.

The rise of gene therapy as a clinically feasible approach for treating human diseases led to a revival of the oncolytic virus therapy approach. The hope of the safe and efficacious use of viruses as anticancer agents has been fuelled by the (largely) incidental evidence of antitumor efficacy as well as by the history of safe use of several live virus vaccines.

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Abbreviations used:
CD, cytosine deaminase; ERA, environmental risk assessments; GMO, genetically modified organisms; LTR, long terminal repeat; MTD, maximum tolerable dose; MuLVs, murine leukemia viruses; PERV, porcine endogenous retrovirus; RCR, replication-component retroviral; RER, relative environmental risk; SVV, Seneca Valley virus.

Moreover the recombinant DNA technology offers effective systems for reverse genetics in many virus families. This has allowed the design of new and potentially more efficacious oncolytic viruses, and even arming the replicative oncolytic viruses with therapeutic transgenes. In parallel, there has been a continued and expanding interest in the use of non-human viruses for oncolytic virus therapy. There are two main reasons for this interest. The first is the absence of immunity against non-human viruses in the human population. This may provide the viral oncolytic agent a head start by allowing the therapeutic virus to be administered without the risk that the pre-existing neutralizing immunity inactivates the vector and frustrates transduction of tumor cells. The other reason is that many non-human viruses have not been associated with diseases in humans. In addition, recombinant DNA technology offers the prospect of overcoming limitations imposed by viral pathogenicity in humans.

These developments brought back the bio-safety issue. With regard to biosafety three risk categories can be distinguished. Firstly, during the preclinical phase, there are the hazards associated with handling the viruses in the preclinical evaluation program. The level of containment required for such activities is dependent on the biosafety classification of the virus that is handled. The guidelines that currently exist for such "contained use" activities require the use of dedicated laboratories. Such guidelines are standardized and widely adopted.

In clinical applications, there are two distinct categories of risks. The first and most visible is the category of risk associated with the (potential) hazard to the patient who receives the viruses as (part of the) therapy. Here the procedure for a proper assessment of the risks and (potential) benefits should not differ greatly from more conventional medical interventions. However, in addition to the patient's risk, the fact that the therapeutic agent is infectious and can potentially be shed from the patient bears the possibility of horizontal transfer of the viral therapeutic agent [4,5]. The latter risk category in the clinical use of viruses is summarized by the term "environmental risk". For the use of oncolytic viruses, the environmental risk encompasses the chance and severity of undesirable effects that result from the clinical use of the therapeutic viruses on humans other than the patient (viz. any person not willfully exposed to the viral therapeutic agent, such as medical personnel, the patient's relatives, or other patients), or on susceptible animals (e.g. pet animals, livestock, poultry, or wildlife). The variety of potential environmental hazards and undesirable consequences is wide, and ranges from pathological effects (e.g. outbreaks of infectious disease on a short time scale to tumor-formation upon exposure to murine leukemia viruses (MuLV) on a long-time scale) to economic consequences (e.g. the risk of a country losing its Aujeszky's disease-free status if suid herpesvirus 1 would be shed from a patient.) The use of oncolytic viruses therefore requires containment procedures to reduce such transfer and minimize the risks associated with it. So far, to the best of our knowledge, no general guidance exists for estimating and evaluating the environmental risks associated with the clinical use of non-human viruses as therapeutic agent, for example in cancer therapy. The absence of guidelines for assessing the environmental risks seems at odds with the specific regulation that is in place for instance in xenotransplantation [6].

The critical shortage of human donor material for transplantation prompted the use of animals as an alternative source of cells, tissues, and whole organs for transplantation into humans, that is, xenotransplantation. Non-human primates and pigs are the two main sources of cells and tissues. The use of non-human primates is constrained by ethical considerations, their small anatomical size, and their limited availability. Pigs are considered the most suitable alternative source. The pigs have large anatomical and physiological similarity with humans such as the size of the organs and the circulatory system. In addition, pigs can be bred in specific pathogen-free and genetically homogeneous herds.

A major concern in xenotransplantation is the potential transfer of animal pathogens to the human recipients. Especially the porcine endogenous retroviruses provide a potential hazard. The risk of cross-species transmission may be increased by the immune suppression that is applied following the transplantations. This led to a precautionary approach and instigation of moratoria on clinical trials involving xenotransplantation in many countries [6].

A CATALOGUE OF NON-HUMAN VIRUSES FOR THERAPEUTIC APPLICATION

To establish the status of the use of non-human replication-competent viruses for therapeutic applications such as oncolytic-virus therapy, we have

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performed a literature study [7]. This yielded a list of 27 different non-human virus species that are being considered for (future) clinical application. Only non-human viruses and vectors that are replication-competent in unmodified cells of their natural host are listed. Replication-defective viruses, that is, viruses and vectors that require special helper functions for their replication, and are therefore replication-incompetent in unmodified cells of their natural host, are excluded. The reason for excluding the latter viruses and vectors is that they have an additional level of containment because they must re-acquire the genetic information to allow functional complementation of the missing function.

The results of the study are summarized in Table 1. The table also summarizes the stage to which the research has progressed for each of the viruses identified. Viruses that have been developed for use as conventional vaccines are excluded from this study. For the development of such vaccine viruses clear guidelines and criteria have been established.

The therapeutic viruses are in various stages of development. At least seven of these have already been evaluated in formal clinical studies (i.e. canary pox virus, infectious bursal disease virus, Newcastle disease virus, Seneca Valley virus (SVV), Sindbis virus, Semliki Forest virus, and murine leukemia virus). Several others have been announced to follow soon. From these data it is reasonable to assume that the next few years will witness a rising number of clinical trials in which non-human viruses are used as therapeutic agents.

BIOSAFETY AND ENVIRONMENTAL RISKS

The widespread clinical use of these viruses may be associated with a small but finite risk of undesirable viral adaptation or zoonotic infections in humans. Cancer patients frequently have a compromised immune function [8]. This may allow the therapeutic viruses to replicate for prolonged periods of time and eventually adapt to humans. If the virus would have, or would acquire, the capacity to spread horizontally from the patient to health care workers or the patients' relatives, it could become established in the human population. Although the authors are not aware of any examples of outbreaks of viruses as a consequence of intentional exposure of humans, recent history has provided several examples of spontaneous cross-species transfer and establishment in a new host. Examples are discussed in detail elsewhere [5] and include, for example, the recent H1N1 "Swine Flu" influenza A virus [9], the SARS-corona virus [10] and in the more distant past, the HIV lentiviruses [11,12].

It is the risk of the transfer and adaptation of non-human viruses to human hosts, which led many countries to impose a moratorium on the clinical use of xenotransplantation, and more specifically the clinical use of porcine tissues. Although there is a wide consensus on a moratorium on xenotransplantation, there seems to be no specific guidance for developing clinical strategies involving the deliberate administration of non-human viruses with a therapeutic intent. The formulation of guidelines to ensure appropriate assessment of risk to the patient and the environment may be suitable to date. Such guidelines could come either as "points to consider" for the investigators or as more formal regulation. In this regard it is relevant to recognize that many of the viruses that are being developed are not by definition "genetically modified organisms" (GMOs). Hence their use may not demand the environmental risk assessments that are part of the formal national or international (e.g. European) GMO regulations. Also, the EMEA ICH Considerations document "Oncolytic viruses" (EMEA/CHMP/ICH/607698/2008) does not provide guidance relating the management of environmental risks: while it advises to consider barrier contraception for the duration of the clinical trial as a standard precaution to prevent person-to-person transmission, it does not address other environmental risks. It merely states "Many of these considerations might fall under the heading of environmental release/risk and regional authorities should be contacted for details."

Nevertheless, the GMO guidelines may provide a scaffold for formulating guidelines for environmental risk assessments (ERA) for the use of non-GMO non-human viruses for clinical trials. The assessment of patient-safety aspects may adopt some of the quality guidelines that have been established for evaluating human vaccine safety. The elements that need to be considered in the ERA are very similar with GMO viruses and non-GMO viruses. Figure 1 shows a number of topics relating to the therapeutic viruses and the intended use that, if pertinent, should be covered in the ERA.

As outlined above, a large number of non-human virus species is being developed for clinical

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Table 1. Non-human viruses: Overview of current oncolytic virus candidates, their progress towards clinical applications, and the relative environmental risk associated with their use in humans

Family	Genus	Species	Stage of research activities[2]	Factors that *increase*/ decrease the relative environmental risk	Relative environmental risk[3]	Reference[4]
Poxviridae	Orthopoxvirus	Raccoonpox virus	2	**No known pathology in mammals, safely used as vaccine, only mild pathology seen in accidental human infections**	Low	[37]
	Leporipoxvirus	Myxoma virus	2		Medium	[38]
	Yatapoxvirus	Yaba-like disease virus	1		Medium	[39]
	Avipoxvirus	Canarypox virus	3	**No productive infection in human cells**	Negligible	[40]
Herpesviridae	Rhadinovirus	Bovine herpesvirus 4	2		Medium	[41]
		Herpesvirus Saimiri	2		Medium	[42]
	Varicellovirus	Bovine herpesvirus 1	1	**Virus circulates world wide, no known pathology in humans despite intensive contact with cattle**	Low	[43]
		Suid herpesvirus 1	1	*Aujeszky's disease-free status can be jeopardized*	Medium	[44]
Baculoviridae	Baculovirus	*Autographa californica* baculovirus	2	**No productive infection in human cells**	Negligible	[45]
Parvoviridae	Parvovirus	Rodent parvovirus H-1 and Minute virus of mice	2	**Causes mainly subclinical infections in rodents**	Low	[46]
		Feline panleukopenia virus[1]	1		Medium	[47]
Birnaviridae	Aribirnavirus	Infectious bursal	2		Medium	[48]

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		disease virus				
Reoviridae	Orbivirus	Bluetongue virus 10	1	**Transmission via insect vector**	Low	[49]
Paramyxoviridae	Avulavirus	Newcastle disease virus	3		Medium	[50]
	Respirovirus	Sendai virus	2		Medium	[50]
Rhabdoviridae	Vesiculovirus	Vesicular stomatitis virus	2	**Transmission via insect vector** *Aerosol-mediated transmission to humans*	Medium	[51]
		Maraba virus	2	**Transmission via insect vector,** *Little information available on natural host and disease associations*	High	[14]
Coronaviridae	Coronavirus	Murine hepatitis virus[1]	2		Medium	[52]
		Feline coronavirus	1		Medium	[53]
Picornaviridae	Enterovirus	Bovine enterovirus	2		Medium	[54]
	Senecavirus	Seneca Valley virus	3	*Very little information available on natural host and disease associations*	High	[19]
	Cardiovirus	Encephalomyocarditis virus	2		Medium	[55]
Togaviridae	Alphavirus	Sindbis virus	2	**Transmission via insect vector**	Low	[56]
		Semliki Forest virus	2	**Transmission via insect vector**	Low	[57]
Retroviridae	Gammaretrovirus	Moloney murine leukemia virus (MuLV)	3	*Little information on amphotropic MuLV in primates, amphotropic MuLV demonstrated to be oncogenic in non-human primates.*	High	[24]
	Spumavirus	Foamy virus	2	**No known disease associations**	Low	[58]

[1]The virus requires artificial modification of the host range as the wild type virus is unable to infect human cells.
[2]The stage indicates the phase to which the research has progressed (1, preclinical, *in vitro*; 2, preclinical, *in vivo*; 3, clinical studies).
[3]The arbitrary relative environmental risk is scaled as Negligible, Low, Medium, High, and Very High. The scale represents the authors' estimate of the environmental risks assigned to the clinical use of the viruses based on the aggregate of biological parameters as described [7]. The wild-type viruses are assessed, and the standard classification is "Medium". The default class was adjusted if there are factors that positively or negatively affect the risk for the environment. If the publically available information was found inadequate, the score was increased to provide caution.
[4]A single reference is included as example of research activities.

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To evaluate the environmental risks involved in clinical use of non-human non-genetically modified viruses it is suggested that the ERA provides information on the following questions:

Virological and biological parameters:

- Is the virus replication-competent in human cells?
- Does the virus produce infectious progeny virus in human cancer cells?
- Does the virus produce infectious progeny virus in human non-cancerous cells?
- Does the virus cause viremia in humans?
- Is the replication of the virus restricted in humans, in such a way that it provides a level of biological containment?
- Is the therapeutic virus an attenuated derivative of a naturally circulating virus?
- Can the therapeutic virus interfere with other viruses that may be present in the treated patient (for example through recombination, (re)activation, immunosuppression) as to cause adverse effects for health or spread?
- Is there evidence of prior exposure in humans and what were the consequences?

Parameters relating to intended clinical use:

- Can the virus be shed from the patient with the proposed clinical use?
- Can the virus shed from the patients infect susceptible host species?
- Can the virus adapt itself in such a way that the capacity of the virus to spread beyond the treated patient is positively affected?

Parameters relating to potential consequences:

- What can be the potential consequences of unintended exposure of non-target humans?
- What can be the biological consequences of unintended exposure of the non-human susceptible hosts?
- What are the potential economic consequences of unintended exposure of the non-human susceptible hosts?

Parameters relating to risk management:

- Is a vaccine available that can be used for prophylactic treatment in humans?
- Is a vaccine available that can be used for prophylactic treatment in the normal host species?
- Are effective antivirals available that can be used for treatment of virally infected humans?

Figure 1. List of topics relating to the therapeutic viruses and the intended use that should be covered in the environmental risk assessments (ERA)

application. Within these virus species there can be marked differences in relevant parameters between different isolates and serotypes. In addition, from many of these viruses, attenuated derivatives were isolated and have been used as vaccines. Vaccine strains can have properties that differ significantly from the wild-type viruses. It is noteworthy that frequently published literature provides insufficient information on the serotype, strain, or isolate that was used in the study. This can frustrate the use and extrapolation of these data.

RELATIVE ENVIRONMENTAL RISK OF THE VIRUSES

The biological characteristics of viruses can differ markedly. To be able to rank the viruses for the potential hazards associated with their clinical use, we have assigned a *relative environmental risk* score to all the virus species that have been considered for clinical use. This score is based on several factors, including the capacity of the virus to replicate productively in human cells, the potential for amplification and shedding of the virus, the potential to be

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transferred horizontally, the occurrence of this virus, and its pathogenicity. In addition, the potential consequences of virus shedding into the natural host species are taken into account. The availability of registered vaccines may be advantageous to protect susceptible hosts in the (unlikely) situation that shedding leads to infection of non-human host species. This classification is intended and should be read as an indicator of the potential environmental hazard associated with the virus, which is independent of the application. The *absolute* or *actual environmental risk* will eventually depend on the application and this can be reduced for instance by containment measures.

The classification uses five categories for relative environmental risks: "Negligible, Low, Medium, High, and Very High". The "Medium" category was used as a start point in the classification, and the assignment was scaled up or down based on factors that could strongly affect the relative environmental risk. The most important of these are included in Table 1. None of the viruses were classified in the "Very High" relative environmental risk category, whereas three viruses are classified as "High". Furthermore, two viruses are scored as "Negligible". The latter concerns the *Autographa californica* baculovirus and the canarypox virus. Three viruses are placed in the "High" risk category: Maraba virus, Seneca Valley virus (SVV), and Murine Leukemia virus (MuLV). The proposed use of the viruses classified as "High" in the relative environmental risk scoring and a brief summary of their biosafety aspects are presented in the following sections. A more comprehensive description of all non-human viruses developed for clinical use, the research activities herewith, and a motivation for the classification has been described elsewhere [7].

Viruses placed in the "High" relative environmental risk category

Maraba virus

Maraba virus belongs to the vesiculoviruses group of the *Rhabdoviridae*. A single strain of Maraba virus (BeAr 411459) was isolated from a pool of 70 female phlebotomine sand flies (*Lutzomyia* spp.) captured from tree trunks in Serra Norte, municipality of Maraba, Para State, Brazil in 1983 [13]. Animals and human sera collected at the time from the same region (the Amazon basin of Brazil) were tested for the presence of neutralizing antibodies. Only a single human sample tested positive for antibodies against Maraba virus, but it should be noted that the region from where the virus was isolated has relatively few human inhabitants [13].

The virus can replicate in sand flies following experimental intrathoracic inoculation and it can be transovarially transmitted in those flies. At present it is not known if the Maraba virus can cause disease in humans. However, it kills newborn mice within 24 h after intracerebral inoculation. It can also be lethal to adult mice, but not if administered intraperitoneally. The Maraba virus is antigenically closely related to the VSV-Indiana, Cocal, and Alagoas viruses, which are known to cause vesicular disease in cattle and swine. There are no records of the Maraba virus being experimentally administered to domestic animals.

Maraba virus as an oncolytic agent

With the aim to expand the current array of safe and potent oncolytic viruses, Brun and colleagues [14] screened a variety of rhabdoviruses on a panel of tumor cell lines. A number of viruses exhibited varying degrees of cytolytic activity, with Maraba virus being the most potent of the 20 viruses tested. The Maraba virus demonstrated good cytolytic activity against various tumor cell lines (37 cell lines from the NCI 60 cell panel). Furthermore, the Maraba virus replicated productively and killed breast, CNS, colon, melanoma, lung, ovarian, prostate, and renal cancer cell lines.

The Maraba virus is efficacious in syngeneic and xenogeneic tumor models. Animals that received six systemic doses of Maraba MG1 (selected mutant, see the following sections) responded to treatment with complete tumor regression and durable cures in 100% of the animals. Complementary to those studies, Brun and colleagues performed tests using immunocompetent animals bearing human ES2 ovarian xenografts [14]. Even at a very low dose (10^4 pfu), animals treated with Maraba MG1 had significant reduction in tumor burden. In these studies, the Maraba MG1 is more efficacious than the wild-type Maraba virus.

Bio-selection and genetic modifications

A system was developed for genetic modification of Maraba virus by reverse genetics [14]. Several recombinant strains of the Maraba virus were developed as potential therapeutic vectors. The authors explored two mutations previously identified in VSV that

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improved VSV replication on BHK-21 cells (resulting in L123W in the M protein, and H242R in the G protein). Similar changes were introduced at the homologous positions in Maraba virus genome, by altering the codon for L123W in the M gene and Q242R in the G gene, respectively. The double mutant (referred to as the Maraba MG1) showed no impairment in replication, but Maraba MG1 is attenuated on primary human skin fibroblasts and it remained strongly lytic on a panel of malignant cell lines [14].

There are no reports on the use of the Maraba virus as oncolytic agent in humans. The Maraba virus was well tolerated following intravenous injection of immunocompetent Balb/C mice [14]. Maximum tolerable doses (MTDs) for the Maraba WT and several attenuated strains were also determined. The MTD of the Maraba MG1 mutant was 100-fold greater than the WT virus. At doses below MTD, mice generally showed transient weight loss and dehydration, which resolved within 3–4 days post-infection. No virus could be detected in the brains of these mice euthanized 12 days later.

Environmental risk assessment/biosafety

It remains to be determined if the Maraba virus can be transmitted to humans who come into close contact with infected animals or by insect vectors. Despite the fact that the virus probably relies on an insect host for transmission between susceptible mammals, we classify the relative environmental risk as "High" based on the uncertainties on the biology and pathology of the Maraba virus in mammalian hosts and humans.

Seneca valley virus

The road for developing a particular virus to a clinically applicable product is usually a long one. A virus that made a remarkably fast progress toward clinical use is SVV. This virus was first identified in 2002 as a contaminant in the cell culture medium [15]. SVV is the first member of a new genus in the *Picornaviridae* called Seneca virus. This genus is proposed to include other porcine picornaviruses that share similarity in sequence and biochemical properties with SVV [15–17]. SVV was isolated at Genetic Therapy Inc. (Gaithersburg, MD) in 2002 from cell culture media as a contaminant during cultivation of PER.C6 cells [15,18]. It is presumed to be introduced via bovine serum or porcine trypsin.

Between 1988 and 2005, 12 picorna-like viruses were isolated from pigs showing a variety of clinical symptoms in various locations across the USA. Subsequent studies demonstrated these to be closely related to SVV. This information, coupled with the isolation of members of SVV in pigs, supports the hypothesis that pigs and possibly other farm animals are natural hosts for SVV.

Analyses of serum samples obtained from the general population, as well as from farmers yielded only a single sample that contained a low-titered neutralizing antibody to SVV. These data indicate that exposure to SVV is not prevalent in the human population.

Although SVV does not infect humans, it can be propagated in human tumor cells showing neuroendocrine features. The cytolytic potential and selectivity of SVV was determined in neuroendocrine and pediatric tumor cell lines and normal cells [19]. SVV was found to be strongly cytotoxic to especially small-cell lung cancer cell lines and pediatric solid tumor cells. The virus may be suitable for intravenous delivery in humans as the virus is not prone to inhibition by components in human blood [15]. These properties are being exploited for developing SVV as an oncolytic agent [15,19,20].

Current status and stage of the research activities

Recently a phase-I trial evaluated the safety of SSV in a dose escalation study in which the virus was administered intravenously to patients with advanced cancers. No dose-limiting toxicity was encountered. There are currently three clinical studies evaluating the safety and activity of SVV, including a study in young patients 3–21 years of age.

Bio-selection and genetic modifications

To evaluate the ability of SVV adaptation to replicate in non-permissive cells, Reddy and colleagues [15] performed experiments where the virus was passaged intentionally three times in non-permissive cell lines A549, H460, and Hep3B. No progeny virus was produced, suggesting that SVV did not change its tropism. In addition, no antibody-escape mutants of SVV were produced in PER.C6 cells when the virus was grown with media containing anti-SVV mouse hyper-immune serum [15]. Although these data suggest that the genome of SVV is stable, it should be noted that similar to other RNA viruses, the genetic variability of picornaviruses is

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very high, and it is likely that new quasispecies are frequently generated [21].

Horizontal transmission and establishment in the human population

To determine whether SVV can be horizontally transmitted, a study was performed in mice, which were injected with SVV and housed mixed with naïve mice. After 30 days, no naïve mice seroconverted, providing evidence for the absence of horizontal virus transmission. Interestingly, analyses of viral sequences from the various isolates fall on an evolutionary time line. This suggests that all the viruses had a recent common ancestor, possibly originating from the early 1980's. Since the viruses were isolated from samples collected in diverse geographic areas of the USA, the results are suggestive of a recent introduction of this virus into the US porcine population [22]. Based on these observations, the introduction of SVV in human population through medical treatment has to be cautiously monitored.

Environmental risk assessment and biosafety

It is clear that SVV has been found in pigs in the USA. However, attempts to infect pigs with two field isolates failed to reveal any pathology. Importantly, phylogenetic studies suggested that the virus may have only recently been introduced into pig population. Although it is possible that SVV exists in porcine populations elsewhere in the world, it is also conceivable that the virus entered the US porcine population from another host. Such alternate host could be a rodent species, since SVV's closest relatives, the cardioviruses, are viruses of rodents.

Despite the availability of preliminary safety data from the initial clinical studies, the limited information on the susceptibility of other mammalian species to SVV, the uncertainties about its natural host, and the absence of shedding data in published literature led us to classify the SVV as "High" in the relative environmental risk score.

Murine leukemia virus

The MuLVs belong to the genus Gammaretrovirus. MuLVs are widely distributed in domestic and feral mice. All mouse strains carry genetic information of MuLV-related viruses (endogenous viruses) in their genomic DNA. Endogenous viruses are the product from rare germline infections and result in Mendelian transmission of the integrated proviruses to all progeny. Most endogenous MuLV proviruses are replication-defective, although some inbred mouse strains carry and spontaneously activate replication-competent endogenous viruses.

The host range of MuLV is controlled in part by the interaction of envelope glycoprotein with the cell surface receptor. On the basis of cell surface receptor specificities, different classes of MuLVs have been identified so far. The ecotropic MuLVs are capable of infecting mouse and rat cells in culture. Non-ecotropic MuLVs may be xenotropic (from *xeno*, "foreign", infecting non-mouse species), amphotropic, or modified polytropic (infecting a range of hosts including mice).

The MuLVs induce leukemias in mice with latencies ranging from 2 to 18 months, depending on the strain of virus and strain of mouse. Neonatal infection is by far the most efficient means of leukemogenesis, whereas infection of adults is not leukemogenic for most viruses. The leukomogenesis is a multistep process and long terminal repeat (LTR) activation of proto-oncogenes probably supplies only one step in the process. For example, Moloney-MuLV induces T lymphoma in mice and rats by provirus insertion and activation of one of a particular set of proto-oncogenes.

Current status and stage of the research activities

Although most activities involve the use of replication-defective retroviral vectors, a replication-competent MuLV is also being developed as vector platform for delivery of the gene encoding the prodrug-activating cytosine deaminase (CD) to tumors. Here the virus's tumor–cell specificity relies on MuLV's dependency on active cell proliferation for productive infection. Once expressed in the cancer cell, the CD enzyme can convert the prodrug 5-FC to the anticancer drug 5-FU, thus sensitizing the tumor to 5-FC.

Pre-clinical studies: replication-competent retroviral vectors assessment *in vivo*

MuLV-based replication-competent retroviral (RCR) vectors have been shown to yield efficient gene delivery both in cell culture and *in vivo*. Logg and colleagues [23] described the development of the RCR vector that harbors an internal ribosome entry site-transgene (GFP) cassette positioned between the ENV gene and the LTR. This vector replicates and efficiently expresses a transgene in

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culture and in solid tumor models *in vivo*. Analyses of high molecular weight DNA harvested from the tumor, spleen, lung, kidney, liver, and heart revealed the presence of the full-length GFP transgene only in the tumor samples, and not in any of the non-tumor tissues. These data show that spread of the RCR vector appears confined to the tumor tissue. Also Tai and colleagues [24,25] showed that transduction by (RCR) vectors is efficient, tumor-selective, and persistent. A single dose of RCR vector expressing the CD prodrug-activating gene, followed by a single cycle of a 5-FC, inhibited growth of pre-established primary gliomas in mice without apparent damage to adjacent normal brain tissue. The authors also reported efficient RCR vector-mediated transduction of malignant gliomas in immunocompetent Fisher rats [26].

Environmental risk assessment and biosafety

The use of RCR vectors raises questions about possible pathogenic effects resulting from the spread of the vector in the host. Although MuLV is known to induce thymic T lymphoma in newborn mice, it is not pathogenic in adult mice [27]. For human studies amphotropic MuLV is required. Initial studies of amphotropic MuLV in rhesus monkeys could find no evidence of pathology in infected animals over a 3-year observation period, despite severe immune suppression at the time of infection and the administration of high doses of replication-competent MuLV [28,29]. A subsequent study, however, revealed that amphotropic replication-competent MuLV can be oncogenic in primates. In this study 3 of 10 rhesus monkeys that received bone-marrow cells infected with replication-competent amphotropic MuLV developed T-cell lymphoma [30,31]. These results suggest that while MuLV is potentially oncogenic in primates, the presence of a normal, functioning immune system reduces oncogenicity to a large extent.

Although replication-defective derivatives of MuLV have been used frequently as gene-transfer vector in clinical gene therapy, relatively little information is available on replication-competent amphotropic-pseudotyped MuLV in primates. Although the use of replication-defective MuLV has been generally safe and well tolerated, it has led to a number of cases where T-cell leukemias developed in the recipient as the result of insertional mutagenesis and activation of proto-oncogene expression. The use of replication-competent MuLV-derived vectors in cancer gene therapy is a novel approach. Despite the fact that MuLV is presumably inefficiently transferred horizontally in humans, the uncertainties surrounding the effects of a replication-competent amphotropic MuLV led us to cautiously classify it as "High" with respect to the relative environmental risk score.

Viruses placed in the "Negligible" relative environmental risk category

The *Autographa californica* baculovirus and the canarypox virus have been placed at the low end of the relative environmental risk spectrum. Their relative risk is scored as "Negligible".

The *Autographa californica baculovirus* has a very narrow host range that is limited to a single moth species, the Alfalfa Looper *Autographa californica*, which occurs in the western half of the North American continent. Although the virus transduces cells of many mammalian species [32,33], no baculovirus genes are expressed and the viral genome is not replicated. In human blood the insect cell-produced viral particles are rapidly inactivated by human complement [34,35]. The very narrow host range, the virus incapacity to replicate in mammalian cells, and therefore, the limited capacity of the virus to be shed from the patient led us to classify the relative environmental risk of baculoviruses as "Negligible".

ALVAC-based canarypox viruses have a host range that is very narrow and limited to canaries (*Serinus* sp.). Also the ALVAC-strain used in clinical studies is strongly attenuated in its natural host. Thus ALVAC-based canary pox vectors are unlikely to become a threat to the environment [36]. Since the virus does not replicate in mammalian cells, the virus does not amplify and shedding is limited to the initial administered dose. The very narrow host range, the virus incapacity to replicate in human cells, and therefore the limited capacity of the virus to be shed from the patient led us to classify the relative environmental risk of canarypox virus as "Negligible".

CONCLUDING REMARKS

Many preclinical studies have provided proof of efficacy of oncolytic virus therapy. A number of the non-human viruses have been tested in early-phase clinical studies. The results so far have demonstrated both the feasibility and the safety of the approach,

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with anecdotal evidence of antitumor efficacy. The aggregate of data demonstrated that the often severe side effects that haunted the initial clinical studies with oncolytic viruses can be overcome. Hence, the field should move forward while maintaining the good safety record and keeping the trust of the public.

Indeed it is reasonable to anticipate that the field will progress and that clinical use of non-human therapeutic viruses will expand in the near future. For activities involving genetically modified viruses, the legal framework permitting such activities is usually embedded in national and international (e.g. European) regulations. The competent authorities are defined, and the procedures are well established and widely known in the field. The procedures for obtaining permission for clinical application of genetically modified viruses require a proper step-wise environmental risk assessment. It seems desirable that a similar step-wise environmental risk assessment is also performed before initiation of clinical applications of viruses that are not considered GMOs.

As clinical application of non-genetically modified viruses does not fall under the regulation pertaining to genetically modified organisms, it is uncertain if there is any environmental risk assessment formally required before such use can be initiated. Either on a national level or on an international level (e.g. European level) the regulatory situation should be clarified.

The potential environmental impact of the use of replication-competent non-human viruses may be difficult to predict. Without reliable estimates of the potential environmental impacts, it may be difficult to obtain consensus on the requirements for containment measures. Practical procedures for risk management in the clinical applications of non-human viruses are essential. Rather than defining a set of strict goals and binding criteria, the authors suggest formulating and listing points to consider in such environmental risk assessment. Such a list could be used for defining practical procedures that facilitate the application while achieving acceptable risk levels. It will be the joint responsibility of authorities and investigators to define such procedures and to find solutions that assist research that can make oncolytic virus therapy a powerful approach for combating cancer.

CONFLICT OF INTEREST

The authors have no competing interest.

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