General Considerations on the Biosafety of Virus-derived Vectors Used in Gene Therapy and Vaccination

Aline Baldo[1],*, Eric van den Akker[2],*, Hans E. Bergmans[2], Filip Lim[3] and Katia Pauwels[1]

[1]*Scientific Institute of Public Health (WIV-ISP), Biosafety and Biotechnology Unit, B-1050 Brussels, Belgium;* [2]*National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands;* [3]*Department of Molecular Biology, Universidad Autónoma de Madrid, 28049 Cantoblanco Madrid, Spain*

Abstract: This introductory paper gathers general considerations on the biosafety of virus-derived vectors that are used in human gene therapy and/or vaccination. The importance to assess the potential risks for human health and the environment related to the use of genetically modified organisms (GMO) in this case genetically modified viral vectors is highlighted by several examples. This environmental risk assessment is one of the requirements within the European regulatory framework covering the conduct of clinical trials using GMO. Risk assessment methodologies for the environmental risk assessment of genetically modified virus-derived vectors have been developed.

1. INTRODUCTION

Due to millions of years of evolution, viruses have become some of the most efficient vehicles for transferring genetic information into eukaryotic cells by binding to their host cells and introducing their genetic material as part of their replication cycle. These features have been further explored and exploited in view of developing efficient methods for the delivery of genes of interest into mammalian cells. To this end viruses have been genetically modified by removing sequences responsible for hazardous properties while keeping sequences important for gene delivery, functionality and effectiveness. Depending on the wild-type virus from which they are derived, vector systems may differ for example in their maximum insert size, their transgene expression, the duration of gene expression or the target cell.

The conduct of clinical trials using genetically modified organisms (GMOs), in this case a genetically modified (GM) viral vector, and the marketing of medicinal products containing or consisting of GMOs are governed in the European Union (EU) by legislation which assesses not only the acceptability and safety for the involved human subjects and the quality control of the gene therapy medicinal product (GTMP) [1], but also several aspects related to the environmental impact of the GMO with regard to the potential risks for human health and the environment. As for all human clinical trials performed in the EU, clinical trials using GMOs or involving medicinal products containing GMOs fall under the scope of Directive 2001/20/EC in which specific provisions regarding the conduct of clinical trials on

human subjects involving medicinal products are established [2].

In addition, these clinical trials must also comply with the legislative provisions on biosafety. The Directive 2001/18/EC applies to the deliberate release of GMOs [3] and requires that an environmental risk assessment (ERA) should be carried out before a release. The objective of the ERA is to identify and evaluate potential adverse effects of the GMO on public health and the environment. Besides clinical trials, an ERA should also be performed as part of the procedure for marketing authorization [4].

In case physical barriers, or a combination of physical barriers together with chemical and/or biological barriers, are used to limit the contact with the general population and the environment, clinical trials and related activities such as the preparation, administration or storage of the medicinal product containing GMOs may comply with EU Directive 2009/41/EC on the 'contained use' which applies to any genetically modified micro-organism [5]. It is worth mentioning that boundaries between deliberate release and contained use are not clear within the context of clinical trials. Patients who received the medicinal product containing GMOs do not stay long in hospitals and once they have left the hospital, the GMO might spread into the environment. At EU level, not all Member States have the same approach to distinguish between aspects relating to deliberate release and contained use of GMOs in the specific case of clinical trials. Some member states such as Belgium and the Netherlands opt for the link between the two specific regulations in the field of biosafety.

2. BIOSAFETY AND RISK ASSESSMENT METHODOLOGY

The objective of an ERA of a deliberate release of a GMO is to identify and evaluate potential adverse effects on

*Address correspondence to this author at the Scientific Institute of Public Health (WIV-ISP), Biosafety and Biotechnology Unit, Rue J. Wytsmanstraat 14, B-1050 Brussels, Belgium; Tel: +32 2 642 52 69; Fax: +32 2 642 52 92; E-mail: aline.baldo@wiv-isp.be

Aline Baldo and Eric van den Akker contributed equally to this work.

human health and the environment under the conditions of the release, on a case-by-case basis. The ERA does not take into account patient safety, which is assessed in other medical and ethical evaluation procedures. However, results from clinical research with patients may inform the ERA, taking into account that the exposure of general population and the environment will usually be significantly lower than the exposure of the patient. The ERA consists of identifying the characteristics of the GMO and its use which have the potential to cause adverse effects for persons (non-patients) directly exposed to the GTMP, *e.g.*, relatives or clinical staff involved in product administration or in patient care, as well as the human population in general, and the environment at large including potential adverse effects for animals, plants and micro-organisms.

While the ERA for medicinal products that are chemicals (chemically derived) is quantitative with a threshold limit, the ERA of GTMP which are biologicals is based on the weight of evidence methodology including both qualitative and quantitative considerations [6]. It consists of the identification and characterization of potential hazards and their probability of occurrence.

The risk assessment methodology of GMOs developed over the past decades is basically similar in many legislative systems and recognizes the following steps: (1) hazard identification, (2) hazard characterization, (3) assessment of likelihood, (4) risk estimation, (5) evaluation of risk management options followed by (6) a conclusion on the acceptability (or not) of the overall impact of the use of the GMO on human health and the environment taking into account the management strategies applied (Fig. **1**). These principles, concepts and methodology are key to the GMO regulatory framework adopted in the EU. An example of an hazard identification and subsequent steps for risk characterization is shown in (Table **1**). The review of van den Akker *et al.* [7] describes a general methodology for risk assessment of recombinant viral vectors, taking into account effects of the insert and the review of Goossens *et al.* [8] describes in details the ERA of modified Vaccinia virus Ankara-based vectors used for clinical trials notified in Belgium.

3. OVERVIEW OF THE STEPS OF GMO RISK ASSESSMENT

Step 1: Hazard Identification

In step 1 the hazards that may be caused by the characteristics of the GMO and its intended application are identified. A hazard is defined as a potentially harmful or adverse effect on human health or the environment. Basically, in this step the question is asked 'what could go wrong?'.

Potentially adverse effects of a GM viral vector may result from altered properties compared to the parental virus regarding specific aspects of the viral life cycle and the interaction of the viral vector and/or the gene products encoded by the insert with the host.

Effects that are to be identified as potentially harmful will vary from case to case and may include, in case of viral vectors:

- increased virulence compared to the parental virus in the human non-target population;

- increased pathogenic effects including toxic and allergenic effects in animals;

- changes in medical practice due to the deliberate release of the GMO in the environment;

- effects on the population dynamics in the natural environment, *e.g.*, effects on the dynamics of populations of species in the receiving environment and the genetic diversity of each of these populations.

Step 2: Hazard Characterization or Evaluation of the Potential Consequences of Each Hazard Identified

The magnitude of the consequences of each potential adverse effect that have been identified in step 1 of the ERA should be evaluated. The magnitude of consequences is likely to be influenced by the modalities of the release including the specific properties of the receiving environment. The health status of the persons likely to be exposed, the route and frequency of administration of the viral vector could also influence the magnitude of consequences.

For each adverse effect that is identified, the consequences need to be described in qualitative terms ranging from *high*, *moderate*, *low* to *negligible* [9]. In case of a high degree of scientific uncertainty about consequences, a worst case scenario [10] may be applied, meaning the consequences are evaluated based on an assumption that the adverse effects will occur to the full extent.

The ERA needs to consider direct and indirect, immediate and delayed effects of the final viral vector. An example of a direct effect could be the accidental exposure of a non-target human to a viral vector which is modified to deliver a human growth factor, the expression of which, in non-target tissues, could have potentially harmful consequences. However, direct effects may be delayed making them more difficult to be linked to the viral vector. For example, oncogenesis could be a direct effect of exposure to integrative viral vectors but it may be difficult to be linked to the occurrence of the vector if the consequences occur several months after the exposure. Indirect effects could occur if there is a recombination or complementation event between the viral vector and other viruses after the release of the viral vector leading to the formation of a new virus able to infect the human non-target population.

Step 3: Assessment of Likelihood of Occurrence for Each Hazard Identified and Characterized or Exposure Assessment

The evaluation of exposure pathways through which viral vectors and their inserted gene products may interact with humans (other than patients receiving treatment with the viral vector), or the environment is of major importance in the ERA. These exposure pathways include the spreading occurring at the site of administration, the biodistribution which corresponds to the dispersion within the patient's body from the site of administration, and the shedding which corresponds to the dissemination in any form into the environment via excreta (urine, feces, sweat, saliva, nasopharyngeal fluids), and skin, blood and semen from the treated patient [11].
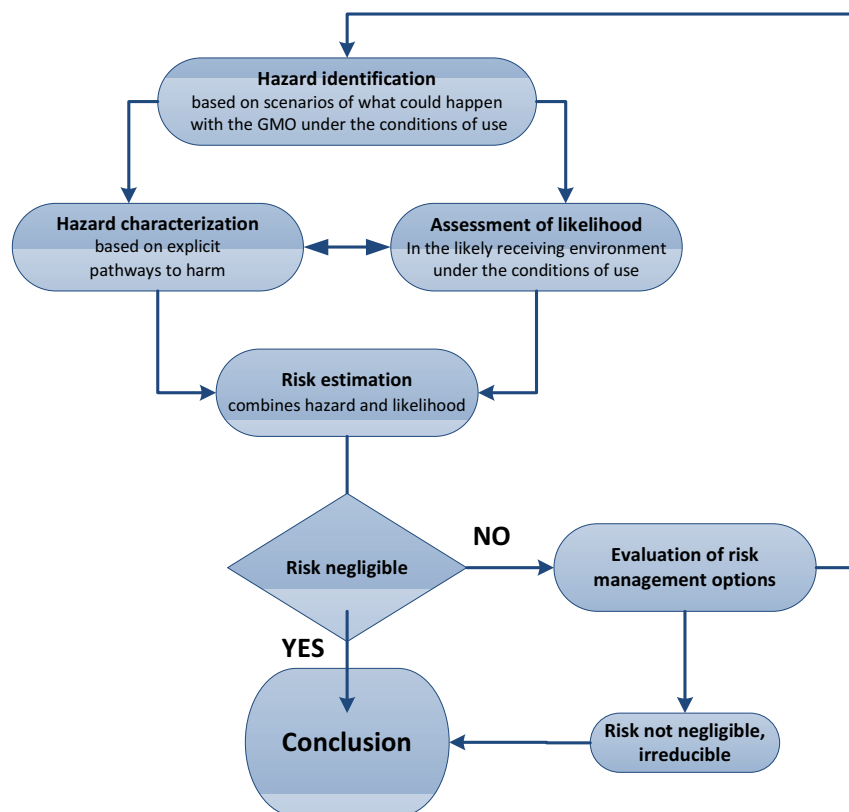
Fig. (1). Standard steps in an environmental risk assessment. For explanation see main text.

Table 1.   **Example of a Hazard Identification[2] and Subsequent Steps for Risk Characterization[3]**

Step 1 Hazard Identification	Step 2 Hazard Characterization	Step 3 Assessment of Likelihood	Step 4 Risk Estimation: Hazard × likelihood	Step 5 Evaluation of Risk Management Options	Step 6 Overall Risk Conclusion
Recombination between the GM[1] replication deficient virus and a wild-type virus	Transfer of the transgene into a replication competent wild-type virus Generation of new recombinant viruses with uncharacterized host range or tissue tropism, infectivity, virulence or latency For this example moderate consequences of adverse effects are assumed (Table 2)	"*low*" due to non-replicability of the GM virus, lack of shedding, low stability in the environment and low incidence of viruses that present sequence homology with GM virus. Risk is not negligible due to small chance that GM virus and wild-type viruses are present in the trial subjects.	"*low*"	Testing of trial subjects for presence of wild-type virus before administration of GM virus	The overall risk is "*negligible*" because due to the applied risk management, the likelihood (step 3) has become "*negligible*"

[1] Direct risks for the patient are not considered.

[2] To scale the magnitude of the likelihood or to estimate the risk the terminology proposed in the guidance notes of the European Commission is adopted [7]: "*high*", "*moderate*", "*low*", "*negligible*".

[3] GM: genetically modified.

Biodistribution in the patient and shedding are among the main factors that determine the probability of exposure through which viral vectors may interact with their environment and hence the probability of transmission. However, dissemination of the viral vector into the environment is not an adverse event *per se*. Indeed, the shedding of the viral vector and the resulting dissemination of the GTMP into the environment and subsequent transmission should not be necessarily considered as a hazard but as a mechanism by which hazard may occur. The impact and possible adverse effect of the dissemination of a viral vector into the environment mostly depend on the characteristics of the vector, *e.g.*, its capacity to replicate, its persistence in the environment, its capacity to infect cells of other persons or animals, but may also be directly related to the inserted gene, or the interaction between viral vector and insert.

The type of viral vector, the dose and the administration route influences the biodistribution and the shedding through associated excreta.

Evaluation of shedding data often consists in analysis of vector genomic sequences by quantitative PCR and/or of infectious viral particles by biological assays [11]. The choice of assay depends on the sample to be tested. PCR has been often used for analysis of blood and related products and a biological assay for other excreta. It is important to take into account that PCR will only detect the presence of the viral vector genome without giving any information about the functionality, *e.g.*, infectivity. Shedding is often short lasting and is observed during the first few days after administration of replication deficient viruses (depending on the case), while shedding of replication competent viral vectors may last for longer periods.

Some procedures might lead to exposure to the viral vector, *e.g.* the production and the preparation of the viral vector, the administration of the GTMP to the patient and waste disposal. Direct exposure may also result from accidental inoculation of the viral vector during the treatment of the patient via droplets or aerosols contacting mucous membrane, non-intact skin or eyes; piercing by needle or injury due to sharps, cutting with broken vials, contact with waste materials or used bandages. Nurses, medical staff and the people visiting the treated patient could also be exposed to contaminated material or spoiled surfaces. These exposure pathways can be reduced or eliminated by application of appropriate risk management strategies.

The characteristics of the environment into which the viral vector is intended to be released and the modalities of the release are also important factors to be considered. For many (gene therapy) viral vectors, there is a widespread immunity in the human population, which is likely to reduce the ability of the GMO to spread into the community. The level of immunity in the community should be considered in the ERA, but the epidemiologic data need to be carefully considered, particularly in immunocompromised people.

It may not be possible to estimate the likelihood of exposure precisely for each hazard that is identified and characterized. Similar to the consequences of each adverse effect, the likelihood of exposure can be expressed qualitatively using a categorical order description (such as "*high*", "*moderate*", "*low*" or "*negligible*").

Step 4: Risk Estimation: Combination of Magnitude of Consequences of Each Hazard and the Likelihood of its Occurrence (Including Identification of Areas of Uncertainties)

An estimation of the risk to human health or the environment posed by each identified characteristic of the GMO which has the potential to cause adverse effects should be made by combining the likelihood of the adverse effect occurring and the magnitude of the consequences, if it occurs. The individual risks are then combined into an overall risk that is at least as high as the highest individual risk. The overall risk should also be described in qualitative terms ranging from "*high*", "*moderate*", "*low*" to "*negligible*". While it is not possible to multiply qualitative terms, a risk matrix is useful as a tool and illustrates the process of risk estimation (Table **2**). In case of a high degree of scientific uncertainty about consequences and likelihood, a worst case scenario may be applied [10], meaning that the consequences are evaluated based on an assumption that the adverse effects occur to the full extent. The worst case scenario is not meant to be a realistic description of what may or will happen. If the worst case scenario leads to the prediction that the risk is negligible, no further data are needed to reduce the scientific uncertainty. If the worst case approach results in a risk that is not negligible, more data or appropriate risk management strategies are needed to resolve the scientific uncertainty about consequences and likelihood.

Step 5: Evaluation of Risk Management Options Aiming at Reducing Risks Identified in the Previous Step

When the overall risk has been defined, it is determined whether application of risk management strategies need to be implemented in order to minimize the likelihood of adverse

Table 2.   Risk Matrix is a Tool that Illustrates the Process of Risk Estimation.

		Likelihood of Occurrence of Adverse Effects			
		Negligible	**Low**	**Moderate**	**High**
Consequences of adverse effects	**Negligible**	Negligible	Negligible	Negligible	Negligible
	Low	Negligible	Low	Low	Moderate
	Moderate	Negligible	Low	Moderate	High
	High	Negligible	Moderate	High	High

effects occurring. Under circumstances that the estimated overall risk is not negligible, relevant protective measures should be applied and considered as adequate to reduce the level of risk to negligible. At the end of the ERA, the previous steps are repeated taking into account the proposed protective measures, in order to ascertain the application of these measures reduces the characterized hazards or their estimated likelihoods, and the resulting risks in such a way that the overall risk is negligible [12, 13]. If no adverse effects were identified risk management is not necessary.

Examples of containment measures that could be applied to decrease the estimated risk:

- The patient could be hospitalized during the administration of the GTMP and several days after the administration in order to avoid the release of the GTMP in the environment. These measures reduce the environmental risk but it is important to prevent the dissemination of the GTMP in the hospital. It should be considered, however, that the patient is free to leave the hospital at any moment, therefore optimal implementation of this measure cannot be warranted.

- Aerosols producing operations should be reduced during preparation and administration procedures and the staff preparing and administering the GTMP should wear personal protective equipment.

- The preparation and the administration of the GTMP may require an emergency plan. This emergency plan should contain procedures that have to be applied in case of accident (*e.g.*, needle piercing, breakage of a vial containing the GTMP) such as information on the efficacy of the disinfection methods proposed and measures taken following a spill or the breakage of a vial containing the GTMP.

- Strict procedures should be provided for medical staff and persons in contact with the patient during the release of the viral vector. These procedures should be posted in the hospital room where the treatment should take place.

- Potentially contaminated non-disposable materials, *e.g.*, the material used for the transport, preparation or administration of the GTMP, instruments, clothing, and surfaces need to be properly decontaminated.

- Contaminated waste should be inactivated using an appropriate method (autoclave or incineration) before disposal.

- Containment measures which are applicable when the patient is outside the hospital should be clearly explained, *e.g.*, bandaging/plaster to contain dissemination from the injection site and education of patient on hygiene.

- Effective prophylaxis, if available, is an important containment and control measure which could be applied if the likelihood of exposure is high.

Some of the viral vectors are derived from wild-type viruses for which a prophylaxis or therapy is available. With respect to the ERA, the availability of effective prophylaxis may be an important containment and control measure, as they could minimize possible adverse effects for persons who come in contact with the patient. Therefore, whether the modification will result in reduced susceptibility of the viral vector to prophylaxis that is effective against the wild-type virus should be carefully considered in the ERA. For example, a prophylactic vaccine that protects against a viral infection by the wild-type virus might not be effective against the viral vector if modifications result in an altered viral vector causing an altered immunogenic profile of the viral vector.

Step 6: Overall Risk and Conclusion

In the final step of the ERA, the overall risk of the viral vector is evaluated taking into account the proposed risk management. Based on the previous steps, a conclusion should be given as to whether the overall risk is acceptable and if the clinical trial can be carried out. This final evaluation should be expressed as a summary of the overall risk related to the specific application.

4. CONSIDERATIONS ON POTENTIAL ENVIRONMENTAL HAZARDS OF VIRAL VECTORS FOR USE IN HUMAN GENE THERAPY

Since viral vectors are derived from wild-type viruses, hazard identification starts with the characterization of the GM virus taking into account the hazard features of the unmodified virus from which it is derived, the intended and unintended alterations in host range or tissue tropism, infectivity, virulence, or latency upon genetic modification and the nature of the inserted genetic material. A comparison of the characteristics of the viral vector with those of the wild-type (non-modified) virus, under corresponding conditions of the release or use, will assist in identifying any particular potential adverse effects arising from the genetic modification.

All these features have to be taken into account and it is important at this stage of the ERA not to discount any potential adverse effect on the basis that it is unlikely to occur.

4.1. Molecular and Biological Characteristics of the Viral Vector

- Risk classification of the parental virus

In 1979, the World Health Organization (WHO) has defined criteria for the classification of micro-organisms into four Risk Groups (RG), taking into account the severity of the disease that the pathogens may cause in immunocompetent humans or animals, their ability to spread amongst the population and the availability of prophylaxis or efficient treatment [14]. Biological agents that are unlikely to cause disease are classified into RG 1 while agents responsible for severe diseases with a high potential of transmissibility and for which no treatment is available are assigned to RG 4. The risk group of the most commonly viruses used in gene therapy are presented in (Table **3**). The risk group of the viral vector may be lower than the risk group of the wild-type virus from which it is derived, if the modification leads to attenuation of the virus. However, the properties of the transgene have also to be taken into account for the allocation of the recombinant vector to a risk group.

- Attenuation of the viral vector; replication deficient viral vectors

Table 3.   Characteristics of the Viral Vectors Most Commonly Used in Gene Therapy and/or Vaccination

Viral vector[1]	RG of the wild-type virus[2]	RG of the viral vector[2,3]	Transmission mode	Persistence	Host range
Adenoviral vector	2	1	Most Ad naturally infect the respiratory tract, transmitted by direct contact, faecal-oral transmission and water-borne transmission (aerosolized droplets), parenteral	Stable, resistant to dehydration, able to persist in aerosols and water	Human, only 2 non-human species that allow replication of human Ad: cotton rat, hamster
Retrovirus vector	MoMLV[4] RG[5] 3 for animals	RG 2 for animals and RG2 maximum for humans	Parenteral inoculation; droplet and aerosol exposure of mucous membranes; contact exposure of broken skin.	Rapidly inactivated, sensitive to dehydration	Depends on the vector
Modified Vaccinia virus (MVA)	1	1	Ingestion, parenteral inoculation, aerosol exposure of mucous membranes, through skin abrasions	High environment stability, high resistance to drying, increased temperature tolerance compared to other viruses	Unknown, broad host range
Adeno-associated viral vector	1	1	Ingestion, inhalation of aerosols or droplets, contact with mucous membranes	High stability	Human
Avipoxvirus	1	1	Ingestion, parenteral inoculation, aerosol exposure of mucous membranes, through skin abrasions	High environment stability, high resistance to drying, increased temperature tolerance compared to other viruses	birds
Lentiviral vector	3	2	Parenteral inoculation; droplet and aerosol exposure of mucous membranes; contact exposure of broken skin.	Rapidly inactivated outside their hosts.	Human
Herpesviral vector (Herpes simplex virus-1)	2	1	Direct contact, by respiratory droplets	Highly susceptible to dehydration, rapidly inactivated outside the host, HSV virus is easily inactivated.	Human

[1]Viral vectors are presented by frequency of use, Adenoviral vector being the most commonly used vector in gene therapy.

[2]Risk classification differ between member states, only the Belgian classification is presented here.

[3]Without taking into account the transgene.

[4]MoMLV: Moloney murine leukemia virus.

[5]RG: risk group.

One way to achieve effective attenuation of the viral vector, which is widely used for most viral vectors used in gene therapy is to reduce self-propagation in target cells while retaining the capacity of introducing genes of interest. To this end, replication deficient viral vectors and conditionally replicating viral vectors have been developed. The origin, nature and stability of the genetic modifications should be well characterized and need to be considered in the ERA as these modifications may significantly alter biosafety features of the GMOs.

One way how attenuating mutations, like replication deficiency, may be lost is recombination of the viral vector with the wild-type parental virus. A replication deficient viral vector may encounter a wild-type virus in the target cell. A conditionally replication competent viral vector may also spread from the injection site or the tumor to organs and to cells where a wild-type virus may be present leading to the formation of a novel virus by recombination or reassortment. As these recombinants may present undefined characteristics distinct from the wild-type virus or viral vector, recombination or reassortment remains one of the major safety concerns during the ERA. Significant efforts have been devoted to render vectors non-replicative as it lowers the probability of contact between the viral vector and the wild-type parental virus, and hence the probability of recombination or reassortment.

Non-replicating vectors lack the genetic information for replication but they retain the capacity for introducing genes of interest into target cells. However, genes could be inadvertently acquisitioned through recombination so that repli-

cability of the vector could be partly restored or even increased. Such an event cannot only occur after administration of the vector, but could occur during the production phase through recombination between vector and genomic helper DNA sequences of the producer cells or helper DNA sequences co-transduced for viral production. For example, replication defective adenoviral vector derived from adenoviral serotype 5 (Ad5) often are generated through deletion of the essential E1A- and E1B-encoding sequences of the viral genome. A well-known approach to produce (replicate and package) replication defective adenoviral vector is the co-transfection of a linearized plasmid containing the recombinant E1-deleted Ad5 vector genome into PER.C6 cells. These helper cell lines have been developed and matched Ad vectors that do not have sequence overlap. They contain the Ad5 E1A- and E1B-encoding sequences. Propagation of matched Ad5 vectors, which lack homology with these Ad5 E1A- and E1B-encoding sequences does not result in the generation of replication competent adenoviruses [15], in contrast to many other helper cell lines used for production of adenoviruses. The construct of replication deficient adenoviral vectors is illustrated in the review of Wold and Toth in the current issue [16].

Other strategies have been designed that limit the probability of recombination. The probability of inadvertent generation of replication competent viruses (RCV) during production can be decreased by separating viral functional elements into different expression plasmids. For example, developments in the design of lentiviral vectors, which mostly have been derived from HIV1 (Human Immunodeficiency Virus 1), have exploited this strategy to generate new lentiviral vector systems with improved biosafety [17, 18]. Biosafety considerations related to the use of lentiviral vectors are further addressed within the current issue [19].

Some wild-type viruses, such as adeno-associated virus (AAV) are naturally replication deficient and need co-infection with other helper viruses to be able to replicate. Along with the fact that AAV are not pathogenic, AAV is therefore considered as appropriate candidate for viral vector development. In the AAV vectors that are currently in use, the entire wild-type viral genome has been deleted, except for the 5' and 3' inverted terminal repeat regions, which are the only *cis* acting, noncoding viral sequences necessary for packaging of a recombinant vector genome into replication defective particles. The production system uses a plasmid construct which contains a mini-Ad genome capable of propagating recombinant AAV vector in the presence of AAV *REP* and *CAP* genes. This construct is missing some of the early and most of the late Ad genes and is incapable of producing infectious Ad [20]. The recombinant AAV genome expressing the gene of interest, adenoviral helper genes, and the AAV coding sequences for the rep and cap proteins are provided on separate plasmids and a cell line is turned into a producer cell line by transfection with these plasmids. Biosafety of AAV vectors is detailed in the review of Dismuke *et al.* [21] in the current issue.

• Considerations for conditionally replication competent viral vectors

Genetically modified replication competent viral vectors (RCVV) retain characteristics that make them able to multi-

ply. These viruses are currently most often being applied in cancer therapy [21] and can be divided into two categories, based on their application as either a tumor vaccine or as an oncolytic virus.

The first category of RCVV comprises viral vaccine strains which are genetically modified by insertion of genes encoding for (immunomodulatory) cytokines employed to stimulate the host immune response to tumor cells or tumor antigens and can be used for cancer therapy but also as vaccines against infectious diseases.

The second category encompasses oncolytic viral vectors, which are viruses that are able to specifically replicate in cancer cells leading to their destruction. As opposed to viral gene therapy which improved cell function (by replacing a defective gene or functionally compensating it with another gene or inactivating a toxic gene or neutralizing its toxic effects), viral oncolysis (also termed virotherapy) can be defined as the killing of specific cells, such as tumor cells, by selective infection, replication, cell lysis, and spread of progeny virus to neighboring cells [22]. Thus, as opposed to (viral) gene therapy, the virus itself is the therapeutic agent, provided that there is efficient replication and cell death. Viral tumor cell lysis can also be combined with gene transfer for increased anti-tumor activity by insertion of therapeutic transgenes into the virus genome generating "armed" oncolytic viruses [23].

The majority of research on oncolytic viruses has focused on adenoviruses. A review article concerning oncolytic adenoviral vectors that have been used in cancer gene therapy and the results obtained with these vectors in clinical trials is included in the current issue [16]. Oncolytic viruses derived from other species have been tested in clinical trials, the most important being herpes simplex virus 1 (HSV-1) [25], vaccinia virus, measles virus, vesicular stomatitis and reovirus [26]. Biosafety features related to the use of vectors derived from Herpesviruses, particularly HSV-1 were detailed in the current review of Lim *et al.* [27].

Several strategies have been developed mediating tumor-specificity and avoiding the dissemination of the RCVV vectors in non-tumor cells. These strategies improve the safety for the patient by preventing damage of healthy tissues [28] and they also enhance the biosafety.

Engineering of vectors to specify target delivery requires the modification of native tropism towards targeting of other receptor molecules, all while retaining innate gene transfer efficiency [29]. Two distinct approaches have been developed to modify RCVV tropism. One method is by targeting viral entry by genetic manipulation of the capsid for naked viruses or the outer virus membrane for enveloped viruses to selectively target ligands that are highly expressed on tumor cells or in the tumor microenvironment [22]. In a variation of this approach an adaptor molecule, for example, the formation of a 'molecular bridge' between the vector and a cell surface receptor is used. For instance in adenovirus, adaptor function is performed by 'bi-specific' antibody molecules that crosslink the Ad vector to alternative cell surface receptors, bypassing the native Ad tropism. However, the exact effects of alterations of the tropism may in some cases be difficult to predict (especially in case of absence of adequate

animal models for certain viral vectors) and one should be aware that possible enhancement of the tropism for normal or tumor cells may influence shedding and/or possible risks to thirds (see considerations for the final GMO).

For post-entry restriction of virus replication to cancer cells, two strategies have been developed. One strategy is achieved through the deletion of viral virulence genes (*e.g.* immune modulators, anti-apoptotic proteins, inducers of cellular proliferation) that are redundant for replication in tumor cells. As a result viral replication is attenuated in normal tissues, but proceeds normally in cancer cells [30]. In order to reduce the probability of the occurrence of wild-type revertants, some vectors contain multiple deletions within their genome. The second and the most established strategy to restrict viral replication to tumor cells, consists in placing the expression of essential viral genes under the control of tumor or tissue-specific promoters, that are preferentially active in tumor cells [22, 31, 32].

Notwithstanding that replication selective oncolytic viruses may present features of targeted specificity, their use raises questions about risks due to the possibility of an increased likelihood of exposure of the environment around the patient, compared to replication deficient viral vectors [21]. As the risk evaluation of RCVV warrants more detailed consideration than will be covered by the brief introduction in this paper, we refer to van den Akker *et al.* in this special issue [7].

Viral replication in human subjects may increase the risk of genomic integration of DNA derived from certain viruses into the host genome with its associated risk of insertional mutagenesis and/or the transactivation of neighboring genome sequences. The risks of integration of retroviral vectors are particularly well known [17, 33] and the biosafety of retroviral vectors and their genotoxicity are detailed in the current issue [34]. The risk of integration is a theoretical concern for any gene therapy vector, and it should be kept in mind that the use of viral vectors at concentrations many orders of magnitude above those encountered in natural infections can have significant implications regarding the risks of integration of even vectors derived from non-pathogenic viruses [35].

The application of replication competent non-human viruses is being evaluated and poses new biosafety risks. A detailed overview of the biosafety of non-human therapeutic viruses used in gene therapy is provided by Hoeben *et al.* [36].

- Stability and survival in the environment

Some viruses are relatively stable, resistant to dehydration and able to persist in the environment whereas other viruses are highly susceptible to dehydration and are rapidly inactivated outside the host. More stable viruses may be resistant to certain disinfectants and may therefore be able to persist in the environment. The persistence of the viral vector could increase the likelihood of exposure. The stability of viral vectors most commonly used in gene therapy is detailed in (Table **3**).

Furthermore, inserted sequences can influence the genomic stability of the viral vector. When inserted sequences enlarge a viral genome to a size where it is not packaged

efficiently, the genome can be prone to rearrangements. This may lead to unexpected changes in the properties of the viral vector.

4.2. Hazard Related to the Characteristics of the Transgene

A comprehensive risk assessment of GM viral vectors carrying gene of interests for gene transfer should take into account the properties of the inserted gene sequences and of the gene product. While the expressed gene product may have intrinsic hazardous properties (*e.g.*, toxic or allergenic properties), its actual hazard in gene therapy trials depends very much on the genetic and physiological context of the parental virus in which it is introduced and of the condition of use (see also paragraph 4.3). The inserted gene may be expressed differently, depending on the activity of the promoter and other regulatory sequences that govern their expression [36]. Gene products that may be considered as potentially hazardous in the context of gene therapy with viral vectors are detailed in the review of Bergmans *et al.* [37]. Moreover, inserts with unknown or novel characteristics produced by synthetic biology could also be considered as potentially hazardous. The implications of the use of inserts produced by synthetic biology are discussed in the current issue in the review of van den Akker *et al.* [7].

Inserted gene sequences could also counteract features introduced into the vector to achieve attenuation, or it could for other reasons enhance the pathogenicity or virulence of the viral vector, as will be discussed in the next paragraph. They could also confer increased probability of recombination due to homology with genes sequences of circulating viruses that are already present in the organism.

4.3. Identification of Hazards Arising from the Final GMO

When the length of the inserted sequences exceeds the maximum packaging capacity of a vector, unexpected and unwanted genome rearrangements may occur. This is only one example showing that apart from assessing the nature and stability of the viral vector and besides assessing the intrinsic properties of the inserted gene, the ERA of the final GMO should also consider the combinatorial interplay between the viral vector and the gene(s) of interest resulting in, for instance, altered virulence, tissue tropism or host range, susceptibility to the immune system, susceptibility to prophylaxis and therapy. Some examples are provided below.

Vectors are generally administered in concentrations far higher than with wild-type and all aspects of this elevated virion titer should be considered in the ERA.

- Altered virulence

Several events could result in inadvertent virulence changes of the recombinant viral vector and may pose increased risk to human health or the environment. These changes could be caused by recombination of the viral vector with wild-type viruses or with viruses with homologous sequences possibly resulting in the reversion of the viral vector to a more virulent strain. Consideration should also be given to inserted genes encoding for virulence factors since the virulence of the viral vector may be increased compared to that of the wild-type virus.

Even therapeutic genes, which are harmless under given conditions, need to be taken into account since genes may encode proteins which only become harmful when expressed at a different level (in a stable or inducible way), in a different tissue or host, or at different stages in development depending on the promoter used.

- Altered tissue tropism and host range

Ideally, the viral vector of choice would transduce the target cells without any transgene expression in adjacent healthy tissue or damage to the adjacent healthy tissue [38]. Different strategies have been designed that either further limit the tropism of viral vectors to one or a few host cell population or on the contrary broadens the tropism of viral vectors to desired target cells. To this end, vectors, so-called pseudotyped viruses, have been developed in which endogenous viral envelope proteins have been replaced by either envelope proteins from other viruses, or by chimeric proteins. Such chimera would consist of those parts of the viral protein necessary for incorporation into the virion as well as sequences meant to interact with specific host cell proteins.

For example, a commonly used retroviral vector in gene therapy trials is the HIV-1 coated with the vesicular stomatitis virus glycoprotein (VSV-G). This VSV G-pseudotyped lentivirus infects most if not all mammalian cells contrary to its wild-type parental virus whose tropism is restricted to human cells expressing the CD4 (receptor).

Modifications of viral surface ligands might also permit the vector to bind specifically or preferentially to novel receptors present solely in target cells, as described above for the strategy used to direct vectors to bind preferentially to tumor cells.

In the ERA, the overall result of all modifications that may influence the viral tropism should be evaluated. Modifications that increase the tropism or the host range of the vector may lead to novel or more severe disease symptoms in human or animals.

- Altered susceptibility to the immune system

Host immune responses play a major role in the clearance of viral infections from the body, and may limit long-term expression and clinical efficacy of viral vectors. Viruses have evolved through various mechanisms to evade the immune system of the host. The deletion of immune evasion determinants can be viewed as innocuous or attenuating. However, if non-target humans are exposed to the viral vector containing a deletion of immune evasion determinant, the loss of immune evasion function (for example, deletion of E3 from adenovirus or the interleukin (Il)-18 binding protein from poxviruses) might result in more effective clearing of the viral vector during an infection and/or an increase of acute responses such as inflammation [12]. Similarly, insertion of genes encoding immune modulatory functions that are not native to the wild-type virus might affect pathogenesis. For example, poxviruses modified to expressed Il-4 were more pathogenic in an animal experiment than the wild-type virus, as this modification led to inhibition of the appropriate immune response for the effective clearance of viral infection [39]. The possible effects (*e.g.*, enhanced proliferation) of a viral vector with impaired immune evasion systems in individuals who may be or become immunosupressed during treatment should also be considered in the ERA.

5. CONCLUSION

The choice of a particular viral vector for gene therapy or vaccination is often determined by the size of the transgene(s) of interest, the required duration and regulation of gene expression, the path of delivery, the target cell type and biosafety issues. This introductory paper gathers general considerations on biosafety of virus-derived vectors that are used in human gene therapy and/or vaccination. While taking into account a commonly accepted risk assessment methodology and highlighting the need to perform a risk assessment on a case-by-case basis, considerations on potential hazards of viral vectors most commonly used have been given and have been illustrated by several examples.

The scope of the current issue focuses on all biosafety aspects that are relevant for the evaluation of the risks for human health and the environment related to the use of viral vectors. By detailing some of the most frequently used viral vectors in gene therapy and vaccination it aims at providing information to researchers, regulatory officers and applicants.

In the following chapters of the current issue, different viral vector systems will be described starting from the virology of the parental virus towards vector properties which concern biosafety.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

PATIENT CONSENT

Declared none.

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