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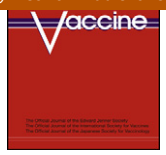

Review

Biosafety aspects of modified vaccinia virus Ankara (MVA)-based vectors used for gene therapy or vaccination

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ABSTRACT

The modified vaccinia virus Ankara (MVA) strain is a highly attenuated strain of vaccinia virus that has been demonstrated to be safe for humans. MVA is widely considered as the vaccinia virus strain of choice for clinical investigation because of its high safety profile. It also represents an excellent candidate for use as vector system in recombinant vaccine development for gene delivery or vaccination against infectious diseases or tumours, even in immunocompromised individuals. The use of MVA and recombinant MVA vectors must comply with various regulatory requirements, particularly relating to the assessment of potential risks for human health and the environment. The purpose of the present paper is to highlight some biological characteristics of MVA and MVA-based recombinant vectors and to discuss these from a biosafety point of view in the context of the European regulatory framework for genetically modified organisms with emphasis on the assessment of potential risks associated with environmental release.

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1. Introduction

The conduct of clinical trials using genetically modified organisms (GMOs) and/or pathogens and the marketing of medicinal substances containing or consisting of GMOs are governed in the European Union (EU) by a comprehensive regulatory framework (see Table 1). Firstly, as all clinical trials performed in the EU, clinical trials using GMOs and/or pathogens fall under the scope of Directive 2001/20/EC on the implementation of good clinical practice in the conduct of clinical trials on medicinal products for human use. Secondly, these clinical trials also fall under the scope of biosafety regulations. In the EU Member States, depending on the way biosafety Directives were implemented and on the type of clinical trial, such clinical trials are regulated by Directive 2009/41/EC on the contained use of genetically modified micro-organisms and/or by Directive 2001/18/EC on the deliberate release into the environment of genetically modified organisms. Finally, the marketing of any medication produced by biotechnology – including medicinal substances containing or consisting of GMOs – has to be authorised by the European Commission once an advice has been given by the European Medicines Agency (EMA). The legislative framework is based on Regulation (EC) No. 726/2004. If a medicinal product contains or consists of GMOs, Regulation No. 726/2004 refers to Directive 2001/18/EC: the applicant should carry out a case-by-case environmental risk assessment in accordance with the principles set out in Annex II and on the basis of information specified in Annex III of Directive 2001/18/EC. He should also provide information on precise instructions and conditions for use and labelling of the product according to Annex IV. This applies to GMO products developed for gene therapy, for therapeutic vaccination or for vaccination against infectious disease.

Several unique features make poxviruses excellent candidates as efficient vector systems for gene delivery or vaccination: (i) large packaging capacity for recombinant DNA; (ii) precise recombinant DNA expression regulated by a strong poxviral promoter; (iii) lack of persistence or genomic integration in the host due to their cytoplasmic replication; (iv) high immunogenicity as vaccine; and (v) ease of vector and vaccine production [5,6]. However, high incidence of complications observed when administering the poxvirus Chorioallantois Vaccine Ankara (CVA) as vaccine during the Smallpox Eradication Programme has generated concerns about the safety of poxviruses [7].

The approach taken to address this issue has been the development of highly attenuated poxvirus strains, such as the modified

Table 1
EU's regulatory framework governing the conduct of clinical trials using GMOs and/or pathogens and the marketing of medicinal substances containing or consisting of GMOs.

Legislation	Main elements	Reference	Web link
Directive 2001/20/EC	This Directive sets out common rules for the authorisation and regulatory follow-up of a clinical trial. It aims at protecting human subjects involved in clinical trials and ensuring that the results are credible, by establishing quality, safety and ethical criteria. Approval of trials is the responsibility of individual EU Member States, who are required to evaluate the products used in clinical studies	[1]	http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32001L0020:EN:HTML
Directive 2009/41/EC	This Directive focuses on the contained use of genetically modified micro-organisms (GMMs), *i.e.* any activity involving GMMs for which specific containment measures are used to limit their contact with, and to provide a high level of safety for, the general population and the environment. The Directive requests Member States to assess on a case-by-case basis the risks contained uses may pose and to implement appropriate containment and other protective measures to avoid adverse effects on human health and the environment. Contained uses are classified in four classes, from no or negligible risk to activities of high risk. The risk classification impacts on the nature of the administrative procedures and notification requirements	[2]	http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:125:0075:01:EN:HTML
Directive 2001/18/EC	This Directive defines the procedure for granting consent for the deliberate release in the environment and placing on the market of GMOs. It provides for a common methodology to assess case-by-case the risks for the environment associated with the release of GMOs. It also introduces compulsory monitoring after GMOs have been placed on the market, as well as compulsory public consultation and GMO labelling	[3]	http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32001L0018:EN:HTML
Regulation (EC) No. 726/2004	This Regulation lays down procedures for the authorisation, supervision and pharmacovigilance of medicinal products for human and veterinary use. For medicinal products derived from biotechnology, it foresees a compulsory centralised authorisation procedure in which the European Medicines Agency is responsible for drawing up opinions on any matter concerning the evaluation of the products	[4]	http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CELEX:32004R0726:EN:HTML

vaccinia virus Ankara (MVA) strain. These attenuated strains, in particular MVA and NYVAC (both derived from vaccinia virus strains) as well as TROVAC (derived from a fowlpox strain) and ALVAC (derived from a canarypox strain), have been used in the past years as recombinant vaccines or gene delivery vectors aiming at preventing or treating human and animal diseases. Several animal vaccines based on this technology are already registered in Europe.

There is now a large amount of information on biosafety issues associated with the use of MVA and MVA-based backbone vectors in the scientific literature and regulatory dossiers. In this article we review the main characteristics of MVA and recombinant MVA vectors and discuss these characteristics from a biosafety point of view.

2. Modified virus Ankara

2.1. MVA generation

MVA corresponds to an attenuated laboratory virus developed by Professor Anton Mayr. It is derived from the Chorioallantois Vaccine Ankara (CVA) strain of the vaccinia virus. The attenuated strain was renamed MVA after the 516th passage of CVA strain on primary chicken embryo fibroblasts (CEF). Genomic studies have revealed that, as a consequence of these long-term passages, the resulting virus lost approximately 15% of its genome compared to the parental CVA strain [8]. The genome of MVA is 178 kb in length and has been sequenced [9]. Genomic changes occuring in MVA have been described in several studies [see, *e.g.* 10–13]. Six large genomic deletions have been identified (three of them located in the left and right end of the genome) as well as many shorter deletions, insertions and point mutations, resulting in gene fragmentation, truncation, or deletions of open reading frames (ORFs). As a result of these deletions and disruptions, MVA no longer encodes many of the known poxviral immune evasion and virulence factors, making the virus defective for replication in human cells and avirulent in test animals. Genes that are affected by the genomic changes include host range genes (such as the *K1L* and *C12L/SPI-1* genes, and all ankyrin-like genes but one), genes encoding immunomodulatory proteins (such as the functional receptors for TNF, IFN-γ, IFN-α/β and CC chemokines, or three of the five kelch-like proteins) and also genes encoding some structural proteins (such as the major protein of the A-type inclusion body). Mutations in viral proteins involved in transcription and replication as well as in morphogenesis and intracellular transport of virus particles might also contribute to the *in vitro* replication restriction of MVA. Different MVA strains or isolates have been generated, depending on the passage number in CEF cells. They all originate from the strain developed by Professor Mayr and some of them have been deposited at the European Collection of Animal Cell Cultures (MVA-572: deposit number V94012707; MVA-575: V00120707; MVA-BN®: V00083008) or at the Collection Nationale de Cultures de Microorganismes, Institut Pasteur (CNCM) (MVA-I721: CNCM I721).

2.2. MVA homogeneity

After passage 570 on CEF cells, MVA was considered homogeneous and genetically stable, and unable to replicate in mammalian cells. However, a recent study [14] has shown that some MVA strains such as the deposited strains MVA-572 and MVA-I721 are actually not as homogeneous as previously thought and contain viral populations or variants able to replicate in some human cell line(s) (human keratinocyte cell line HaCat, human embryo kidney cell line 293, human bone osteosarcoma cell line 143B, and human cervix adenocarcinoma cell line HeLa) and in immune deficient mice. It was found that these variants have an altered genotype compared to the original parental MVA strain. They are characterised by the presence of loci (site II and/or V) that are deleted in non-replicating MVA strains and are associated with mammalian host range genes [9] which could explain the increased ability of these variants to replicate in some lines and enrich from the parental MVA strain. Point mutations have also been identified in some variants isolated from immune suppressed mice but most of the point mutations identified affect genes with unknown function. Only a minority of such variants have been observed, explaining why they have not previously been detected by PCR and nucleotide sequencing alone. In addition, the abovementioned study revealed that MVA-BN® (Bavarian Nordic's vaccine (IMVAMUNE®)) failed to replicate in any of the human cell lines tested or in immune-suppressed mice, suggesting that MVA-BN® represents a significantly homogeneous MVA strain.

Most of the MVA strains are actually polyclonal and contain a minority of variants that may differ in their replication profile, and therefore in their attenuation profile. These variants are similar to replicating viruses and are able to replicate in generally considered non-permissive cells for MVA [15]. This finding could have a clear safety impact on the development of MVA as vaccine or viral vector. However, it should be noted that even if replicating variants could lead to the isolation of virulent strains *in vitro*, this has not been observed in any preclinical animal testing or in any human clinical trial undertaken so far [15]. Nevertheless, these findings indicate that the homogeneity and replication competence of the MVA strain used in a clinical trial should be addressed during the risk assessment. Since the attenuation of MVA has been associated with the acquisition of large genomic deletions together with multiple shorter genomic changes, DNA extraction from virus-infected cell cultures followed by PCR analysis and sequencing can be used to characterise the genotype of the MVA strain involved. However, these methods have some limitations for the assessment of complex heterogenic mixtures of viruses potentially containing a small amount of viral populations with an altered genotype and phenotype compared to the original parental MVA strain. One way to assess the presence of these MVA variants is to undertake infection assays using immune-suppressed mice (incapable of producing mature B and T cells and highly susceptible to replicating viruses) in order to recover potential replicating viruses from organs and tissues of these infected mice, combined with PCR analysis and/or sequencing to analyse their genome [14].

2.3. MVA host range and expression effects

A series of studies have been undertaken to determine which cell lines were able to support MVA replication and propagation. It has been reported that MVA growth is restricted to a few cell lines. Nevertheless, only a limited number of mammalian cell lines have been evaluated for MVA multiplication suggesting that it might be possible that other cell lines than those tested could support MVA replication. The known permissive, semi-permissive and non-permissive cells and the corresponding reference studies are listed in Table 2. As already mentioned (see Section 2.2 above), the sensitivity of human cell lines (HeLa and 293) against MVA differs according to studies. This is explained by the fact that the MVA strains used in these studies were polyclonal and contained a minority of variant strains able to replicate in these otherwise considered non-permissive cells.

It has been shown that cytopathic effects, a common feature observed upon infection with wild-type vaccinia Virus (and which include induction of early cell rounding, damage to the host genome and RNA, inhibition of host protein synthesis, and eventually, death of the infected cells) are induced only very moderately with MVA strains [21,22]. Current research also focuses on deciphering

Table 2
Cell lines susceptibility to MVA.

Cell line	References						
	Altenburger et al. [16]	Meyer et al. [8]	Sutter and Moss [17]	Carroll and Moss [18]	Drexler et al. [19]	Blanchard et al. [10]	Okeke et al. [20]
Chick embryo CEF		P		P	P	P	
Chick fibroblast LSCC-H-32		P					
Quail embryo QT35				P			
Syrian hamster cell line BHK-21				P	P		P
Rat cell line IEC-6							P
Monkey kidney fibroblast CV-1	P			SP	P		
Monkey embryonic kidney MA104		P					
African green monkey cell line BSC-1				SP			
African green monkey cell line CV-1				SP			
African green monkey cell line Vero		SP					SP
Human cell line A549							SP
Mouse cell line NMULI							SP
Bovine embryonic lung		SP					
Human cell line 293			NP	NP	SP		NP
Human cell line HeLa		NP	NP	NP	SP	SP	
Human cell line SW 839				NP			
Human cell line Caco-2							NP
Human cell line FHs74int							NP
Human cell line Hutu-80							NP
Rat cell line H411E							NP
Rhesus monkey cell line FRhK-4				NP			
Chinese hamster cell line CHO				NP			NP
Chinese hamster cell line CHL				NP			
Pig cell line PK(15)				NP			NP
Rabbit cell line RK13			NP	NP			NP
Rabbit cell line RAB-9				NP			
Rabbit cell line SIRC				NP			
Mouse DBT			NP				
Mouse BALB3t3		NP					
Bovine MDBK			NP				
Equine dermal			NP				
Human fibroblast MRC5			NP			NP	
Human HRT 18			NP				
Human Hep-2			NP				
Human melanoma SK 29 MEL 1					NP		
Human embryonic lung LC5					NP		
Human astrocytoma 85 HG 66					NP		
Human glioblastoma U 138					NP		
Human T-cell lymphoma C 8166					NP		
T-cell lymphoma HUT78					NP		
B-cell SY 9287					NP		
FS-2						NP	

NP: non-permissive; P: permissive; SP: semi-permissive.

distinct immune cell responses upon MVA infection as it may help in the design of innovative vaccine strategies. Dendritic cells (DCs) are important targets of MVA infection. Upon MVA infection, DC morphology, gene expression profiles, and maturation state are modified [23]. It has been suggested that apoptosis of human DCs upon MVA infection may be responsible for the high immunogenicity of MVA vectored vaccines [24]. Less data is available about the sensitivity of primary epithelial and muscle cells that are likely to be some of the first cells, along with DCs, that may be targeted by the vector after mucosal or intramuscular vaccination.

2.4. History of safe use

MVA was originally developed in the 1970s as a vaccine against smallpox, a human disease caused by two variola viruses, variola minor and variola major, and responsible for millions of deaths. MVA-571 (the 571 serial passage strain on CEF cells) used as a pre-vaccine followed by conventional smallpox vaccination was administered in more than 120,000 individuals in Germany including high-risk subjects such as patients with nervous system disorder, allergy or skin disease, chronic disease, infants and children. Contrary to what had been observed with other vaccinia strains, no serious adverse events were reported during the vaccination campaign with MVA. Only mild or moderate side effects were associated with the use of this vaccine, such as local reaction (redness), fever (in ~2% of vaccinees), "flu-like" symptoms (in ~4% of vaccinees) [25–27]. MVA has since been evaluated in animal models and in human studies (under normal or immune-suppression conditions) and was found to be safe and immunogenic without developing clinical disease [28].

More recently, the strain MVA-BN® has been developed to generate a new smallpox vaccine (third generation vaccine). MVA-BN® is derived from the licensed MVA used in Germany by additional passages in CEF cells for extensive plaque purification, and was shown to be a more stable and homogeneous MVA strain [14]. MVA-BN® and recombinant MVA-BN®-based vectors have been administered to more than 3400 human subjects [29] including high-risk populations (*e.g.* people diagnosed with atopic dermatitis or infected with HIV) in which replicating vaccines are contraindicated [15]. In addition to extensive preclinical studies in animal models, the increasing amount of clinical safety data available for

MVA-BN® (Phase I and Phase II clinical studies) strongly suggests the safety character of this strain [15,30,31].

2.5. Site of replication

MVA is a large complex enveloped virion containing a linear double-stranded DNA genome of 178 kbp. MVA as well as other members of the *Poxviridae* family are unique among DNA viruses in that they replicate in the cytoplasmic compartment of the cell [32]. Compared to other DNA viruses, the possibility for integration of their genetic material into the host chromosome is therefore extremely low [33].

2.6. Biodistribution and dissemination

Biodistribution is defined as the dispersion of the vector within the patient's body from the site of administration. Knowledge of the biodistribution of the viral vector is crucial to evaluate the risk associated with dissemination into the environment and possible transmission to people in close contact with the patient. Indeed, the presence of viral vectors in organs might be indicative of potential shedding through associated excreta. It has been shown that the type of the viral vector involved and the administration route influence the spreading inside the body. It is therefore important that biodistribution is assessed for each viral vector used.

Only a few data about MVA biodistribution could be collected from the published literature. One major study addressed the fate of MVA vector in BALB/c mice after intraperitoneal inoculation in comparison with that of the replication-competent Western Reserve (WR) strain using luciferase-expressing viruses [22]. By measuring the level of luciferase expression in target tissues (spleen, liver, ovaries), it was shown that MVA is able to reach target tissues other than the site of administration and retained the capability to efficiently infect the same tissues as the WR strain. However, the luciferase activity of the MVA vector was decreasing with time falling to a background value at 48 h post inoculation. These results were supported by further studies performed on immune-suppressed mice and macaques [34–37]. The observed decay of luciferase activity suggesting a rapid viral clearance of MVA from the tissues is consistent with the fact that MVA is an attenuated replication-defective strain unable to produce virus progeny.

Another study of Ramirez and co-workers reported that MVA biodistribution depends on the route of administration used. Intraperitoneal or subcutaneous inoculation of luciferase-expressing MVA resulted in virus spread in almost all tissues studied (non-lymphoid tissues, draining and non-draining lymph nodes, spleen) while nasal administration resulted in a more restricted distribution (nasal associated lymph tissue, lungs and graining lymph nodes draining the lungs). Vaginal or rectal immunisation did not support any viral spread in the analysed organs [38].

2.7. Survival in the environment

The *Poxviridae* family is a very diversified family of viruses. Although there are considerable differences between viruses, they share some major characteristics, such as a high environmental stability and an extraordinary high resistance to drying enhanced by the materials in which the virus is released into the environment (dermal crust, serum, blood, other excretions) [39].

2.8. Reconversion to wild type

As mentioned before, MVA corresponds to a highly attenuated strain that has lost approximately 15% of the initial vaccinia genome (see Section 2.1). Although the risk of reconversion to wild type can be considered as negligible, it has been suggested that some of the disrupted or deleted genes could be rescued by recombination in case of co-infection of a MVA-based vaccine and a naturally occurring orthopoxvirus (OPV) [40] (see also Section 3.2 below). Such an event, however, is considered as extremely rare. Moreover, revertance of MVA to a replication-competent phenotype is expected to be highly unlikely because MVA's replication restriction and attenuation is most probably based on a multitude of missing or only partly functional gene products [13].

3. Recombinant MVA vectors

In addition to its use as a vaccine against smallpox, MVA is since the early nineties considered as a suitable backbone for the development of gene therapy recombinant vectors. Since the replication defect occurs at a late stage of virion assembly the gene expression remains unimpaired in non-permissive cells making MVA an efficient expression vector but incapable of causing disseminated infection in mammals [17]. Actually, MVA provides a quite high level of gene expression and has proven to be immunogenic when carrying heterologous antigens in animals and humans [11].

MVA is now being used frequently as a viral vector backbone for the development of recombinant vaccines for infectious diseases and cancer or gene delivery systems. These studies provide some good indications on the clinical safety as well as on the clinical efficacy of recombinant MVA vectors. The first study reporting safety data on immunisation with recombinant MVA in humans dates back to 2003 [41]. Moorthy and co-workers reported the safety profile of a MVA vaccine against *Plasmodium falciparum* malaria. Other studies testing MVA vaccines for prophylaxis or immunotherapy against AIDS, tuberculosis, human papilloma virus-associated cancer, melanoma and other cancers have since then been completed and published (Tables 3 and 4). For instance current research investigates how MVA interacts with dendritic cells (DCs), what mechanisms are involved in their maturation of DCs, and how these cells generate a potent T-cell response to the vector. This is of importance because MVA appears an interesting candidate for the development of vaccines in settings where a T-cell response is required to control disease. A recent study demonstrated how dendritic cells exposed to MVA-based HIV-1 vaccine induce highly functional HIV-1-specific CD8(+) T-cell responses in HIV-1-infected individuals [42].

3.1. Transgene

As shown in the previous sections, MVA is a well characterised viral vector and information concerning the safety of MVA and MVA-backbone can be retrieved from numerous preclinical and clinical studies both in human and in animals. When recombinant MVA-based vectors are used, risk assessment should also focus on the potential risks associated with the transgene. The inserted gene(s) and the corresponding sequence(s) should be clearly described. Transgenes of concern are for example genes that modulate the immune response or that code for toxins. The potential effect of the transgene on the biological properties of the MVA vector should also be considered: the host species, the cell tropism, the possibility of recombination, the virulence of the virus or its biodistribution may actually be modified. The absence of change in the biological properties of the vector after insertion of a foreign gene should be assessed and if necessary, confirmed by *in vitro* and *in vivo* studies.

The stability and integrity of the transgene throughout the manufacturing process is another aspect that should be addressed during risk assessment. It needs to be demonstrated that the integrated sequences have not undergone any rearrangements or

Table 3
Clinical studies using recombinant MVA vector as prophylaxis or therapeutic vaccines against viral, bacterial and parasitic diseases (until 2010).

Target disease	Antigen	Clinical trial (number of trials)	Reference(s)
HIV	HIVA (HIV-1 clade A-derived p24/17 gag)	Phase I (4)	[43–47]
HIV	HIVA (HIV-1 clade A-derived p24/17 gag)	Phase I/II (1)	[48]
HIV	HIV-1-LAI nef (clade B)	Phase I/II (1)	[49]
HIV	HIV-1-LAI nef (clade B)	Phase II (1)	[50]
HIV	Env/gag/pol (clade CRF_A/E)	Phase I (1)	[51]
Malaria	ME-TRAP	Phase I (5)	[41,52–55]
Malaria	ME-TRAP	Phase IIa (1)	[56]
Malaria	ME-TRAP	Phase IIb (1)	[57]
Malaria	ME-TRAP/CS	Phase I (2)	[58,59]
Malaria	CS	Phase I (1)	[60]
Smallpox	–	Phase I (1)	[31]
Smallpox	–	Phase I/Ib (1)	[61]
Tuberculosis	85A	Phase I (6)	[62–67]
Tuberculosis	85A	Phase I/IIa (1)	[68]

CS: circumsporozoite protein; TRAP: thrombosporin related adhesion protein; ME: multiple epitope.

mutations, or have been lost. MVA, such as all orthopoxviruses, encodes its own DNA polymerase which displays a proofreading function. The intrinsic mutation rate of poxviruses and therefore MVA, should be similar to that of other replication systems with proofreading DNA polymerases. Nevertheless, it has been shown that the stability and integrity of the transgene can be affected by several factors. For instance, the formation of spontaneous mutations in recombinant MVA depends on the insertion site of the transgene. In that respect, it has been suggested that recombinant MVA stability could be increased by avoiding transgene insertion at MVA del II site or other sites between non-essential genes, or by eliminating long homonucleotides runs by silent codon alteration to reduce the risk of frameshift mutations [81]. It has also been shown that the stability of the transgene may vary according to the characteristics of the transgene [81], the MVA isolate itself [82] or the cell lines used for viral vector multiplication [83]. In the latter case it was shown that a gene encoding influenza virus haemagglutinin inserted into a MVA vector was stable after several passages on African Green Monkey derived Vero cells but unstable in rat derived IEC-6 cells.

The genetic stability of each recombinant vector should therefore be confirmed by several passages (number that covers the passage from the Master Seed Virus to the Production Batch) at a low multiplicity of infection in CEF cells and hybridisation with a DNA probe specific for the inserted gene.

3.2. Recombination

Orthopoxviruses (OPVs) are widely distributed in the ecosystem, and since recombination among OPVs has been reported to occur at high frequencies [84,85], naturally occurring OPVs may actually represent a pool of viruses available for putative recombination with recombinant MVA vectors during co-infections. Indeed, it has been shown that poxvirus recombination, which is inextricably connected to replication, only requires 12 kb end sequence homology [86]. Since replication is only blocked at a late stage in non-permissive hosts, even the highly attenuated MVA could undergo homologous recombination in non-permissive hosts co-infected with other OPVs [83]. Most specifically, post exposure therapies of MVA to treat pre-existing OPV infection in animals [87] correspond to situations where the risk of co-infection between vaccine strains and naturally circulating relatives may exist [83].

In a recent publication, recombination ability was addressed by co-infecting BHK-21 cells with a MVA vectored influenza A vaccine and a Norwegian cowpox isolate (CPXV). Vero cells that are permissive only for CPXV and not for MVA were used to select CPXV hybrid viruses expressing the Influenza haemagglutinin (HA) gene [83]. It was shown that MVA and CPXV undergo recombination *in vitro*, establishing new CPXV hybrids expressing the HA gene initially present on MVA. The experimental conditions were set up to isolate only CPXV-HA hybrids suggesting that other recombination events did certainly happen but were not reported.

The possibility of recombination should therefore be evaluated for each recombinant vector in the context of its use, by taking into account the susceptibility of the target species to other OPVs as well as any epidemiological data concerning the presence or the absence of OPVs in the area where the vector is planned to be administered. For example, during the development of the vaccinia-rabies glycoprotein recombinant virus for vaccination of red fox, the susceptibility of red fox to cowpox virus was investigated and serological studies were undertaken to investigate the presence of circulating orthopoxviruses in the target population [88].

In situations where a possibility of recombination exists, for example when natural OPVs are expected to be present in the main target cells, the potential consequences of recombination and the

Table 4
Clinical studies using recombinant MVA vectors for prevention and treatment of cancer (until 2010).

Target disease	Antigen	Clinical trial	Reference
Cervical cancer	Transcriptional activator HPV E2	Phase I/II	[69]
Cervical cancer	Transcriptional activator HPV E2	Phase II	[70]
Melanoma	Human tyrosinase	Phase I/II	[71]
Melanoma	Tyrosinase	Phase I	[72]
Melanoma	7 Melanoma tumour antigen cytotoxic T lymphocyte (CTL) epitopes	Phase I	[73]
Breast cancer	MUC1	Phase I	[74]
Breast cancer	Oncogenic growth factor receptor HER-2	Phase I	[75]
Colorectal cancer	Tumour antigen 5T4	Phase I/II	[76]
Colorectal cancer	Tumour antigen 5T4	Phase II	[77]
Prostate cancer	MUC1/IL2	Phase II	[78]
Lung cancer	MUC1/IL2	Phase II	[79]
Renal cell carcinoma	Tumour antigen 5T4	Phase II	[80]

resulting associated risk should be assessed on a case-by-case basis taking into account the characteristics of the recombinant vector (the transgene, especially) and of the naturally occurring OPVs involved. Recombination could result in a modified recombinant vector containing previously deleted genes (and therefore recovering replicative properties) or, alternatively, in the transfer of the transgene into replication competent OPVs. Additional *in vitro* or *in vivo* studies could be designed to investigate the genetic and biological properties of the potential recombining progeny viruses. It must be noted that the probability of recombination between MVA-based vectors and other OPVs is expected to be higher in the case of wildlife or domestic animal vaccination since animals represent a wide reservoir for natural OPVs.

4. Considerations for risk assessment and risk management

4.1. Risk classification

Microbiological agents are categorised into four Risk Groups (Risk Groups 1–4) according to their impact on human health and the environment. Several factors such as the severity of the disease caused, the transmissibility and the availability of effective treatment or vaccine are taken into account to assign an agent to a specific Risk Group. Biological agents that are unlikely to cause disease are classified into Risk Group 1 while agents responsible for severe diseases with a high potential of transmissibility and for which no treatment is available are assigned to Risk Group 4.

According to its high attenuation profile and its history of safe use, MVA is generally classified in Risk Group 1. However, this classification is only valid if the MVA strain is genetically stable, homogenised and is characterised by (i) a high degree of attenuation in mammalian cells; (ii) a host-range restriction (inefficient propagation in mammalian cells: no viral particles are produced); and (iii) a cytoplasmic localisation (no genome integration). The presence of the six deletions in the genome of MVA responsible for its high attenuation and host-restriction can be confirmed by polymerase chain reaction using specific primers.

In case of a recombinant MVA vector, the risk classification should also take into account the potential risk associated with the transgene product. Specific classes of genes including cytokine coding genes and virulence genes are inherently associated with a higher risk (see also [89]). For instance the vaccinia virus gene *K1L*, which is naturally deleted in MVA but has been used as a marker for selection and isolation of recombinant MVA, extends the host range to rabbit kidney cells [90] and encodes viral functions that impair important anti-viral defence mechanisms of the infected host [91].

4.2. Environmental risk assessment

Shedding corresponds to the dissemination of a viral vector in any form into the environment *via* excreta (urine, faeces, sweat, saliva, nasopharyngeal fluids), blood and semen from the treated patient [92]. It is a major concern in the environmental risk assessment since shedding determines the likelihood of exposure of the recombinant vector to contact persons (third parties: untreated persons/animals) and to the environment. Shedding analysis generally consists of the detection in excreta of vector sequences by PCR using vector-specific primers and/or detection of infectious viral particles by biological assays (*in vitro* culture of shed material) [93].

There is currently limited information available from the literature concerning vector shedding in general. Shedding studies are indeed rarely reported in publications on clinical trials. Data concerning shedding associated with MVA-based vectors should therefore be collected and discussed primarily in the context of the environmental risk assessment of regulatory dossiers.

To be sufficiently representative, shedding studies should be designed (determination of the sample collection, sampling frequencies and study duration) by taking into account the dose, the route of administration but also the characteristics of the vector [92]. The capacity of replication is, for example, an important factor to consider. Attenuated replication-deficient vectors do not persist for a long period of time in tissues, suggesting that shedding should be of short duration. Biodistribution studies revealed that MVA does not persist more than 48 h inside the body [6,34]. In one study reporting the results of a Phase I immunotherapy with a MVA expressing human MUC1, urine samples collected 4 h post-injection and on day 8 appeared to be negative for the presence of vector sequences [74]. The sampling should therefore focus on the first few hours following administration and a last control should be performed a few days after administration.

Spreading occurring at the site of administration is another concern in the environmental risk assessment. Especially when the product is administered subcutaneously, viral particles are often found on the skin close to the site of administration. This issue can easily be addressed by cleaning any residual vectors present at the site of injection after each administration [36,76]. To this end, upon injection, the injection site is disinfected using 70% alcohol, isopropanol swabs or other suitable disinfecting agent and covered with a wound dressing to capture any leakage of GMO shortly after the injection. After a standard medical follow-up of the subject during 4 h the wound dressing is removed and replaced by a new disposable dressing before the subject is released from the hospital setting. The original wound dressing is collected together with any other material and/or objects that have been in contact with the GMO material and are destroyed as hazardous medical waste. The second wound dressing can be removed and discarded as normal household waste. The inoculation of MVA vaccine *via* the intramuscular route also eliminates the development of skin pock lesion, reducing the shedding *via* those lesions [15].

It is important to point out that shedding/spreading of the recombinant vector and the resulting dissemination into the environment is not an adverse event *per se*. Significant shedding/spreading will result in greater environmental exposure but its impact will mostly depend on the characteristics of the recombinant vector itself, *i.e.* its capacity to replicate, its survival in the environment, its transmissibility and also the safety profile of the transgene. The shedding/spreading of vectors such as MVA (as long as no potentially harmful transgene is inserted) which are unable to produce new viral progeny and to propagate in most mammalian cells should only lead to limited environmental impact.

4.3. Containment and worker protection measures

When MVA or MVA-based recombinant vectors are used under contained conditions (laboratories, hospital rooms, animal husbandries, production facilities), appropriate containment and other measures to protect human health and the environment shall be implemented as a result of a risk assessment taking into account in particular the characteristics of the microbiological agent manipulated and the nature of the activity. Four basic containment levels are defined in the European legislation (level 1 to level 4, with level 4 being the most stringent) describing the required practices, safety equipment and design criteria of the facilities. The extreme attenuation and the history of safe use of MVA allow handling this virus under containment level 1 in the clinical setting. A containment level 2 should nevertheless be recommended for the manufacturing/production of MVA-based vectors in order to implement adequate precaution measures (physical containment devices and personal protective equipment).

When recombinant MVA vectors are used, the containment level will depend also on the nature of the transgene as it

might ultimately influence the level of risk associated with the recombinant vector (see Section 4.1).

4.4. Laboratory-acquired infections

No laboratory-acquired infections resulting from exposure to MVA strains or to recombinant vectors derived from these strains have been reported in the scientific literature or to the US Centers for Disease Control and Prevention (CDC).

4.5. Waste treatment

Compared to other enveloped virions, poxviruses have a low content of lipids in their envelope: they are therefore less sensitive to organic solvents. Nevertheless, they stay quite susceptible to a variety of chemical disinfectants, such as formaldehyde, glutaraldehyde, ethanol, isopropanol and peracetic acid (PAA) [94]. It has been shown that MVA presents the same disinfection susceptibility profile as the infectious vaccinia Lister Elstree strain [94,95]. In addition to chemical disinfection, steam sterilisation remains very effective to inactivate these viruses [39].

It is recommended that liquid and solid waste (potentially) infected by MVA as well as disposable materials are inactivated before removal in accordance with the regulation in force. Since studies have revealed the presence of residual vectors at the injection sites [36,76], these sites should systematically be cleaned after administration to reduce all risk of vector dissemination.

5. Conclusions

Initially developed in response to the need for a safer vaccine against Variola in the 1970s, MVA is now widely used as recombinant vector for vaccination against various pathogens or as delivery vehicle for gene therapy. Indeed, MVA presents several advantages. It is an attenuated virus that has undergone several deletions including deletion of some mammalian host range genes, which have significantly reduced its virulence and pathogenesis in both healthy and immuno-compromised humans and animals. MVA is not able to propagate in human and in most mammalian cells thus reducing the risk associated with its potential dissemination. In addition, the MVA genome cannot interact with the genome of the infected cells since it remains localised in the cytoplasm which limits the risk of integration. Since 1970, various MVA-based vectors have been administered to thousands of individuals without any major side effects reported.

Although these advantages make MVA vectors potentially safer when compared to other vaccinia strains, we have presented in this document several issues which should be considered carefully when performing the risk assessment of MVA and MVA-based vectors. These biosafety issues and suggested related recommendations are summarised in Table 5.

As shown in Table 5, risk assessment of MVA-based vectors should consider the intrinsic characteristics of the MVA strain (in particular its homogeneity), the characteristics of the transgene (and its potential impact on the properties of the whole recombinant vector) and the possibility of recombination with natural orthopoxviruses. This information is essential to evaluate the potential risk for people manipulating the MVA product or the risk associated with transmission to third parties and dissemination into the environment after shedding or spreading of the viral vector. In that respect, it is recognised that although MVA disseminates quickly into the organism after administration, shedding should be limited to the first hours following administration because the dissemination in the body is followed by rapid viral clearance of the vector due to its replication-deficient property. In addition, the risk of dissemination of potential shed material can be considered negligible since MVA corresponds to a highly attenuated vector unable to propagate in most mammalian cells. Nevertheless, it is important to consider that to date there is a lack of available information on MVA shedding in the literature. Therefore, abovementioned information is critical in guiding, on a case-by-case basis, the design of shedding studies in support to the risk assessment of MVA-based vectors.

Table 5
Biosafety aspects associated with the use of MVA and MVA-based vectors.

	Biosafety issues	Recommendations
MVA homogeneity	Potential presence of variants (minor population) able to replicate in mammalian cell lines	The homogeneity of the MVA strain should be evaluated during the risk assessment, by performing when necessary *in vitro* or *in vivo* infection studies (such as infection of human cell lines or immune-deficient mice with MVA strains) in addition to traditional PCR and sequencing methods
Transgene	Transgene may present hazardous properties or change the vector properties	Risk assessment should take into account the characteristics of the transgene (nature, stability, condition of expression), the construction/production process and the characteristics of the final recombinant vector (absence/presence of new properties compared to the MVA-backbone) and possible or known side effects related to the expression of the transgene
Recombination	Establishment of new vector with novel genetic and biological properties (genes that are interrupted or deleted in MVA could be rescued during recombination; transgene could be transferred to replication competent orthopoxviruses)	Epidemiological data concerning the occurrence of natural OPVs in the area of administration should be analysed to consider the necessity for *in vitro* or *in vivo* co-infection studies (between the recombinant vector and the potential natural OPVs)

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