EXPERT REVIEWS

Candidate influenza vaccines based on recombinant modified vaccinia virus Ankara

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Recombinant modified vaccinia virus Ankara (MVA) is attractive and promising as a novel viral vector for the expression of foreign genes of interest because it possesses unique properties. In particular, its excellent safety profile and the availability of versatile vector technologies have frequently made MVA the vaccinia virus of choice for preclinical and clinical studies. Owing to its avirulence and deficiency to productively replicate after *in vivo* inoculation, MVA can be used under biosafety level 1 conditions. In addition to a better safety profile than replication competent vaccinia viruses, the use of MVA leads to similar levels of gene expression and has better immunostimulatory properties and improved efficacy as a recombinant vaccine. In animal models, recombinant MVA vaccines were immunogenic and induced protective immunity against various infectious agents, including viruses, bacteria and parasites. Here we review the progress that has been made in the development of recombinant MVA as a viral vector and candidate pandemic influenza H5N1 vaccine. Specifically, we will focus on the preclinical evaluation of recombinant MVA vector as pandemic influenza A/H5N1 vaccine candidates and discuss the possible future approaches for the use of these novel MVA-based vaccines.

Influenza viruses are a major cause of upper respiratory tract infections and are responsible for epidemic outbreaks during the winter months. These seasonal influenza epidemics are caused by currently circulating human influenza A and B viruses. For certain patients who are at a high risk of complications due to influenza, annual vaccination against influenza is recommended. Currently used inactivated vaccines are mostly efficacious and reduce morbidity and mortality provided that the strains used for vaccine production match the epidemic strains. Occasionally, novel subtypes of influenza A viruses are introduced into the human population. These new subtypes originate from the avian reservoir of all subtypes of influenza A viruses [1]. Since the human population has never been exposed to these novel subtypes, these viruses can replicate in their new host without the interference of pre-existing virus-neutralizing antibodies. Under these circumstances, new viruses can cause worldwide influenza epidemics, also known as pandemics, with considerable morbidity and mortality in the human population. In the last century, three pandemics occurred, caused by influenza A viruses of the H1N1, H2N2 and H3N2 subtypes [2].

Currently, influenza A viruses of the H5N1 subtype pose a pandemic threat because transmissions from infected birds to humans have been reported during outbreaks of highly pathogenic avian influenza (HPAI) in poultry since 1997 [3–5].

Since 2003, 387 human cases have been reported, of which more than 60% were fatal [101]. So far, these viruses do not spread efficiently from human to human, although sporadic clusters of human-to-human transmissions have been described [6–8].

When the HPAI viruses acquire the necessary adaptations to cause sustained human-to-human transmission, they may cause a future pandemic outbreak [9]. To limit the impact of such an outbreak, the availability of safe and effective vaccines is desirable and considered a high priority by the WHO [102]. Major efforts have been made to prepare such H5N1 vaccines. However, there were a number of issues that complicated the development of such vaccines, including poor vaccine immunogenicity, long response time, limited production capacity and antigenic variation of circulating strains [103].

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Some important developments have been made during the last decade. A review by Kreijtz *et al.* addresses some of these issues, such as the development of rapid procedures to produce vaccine strains (reverse genetics), cell culture technology to produce vaccine independent of embryonated chicken eggs and the development of adjuvants that increase the immunogenicty of conventional vaccine preparations and that would facilitate dose sparing [10]. Here, we discuss modified vaccinia virus Ankara (MVA) as a viral vector for the delivery of influenza virus antigens as a promising technology that addresses most of the issues outlined above.

MVA: a replication-deficient poxvirus vector

Modified vaccinia virus Ankara is an attenuated strain of vaccinia virus that was originally developed for use as safer vaccine during the last decades of the smallpox eradication campaign [11,12]. Indeed, MVA was chosen by the Bavarian State Vaccine Institute in Munich (Germany) as a basis for the evaluation of new vaccine preparations and vaccination strategies against smallpox [13,14]. From 1968 to 1988, MVA immunizations were administered to more than 120,000 individuals in Germany without significant adverse events. The excellent safety profile is also observed in the more recent clinical trials aiming at the approval of MVA as a next-generation smallpox vaccine [15–17]. The extraordinary safety profile was exemplified in studies with immunocompromized macaques. Macaques from which T lymphocytes were depleted by treatment with anti-thymocyte globulin or that received a total-body irradiation were subsequently vaccinated with a high dose of MVA. In these severely immunocompromised animals, the virus did not replicate and did not lead to generalized infection normally seen with wild-type, replication-competent vaccinia viruses [18].

After genetic modification of MVA (i.e., insertion of foreign genes under the control of a vaccinia virus promoter), its potential as viral vector was recognized after demonstrating that MVA can efficiently express foreign genes of interest in nonpermissive human cells [19]. At present, recombinant MVA serves as a vaccine-development platform due to its clinical safety and its potency to induce robust immune responses against heterologous antigens [20–22]. Various recombinant MVA vaccines are currently being evaluated in clinical trials, mostly aiming for prophylaxis or therapy of infectious diseases and cancers against which no vaccine is available [23–27]. The wealth of information that has been obtained from a vast and ever-increasing body of basic and clinical research with MVA vector vaccines has provided answers to important questions related to the development and use of viral vector vaccines. One of these questions relates to the influence of pre-existing antivector immunity and the possibility to repeatedly administer the same vector expressing the same or other antigens. Of course, the potential interference with vaccination by pre-existing antivector antibodies is a concern. However, it is of special interest to note that MVA vector vaccines differ in this respect from many other viral vectors that are not effective in the presence of pre-existing antibodies and that induce antibodies against the vaccine antigen of interest inefficiently upon a second administration of the same vector.

Preclinical evaluation of MVA vectors already demonstrated its capacity to repeatedly boost immune responses directed to the recombinant antigens. This suggests that immunization with nonreplicating MVA resembles immunization with inactivated vaccines more than with replicating live vaccines, which are more sensitive to antivector immunity [28]; for a review of this see [21,22]. Recently, the first encouraging data have been obtained from the therapeutic immunization of humans [29]. This Phase II clinical trial in colorectal cancer patients tested the immunogenicity of six consecutive applications of a MVA vector encoding the tumor antigen 5T4. Despite efficient induction of MVA-specific antibodies already peaking to high levels after the second vaccination, the antibody responses to 5T4 were boosted after each vaccination, with the highest levels found after the fifth and sixth immunization. Thus, vector-specific antibodies do not seem to have a major impact on the induction of antibody responses specific for the target antigen by repeated administration of recombinant MVA vaccines.

Yet, pre-existing antivector immunity may have a greater influence on target antigen-specific CD8$^+$ T-cell responses. Data from MVA vector immunizations with simian immunodeficiency virus antigens in the macaque model firstly suggested that three prior applications of MVA vaccine limited the levels of Gag epitope-specific CD8$^+$ T cells induced by a fourth immunization [30]. MVA delivery of the Ebola virus glycoprotein (GP) in the mouse model demonstrated that prevaccination with replication-competent vaccinia virus inhibited cellular (cytotoxic T cell) but not humoral immune responses to GP [31]. Interestingly, such hindrance of MVA immunogenicity by pre-existing vaccinia-specific immunity could be largely overcome by priming with a GP-specific DNA vaccine [31] or by the use of a new oral vaccination with recombinant MVA attached to TMPEG-modified cationic liposomes [32].

With regard to the induction of T-cell immunity, evidence is accumulating that recombinant MVA vaccines can induce more balanced antigen-specific CD4$^+$ and CD8$^+$ T-cell responses in animal models than other poxviral or adenoviral vectors that elicit either dominant CD4$^+$ or CD8$^+$ antigen-specific T-cell responses [33,34]. Typically, MVA-induced CD4$^+$ T-cell responses are being characterized predominantly as Th1 like [35], which fits well with the recent finding that MVA vector vaccination can be used to protect against allergic sensitization [36]. Strong CD8$^+$ T-cell responses directed against the target antigens were consistently found with various heterologous vaccine prime–MVA boost protocols (first shown with DNA vaccines) [23,24,37,38]. To most efficiently elicit CD8$^+$ T-cell responses with MVA vectors, the delivery of full-length antigen was found to be superior to the expression of peptide antigens or rapidly degradable proteins [39]. These data suggest that the particular importance of cross-priming in MVA-mediated antigen presentation and appears to correlate with recent clinical findings from HIV-1-specific DNA/MVA prime–boost vaccinations in humans [23,40]. Recombinant MVA vaccines expressing HIV proteins as antigens were highly immunogenic, in contrast to more disappointing responses that were elicited by MVA expressing a HIV-1

fusion protein consisting of a string of CD8+ T-cell peptide epitopes [40]. Finally, a long-standing observation is that non-replicating MVA vectors seem to be paradoxically immunogenic in comparison with fully replication-competent vaccinia viruses, which are able to deliver overall drastically higher amounts of antigen upon administration *in vivo* [41,42]. Further evidence supports the notion that MVA has particular immunostimulatory properties [43–45]. Recent experiments in mouse models revealed the *in vivo* synthesis of substantial amounts of type I interferon shortly after MVA vaccine administration and an activation of dendritic cells by both Toll-like receptor (TLR)-9-dependent and TLR-independent pathways [46,47]. Moreover, MVA infection of human monocyte-derived cells can induce or upregulate the expression of genes for host molecules involved in antigen uptake, cytokines, cytokine receptors, chemokines and chemokine receptors [48,49]

New developments in MVA vector generation & vaccine production

The generation of MVA vectors is straightforward, requiring genomic insertion of heterologous gene-expression cassettes. In most cases, this is achieved by homologous recombination between the MVA genome and DNA from a plasmid that carries recombinant gene sequences being placed under the control of poxvirus-specific promoters. Subsequently, the MVA vector viruses are to be clonally isolated, a procedure that is helped by well-established selection techniques [50]. Concerning influenza candidate vaccines, a very rapid generation of recombinant MVA might be desirable. Recent advances in methodology are likely to significantly shorten the time window required to obtain MVA vectors. One particularly well-performing protocol takes advantage of selective propagation on rabbit kidney RK-13 cells [51]. Another elegant method is based on the achievement to clone and engineer the entire vaccinia virus genome within a bacterial artificial chromosome (BAC) [52] and application of this BAC technology might well be an additional viable route to a more rapid and efficient generation of MVA recombinant vaccines [53].

The first MVA vaccines to be used in humans were propagated in embryonated chicken eggs or in cultures of primary chicken embryo fibroblasts (CEFs) [14]. As of today, CEF cultures are still the preferred and sole substrate for the production of MVA (vector) vaccines and, among vaccine producers, there is considerable experience in the manufacture and use of other live virus vaccines against human infections (e.g., measles and mumps). Due to the highly active development of MVA as new third-generation smallpox vaccine, MVA propagation in CEF has been adapted to a large-scale process by Bavarian Nordic [104]. In its manufacturing facility in Kvistgard, Denmark, this company reportedly uses CEF cells grown in suspension in Wave Bioreactor® bags aiming for a production capacity of up to 60 million doses of MVA vaccine per year. In addition to primary CEF culture, the potential use of cell lines could be of great interest to establish a robust and commercially viable manufacturing process with a controlled seed-lot system, full characterization of the production cells and a lower risk of

introducing adventitious agents. For example, the CEF cell line DF-1 can be used to efficiently generate and amplify MVA vector viruses at the laboratory scale [54]. In addition, although MVA cannot productively replicate in most cell lines of mammalian origin, the virus has been found to efficiently multiply in the hamster kidney cell line BHK-21 [55,56] and, more recently, also in rat IEC-6 cells [57]. However, it still remains to be determined if these or other continuous cell lines fulfill the technical and regulatory requirements for development of a commercial manufacturing process for MVA vector vaccines.

MVA induces protective immunity against influenza virus infection

The potential of recombinant MVA to induce protective immunity was already demonstrated 15 years ago. A recombinant MVA was constructed that expressed the hemagglutinin (HA) and NP genes of influenza virus A/PR/8/34 (H1N1) under the control of the synthetic early/late vaccinia virus promotor *sP* [41]. A single immunization with 10^8 plaque-forming units (PFU) of MVA-HA-NP induced strong antibody responses that could be boosted by subsequent immunizations. Furthermore, a single immunization with as low as 10^5 PFU of MVA HA-NP afforded protection against a lethal challenge infection with influenza virus A/PR/8/34 4 weeks later. The protective immunity not only correlated with the induction of virus-specific antibodies but also with anamnestic cytotoxic T lymphocyte (CTL) responses detected in MVA-HA-NP-primed mice. Interestingly, the same MVA vector vaccine also provided protection against influenza challenge upon oral delivery [58]. The enteric administration of two doses (10^8 PFU) MVA-HA-NP elicited serum anti-H1 IgG and mucosal anti-H1 IgA antibodies and protected the upper and lower respiratory tract upon influenza virus challenge. Furthermore, vaccination with this MVA-HA-NP vaccine afforded enhanced recovery from infection with a heterosubtypic (H3N2) influenza A virus strain, which correlated with the induction of cross-reactive CTL responses in the vaccinated mice. More recently, recombinant MVA were constructed that express the HA genes of influenza A/H5N1 viruses A/Hong Kong/156/97 (A/HK/97) and A/Vietnam/1194/04 (A/VN/04), which originate from clades 0 and 1 of A/H5N1 viruses, respectively [59]. Expression of HA was under control of the vaccinia virus promoter *PsynII*. Initially, these MVA recombinants were evaluated in mice and, upon a single immunization with 10^8 PFU, both constructs proved to be immunogenic. However, higher antibody titers were achieved with the recombinant MVA expressing the HA of A/HK/97 (clade 0). After a second immunization, the homologous antibody titers against A/VN/04, in particular, increased, which crossreacted with influenza virus A/HK/97 but not with influenza virus A/Indonesia/5/05 (A/IND/05), a virus belonging to yet another clade (clade 2.1). However, MVA-HA-VN/04-vaccinated mice were not only fully protected against infection with the homologous strain but also against infection with influenza viruses A/HK/97 and A/IND/05. Protective immunity was assessed by scoring clinical

signs of infections (e.g., weight loss), virus titers in the lungs and immunohistochemistry. By contrast, vaccination with MVA-HA-HK/97 only afforded protection against homologous challenge infection. Since promising results were obtained with MVA-HA-VN/04, this vaccine candidate was further evaluated in a nonhuman primate model [60]. To this end, cynomolgus macaques were immunized twice with MVA-HA-VN/04 and then challenged with influenza virus A/Vietnam/1194/04 (clade 1) or A/Indonesia/5/05 (clade 2.1) to assess the level of protective immunity.

Immunization with MVA-HA-VN/04 induced antibodies and prevented replication of both viruses used for challenge infection in the upper and lower respiratory tract and the development of fever and severe necrotizing broncho–interstitial pneumonia. Furthermore, vaccination was well tolerated and did not provoke a rise of body temperature in vaccinated animals. Therefore, MVA-HA-VN/04 is a promising vaccine candidate for the induction of protective immunity in humans against highly pathogenic H5N1 avian influenza viruses that originate from clades of antigenically distinct viruses. Based on these promising results obtained in mice and macaques, further development of recombinant MVA as pandemic influenza vaccine candidates seems warranted.

Collectively, recombinant MVA expressing selected influenza virus proteins are promising and attractive vaccine candidates for the induction of protective immunity against pandemic influenza. The production of vaccine seed strains is fairly easy and straightforward and production of vaccines might even be performed in a flexible way independent of embryonated chicken eggs in CEF. This may seem contradictive; however, CEF cells can be produced in advance and cryopreserved until use. If this is achievable at a large scale, production can start without delay as soon as the seed virus becomes available and independent of a source of embryonated chicken eggs, which

Table 1. Advantages and disadvantages of recombinant modified vaccinia virus Ankara vaccines and implication for pandemic influenza vaccine development.

Advantage	Implication for influenza vaccine production	Disadvantage	Implication for influenza vaccine production
Production			
Independent of embryonated chicken eggs, in (stockpiled) CEF cells	Flexible vaccine production	Use of primary/secondary CEF cells	Higher risk for adventitious agent contamination
Safe, BSL-1 conditions	Ease of manufacturing		
Option to upscale	Increase production capacity		
Efficacy			
Induction of strong antibody responses	Use of adjuvant is not required	High dose required	Increased costs
Induction of cross-reactive antibodies	Protection against antigenically distinct variants		
Induction of T-cell responses	Possibility for broadly protective immunity		
No interference by pre-existing vector immunity	Allows for repeated vaccination and induction of antibodies to multiple influenza virus antigens		
Safety			
Replication deficiency and avirulence	Acceptable safety profile		
Administration to immunocompromized individuals	Vaccination of these high-risk patients possible		
Multivalent vaccines possible	Induction of virus-specific antibodies and T cell Expression of multiple HA genes to induce broad protective antibody responses		
Stability			
Record for stability as lyophilized vaccine >4 weeks at 37°C*	Stockpiling of vaccines possible		

*Taken from [61].
CEF: Chicken embryo fibroblast; HA: Hemagglutinin.

may be in short supply when HPAI viruses are circulating. Furthermore, the use of recombinant MVA expressing the HA gene of A/H5N1 influenza viruses was highly immunogenic. A major advantage of MVA as a vaccine candidate over other vaccine preparations currently under evaluation is that adjuvant systems are not required for high immunogenicity, which will most probably increase their acceptance. Although not studied in great detail, a single dose of recombinant MVA vaccine may be sufficient to protect against infection with a (homologous) virus. However, more research is required to assess the minimal dose and number of vaccinations required to afford protection against homologous and heterologous virus strains. Nevertheless, two immunizations with a high dose of a candidate MVA-based H5 vaccine induced protective immunity against multiple clades of influenza virus A/H5N1 strains in mice and macaques. This indicates that the MVA-H5 vaccine can induce broad protective immunity. Since the immunogenicity of the MVA-based vaccines is not affected substantially by pre-existing antibodies against the vector and repeated vaccinations can induce antibody responses to the target antigens, this approach could possibly be used for repeated vaccination against seasonal influenza virus strains. Based on their favorable properties (Table 1), we conclude that recombinant MVA is an attractive platform for the development of next-generation influenza vaccines.

Expert commentary

During the last decade, the transmission of HPAI A viruses of the H5N1 subtype from infected poultry to humans has raised our awareness of our inadequate preparedness for the next influenza pandemic. The timely availability of safe and effective vaccines would be a cornerstone in controlling the impact of an influenza pandemic. However, the timely delivery of sufficient doses of effective and safe vaccines is a point of concern and has spurred the development of adjuvants that improve the immunogenicity of vaccines and that allow dose sparing and the development of novel production technologies. Recombinant MVA was recently evaluated preclinically as a viral vector for the delivery of the influenza A/H5N1 virus HA. The use of MVA-H5 induced protective immunity in mice and macaques against challenge infection with homologous and heterologous A/H5N1 influenza virus strains. Based on these results and

the unique properties attributed to MVA, recombinant MVA expressing the influenza A/H5N1 virus hemaglutinin is a promising influenza vaccine candidate that could address most issues raised in association with pandemic influenza vaccine development, including timely delivery, production capacity, efficacy and safety. The promising results obtained in animal models warrant further evaluation and development of recombinant MVA-H5 as a pandemic influenza vaccine candidate.

Five-year view

Further evaluation of MVA-H5 in animal models and clinical Phase I/II/III trials is needed to confirm its suitability for use in humans. Based on the increasing clinical experience with MVA as a candidate third-generation smallpox vaccine and as an experimental vector vaccine against various other human diseases, it can be anticipated that a recombinant MVA-H5 vaccine will perform as is expected in humans and will be well tolerated. In addition, dose-finding experiments will assess the minimal dose required for the induction of protective immunity. Furthermore, the possibility to induce protective immunity by a single immunization with the MVA-H5 vaccines needs to be explored, which of course would be an ideal vaccination regimen in the face of a pandemic outbreak. In the long run, the MVA technology may be at the basis of a new generation of safe (pandemic) influenza vaccines that are immunogenic without the use of an adjuvant. To this end, the feasibility to produce MVA-based vaccines in a flexible way in CEFs at a large scale needs to be demonstrated.

Financial & competing interests disclosure

Key issues

- An influenza pandemic caused by influenza A viruses of the H5N1 subtype is imminent.
- To limit the impact of a future influenza pandemic, the timely availability of sufficiently efficacious and safe vaccines is highly desirable.
- Current influenza vaccine production capacity is limited, although the availability of adjuvants facilitates dose sparing and increase vaccine efficacy.
- Modified vaccinia virus Ankara (MVA)-based vaccines have been proven to be safe and efficacious and constitute a promising technology for the development of recombinant vaccines.
- A recombinant MVA expressing the hemagglutinin gene of influenza A/H5N1 viruses was immunogenic in mice and macaques and afforded protection against challenge infection with homologous and heterologous highly pathogenic avian influenza A/H5N1 viruses.
- The development and use of recombinant MVA-H5 could address most of the issues related to the production of a pandemic influenza vaccine and further clinical evaluation seems warranted.

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