MAJOR ARTICLE

Recombinant Modified Vaccinia Virus Ankara–Based Vaccine Induces Protective Immunity in Mice against Infection with Influenza Virus H5N1

Joost H. C. M. Kreijtz,[1] Yasemin Suezer,[2] Geert van Amerongen,[1] Gerrie de Mutsert,[1] Barbara S. Schnierle,[2] John M. Wood,[3] Thijs Kuiken,[1] Ron A. M. Fouchier,[1] Johannes Löwer,[2] Albert D. M. E. Osterhaus,[1] Gerd Sutter,[2] and Guus F. Rimmelzwaan[1]

[1]Department of Virology, Erasmus Medical Center, Rotterdam, the Netherlands; [2]Paul-Ehrlich-Institut, Langen, Germany; [3]National Institute for Biological Standards and Control, Potters Bar, United Kingdom

Since 2003, the number of human cases of infections with highly pathogenic avian influenza viruses of the H5N1 subtype is still increasing, and, therefore, the development of safe and effective vaccines is considered a priority. However, the global production capacity of conventional vaccines is limited and insufficient for a worldwide vaccination campaign. In the present study, an alternative H5N1 vaccine candidate based on the replication-deficient modified vaccinia virus Ankara (MVA) was evaluated. C57BL/6J mice were immunized twice with MVA expressing the hemagglutinin (HA) gene from influenza virus A/Hongkong/156/97 (MVA-HA-HK/97) or A/Vietnam/1194/04 (MVA-HA-VN/04). Subsequently, recombinant MVA–induced protective immunity was assessed after challenge infection with 3 antigenically distinct strains of H5N1 influenza viruses: A/Hongkong/156/97, A/Vietnam/1194/04, and A/Indonesia/5/05. Our data suggest that recombinant MVA expressing the HA of influenza virus A/Vietnam/1194/04 is a promising alternative vaccine candidate that could be used for the induction of protective immunity against various H5N1 influenza strains.

Since the first human cases of H5N1 infections in 1997, influenza viruses of this subtype caused outbreaks of avian influenza worldwide associated with an accumulating number of bird-to-human transmissions. As of 19 November 2006, 258 human cases were recorded, of which 154 proved to be fatal [1]. In addition, the H5N1 virus infections have spread from Southeast Asia to other continents [2].

Because these viruses not only infect avian species but also various mammalian species [3–6], including humans [7], there is a risk of the emergence of a new pandemic strain, either through adaptation of the avian viruses to replication in mammalian species or through the exchange of gene segments with normal epidemic influenza A viruses. For these reasons, the development of effective and safe H5N1 vaccines is considered a priority [8].

However, the development of such vaccines and the production of sufficient quantities of vaccine doses is not straightforward: at present, the combined vaccine production capacity of all manufacturers is not sufficient to timely provide for a worldwide vaccination campaign. There is a clear need for alternative vaccine delivery systems and production technologies that could help to overcome this problem.

Because different antigenically distinct clades of H5N1 viruses have been identified recently [8], an ideal vaccine would also induce cross-protective immunity against these antigenic variants. Recently, conventional

Received 15 November 2006; accepted 20 December 2006; electronically published 17 April 2007.

Potential conflicts of interest: none reported.

Financial support: Netherlands Influenza Vaccine Research Center; Netherlands Organization for Health Research and Development (ZonMW; grant 91402008); European Commission (grants QLK2-CT2002-01034 NOVAFLU and LSHP-CT-2006-037536 MVACTOR); the Forschungs-Sofortprogramm Influenza of the Federal Government of Germany.

Reprints or correspondence: G. F. Rimmelzwaan, Dept. of Virology, Erasmus Medical Center, PO Box 1738, 3000 DR Rotterdam, The Netherlands (g.rimmelzwaan@erasmusmc.nl).

The Journal of Infectious Diseases 2007;195:1598–606

0022-1899/2007/19511-0008$15.00
DOI: 10.1086/517614

inactivated vaccine preparations have been evaluated, such as whole-inactivated virus (WIV) and split-virion vaccines [9–11]. In addition, vaccines based on recombinant hemagglutinin (HA) expressed by baculoviruses have been tested [12–14]. In immunologically naive individuals, these vaccines were poorly immunogenic and appreciable antibody responses were only induced when a high dose or a combination with an adjuvant such as alum was used [9–11].

Clearly, additional development efforts are urgently needed to overcome a catastrophic shortage of vaccine in the case of a H5N1 influenza pandemic. New promising influenza vaccine candidates include recombinant DNA–based vaccines and adenoviral vector vaccines [15–18]. However, the efficacy of these experimental vaccines in humans still needs to be confirmed, and, at present, they are not considered widely acceptable for use in human populations [16].

In the present study, we evaluated another candidate vector vaccine based on a replication-deficient poxvirus vaccine strain: modified vaccinia virus Ankara (MVA). MVA had been tested originally in >120,000 individuals and proved to be a safe and effective vaccine against human smallpox [19]. More recently, recombinant MVA expressing foreign genes proved successful in evoking immune responses and providing protection against diseases caused by viruses, bacteria, parasites, or tumors from which the antigens were derived [20–26].

The advantages of using MVA vector vaccines include their established safety profile in humans, their efficacy on delivery of heterologous antigens in clinical trials, and the availability of technologies for large-scale production under the requirements of good manufacturing practice [20, 24, 27, 28]. Other properties are good immunogenicity, extreme host-range restriction, possibility of long-term storage (stockpiling), and easy production at biosafety level 1 (BSL-1) conditions in chicken embryo fibroblasts (CEF) and baby hamster kidney cells [20, 23, 28–31].

Here, we describe the construction and evaluation of 2 different recombinant MVA viruses expressing the HA genes of H5N1 influenza viruses A/Hongkong/156/97 (A/HK/156/97) or A/Vietnam/1194/04 (A/VN/1194/04). These recombinant viruses were evaluated as vaccines in a mouse model to assess the induction of protective immunity against 3 different H5N1 viruses. A 2-dose immunization regimen induced strong antibody responses that partially cross-reacted with heterologous H5N1 strains. The elicited antibody responses correlated with protection against challenge infection with homologous and heterologous influenza virus strains. Thus, MVA can be considered as a promising alternative vaccine candidate for the induction of protective immunity against H5N1 influenza viruses.

MATERIALS AND METHODS

Vaccine preparation. The influenza H5N1 viruses A/HK/156/97 and A/VN/1194/04 were propagated in Madin Darby canine kidney (MDCK) cells, and the viral RNA was extracted from the culture supernatants using an RNA isolation kit (Roche). Subsequently, cDNA was synthesized from the vRNA using Superscript reverse-transcriptase (Invitrogen) and the AGCAAAAGCAGG oligonucleotide (Eurogentec) as primer. Next, the HA genes were amplified by polymerase chain reaction (PCR) using Pfu (Stratagene) as heat-stable DNA polymerase. Primer sequences were extended with the *NotI* and *XhoI* restriction sites to facilitate directional cloning into the plasmid pBluescriptSK$^+$ (Stratagene). Primer sequences are available on request. Subsequently, HA gene sequences were excised from these plasmids by *NotI/XhoI* digestion, treated with Klenow polymerase to generate blunt ends, and cloned into the *PmeI* site of MVA expression plasmid pIIIdHR-PsynII to generate the MVA vector plasmids pIII-HA-HK/97 and pIII-HA-VN/04.

On transfection in MVA-infected cells, these plasmids' direct insertion of foreign genes into the site of deletion III within the MVA genome [31] and allow transcription of the HA target genes under control of the vaccinia virus–specific promoter PsynII [32]. Recombinant viruses MVA-HA-HK/97 and MVA-HA-VN/04 were generated in primary CEFs on transfection with 1 μg of plasmid DNA, infection with 0.05 infectious U/cell MVA isolate F6 [32], and plaque selection on RK-13 cells [28]. The recombinant MVA genomes were analyzed by PCR to verify HA gene insertion and genetic stability. The production of HA antigens by the MVA vector viruses was confirmed by Western blot analysis of CEF cell lysates harvested at various time points after infection with MVA-HA-HK/97 or MVA-HA-VN/04 (data not shown). One-step and multiple-step growth analysis in CEFs demonstrated that the replication capacities of MVA-HA-HK/97 and MVA-HA-VN/04 were comparable to nonrecombinant MVA (data not shown).

To generate vaccine preparations, the viruses were amplified in CEFs, purified by ultracentrifugation through sucrose, and reconstituted in 1 mmol/L Tris/HCl (pH 9.0). MVA vaccines were used at a dose of 10^8 pfu diluted in 100 μL of PBS.

Whole-inactivated NIBRG-14 virus, a reassortant vaccine strain based on influenza virus A/VN/1194/04 made by reverse genetics was used as positive control. The lyophilized whole-virus antigen was reconstituted in distilled water at a concentration of 2 μg of HA/50 μL and mixed 1:1 with the adjuvant Stimune (Specol; Cedi-Diagnostics) [33]. Control mice were inoculated with PBS.

Influenza viruses. Influenza viruses A/HK/156/97, A/VN/1194/04, and A/Indonesia/5/05 (A/IND/5/05) were inoculated in the allantoic cavity of 11-day-old embryonated chicken eggs. The allantoic fluid was harvested after 2 days. Infectious

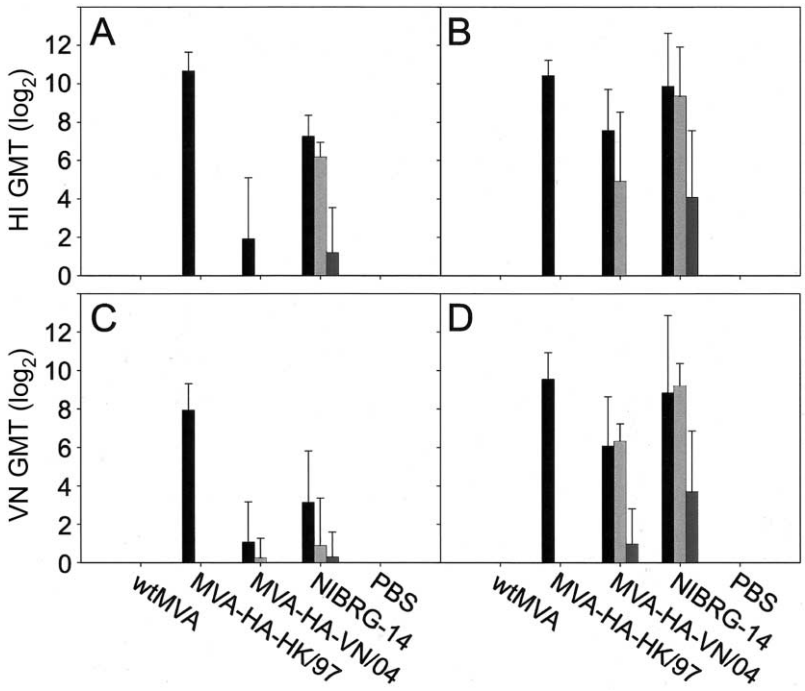

Figure 1. Antibody responses induced by vaccination. Antibody titers against the 3 challenge viruses—influenza virus A/HK156/97 *(black bars),* A/VN/1194/04 *(light gray bars),* and A/IND/5/05 *(dark gray bars)*—were measured by hemagglutination inhibition (HI) assay 28 days after the first immunization *(A)* and 28 days after the second immunization *(B).* Antibody titers against the 3 different challenge viruses were measured by virus neutralization (VN) assay 28 days after the first immunization *(C)* and 28 days after the second immunization *(D).* Data are geometric mean titers (GMTs) (log_2). MVA, modified vaccinia virus Ankara; wtMVA, wild-type MVA.

virus titers were determined in MDCK cells as described elsewhere [34].

Mice. Female specified pathogen–free 6–8-week-old C57BL/6J mice were purchased from Charles River. Mice were divided in 5 groups of 18 and immunized with PBS, MVA-HA-HK/97, MVA-HA-VN/04, wild-type MVA (wtMVA), or Stimune-adjuvanted NIBRG-14. Immunizations were performed intramuscularly: 50 μL in the left hind leg and 50 μL in the right. Four weeks later, blood samples were collected, and mice were immunized again as described above. After another 4 weeks, blood samples were again collected, and each of the 5 vaccine groups was divided into 3 subgroups of 6 mice each. The subgroups of each vaccine group were inoculated with 10^3 $TCID_{50}$ of influenza virus A/HK/156/97, A/VN/1194/04, or A/IND/5/05 in 50 μL of PBS by the intranasal route. A challenge dose of 10^3 $TCID_{50}$ of the respective H5N1 viruses was used because this resulted in the infection and significant loss of body weight in >90% of the mice reproducibly. Six nonimmunized mice were used as negative controls and were inoculated with 50 μL of PBS. Mice were weighed every day until day 4 after infection and then killed by exsanguinations. After euthanasia, brains, lungs (inflated with formalin), spleens, and intestines were taken out.

Mice in all groups were properly age matched at the time point of challenge infection. The experimental protocol was approved by an independent animal ethics committee before the start of the experiment. Intramuscular immunizations, intranasal infections, blood sampling, and euthanasia were performed under anesthesia with inhalative isoflurane. The mice were housed in filter-top cages and had access to food and water ad libitum. During the 5 days of infection with the H5N1 influenza virus, mice were placed in filter-top cages in BSL-3 containment facilities. One BSL-3 isolator unit was used per virus.

Virus titers in organ tissues. Organs were snap frozen using a dry ice/ethanol bath and were stored at −70°C. Organs were homogenized with a Polytron homogenizer (Kinematica AG) in transport medium (Hanks' medium [MEM]), glycerol, 100 U/mL penicillin, 100 μg/mL streptomycin, polymyxin B, nystatin, gentamicin, 7.5% NaHCO3, and 1 mol/L HEPES). Quintuplicate 10-fold serial dilutions of these samples were used to determine the virus titers on confluent layers of MDCK cells.

Serological analysis. After treatment with cholera filtrate and heat inactivation at 56°C, the serum samples were tested for the presence of anti-HA antibodies. For this purpose, a hemagglutination inhibition (HI) assay was used following a standard protocol of 1% turkey erythrocytes and 4 HA U of either influenza virus A/HK/156/97, A/VN/1194/04, or A/IND/5/05 [35]. For this purpose, reverse genetics viruses were produced from which the basic cleavage site was removed. The

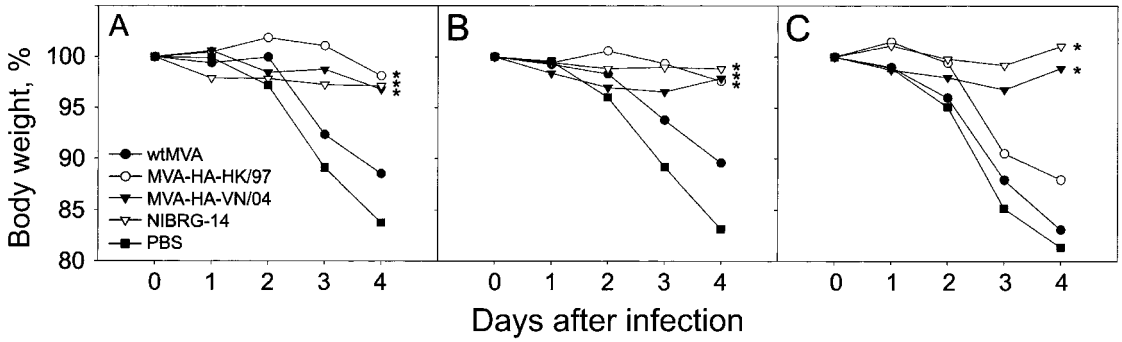

Figure 2. Weight loss in mice intranasally infected with 10^3 $TCID_{50}$ of influenza virus A/HK156/97 *(A),* A/VN/1194/04 *(B),* or A/IND/5/05 *(C).* Mean weight loss is expressed as the percentage of the original weight before infection. *Statistically significant difference ($P < .05$). MVA, modified vaccinia virus Ankara; wtMVA, wild-type MVA.

use of these reverse genetics viruses was validated, and titers obtained were comparable with those against the wild-type strains (data not shown). Serum samples were also tested for the presence of virus-neutralizing antibodies specific for the 3 influenza viruses using a micro virus neutralization (VN) assay with 100 $TCID_{50}$ of the respective viruses that were produced by reverse genetics as described above [36]. Hyperimmune serum obtained from a swan immunized twice with inactivated H5N2 influenza virus A/Duck/Potsdam/1402/86 (Intervet) was used as a positive control against the 3 different influenza A viruses.

Histopathological analysis. Formalin-inflated lungs were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin-eosin for histological evaluation. Sequential slides were stained using an immunoperoxidase method with a monoclonal antibody (clone HB65 IgG2a [American Type Culture Collection]) directed against the nucleoprotein of influenza A virus. A goat anti–mouse IgG2a horseradish peroxisade (Southern Biotech) was used as the secondary antibody. The peroxidase was revealed using diamino-benzidine as a substrate, resulting in a deep red precipitate in the nuclei of influenza A virus–infected cells and a less intense red staining of the cytoplasm. The sections were counterstained with hematoxylin.

Statistical analysis. Data for viral titers and antibody titers were analyzed using the 2-sided Student's *t* test, and differences were considered significant at $P < .05$.

RESULTS

Serological results. On a single vaccination with MVA-HA-HK/97, mice developed antibody responses against the homologous virus strain with geometric mean titers (GMTs) of 1629 and 239 measured in HI and VN assays, respectively. These antibodies, however, did not cross-react with the influenza virus strains A/VN/1194/04 and A/IND/5/05 (figure 1). Four weeks after the booster vaccination, the homologous antibody GMTs in the HI and VN assays were 1370 and 744, respectively. Again

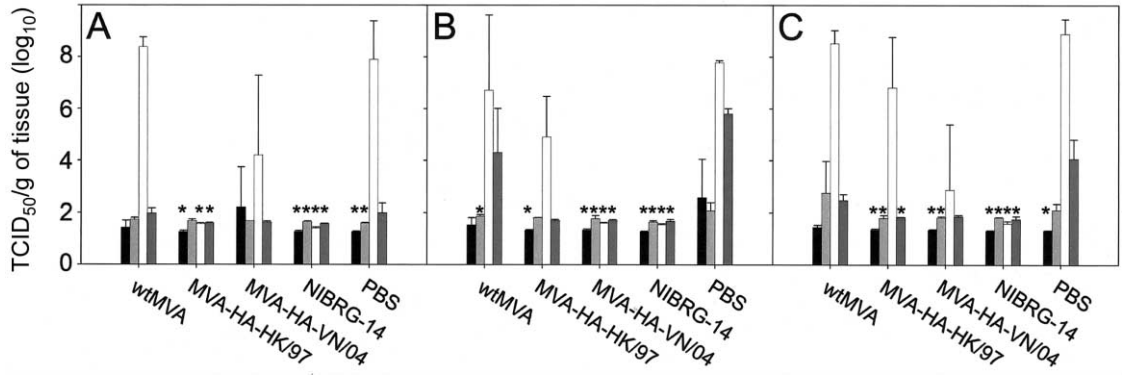

Figure 3. Virus titers in organ tissue at day 4 after infection with either influenza virus A/HK156/97 *(A),* A/VN/1194/04 *(B),* or A/IND/5/05 *(C).* Results are shown for the wild-type modified vaccinia virus Ankara (wtMVA)–, MVA-HA-HK/97–, MVA-HA-VN/04, Stimune-adjuvanted NIBRG-14–, and PBS-immunized mice. Titers were measured in brains *(black bars),* intestines *(light gray bars),* lungs *(white bars),* and spleens *(dark gray bars)* and presented as $TCID_{50}$ per gram of tissue (log_{10}). *Average virus titer below the cut-off value and all mice tested negative by virus isolation.

Table 1. Positive virus isolation from tissues and weight loss in individual mice after challenge infection.

	A/HK/156/97					A/VN/1194/04					A/IND/5/05				
Vaccine	Brain	Intestines	Lung	Spleen	>10% WL	Brain	Intestines	Lung	Spleen	>10% WL	brain	Intestines	Lung	Spleen	>10% WL
wtMVA	1/6	1/6	6/6	4/6	5/6	2/6	0/6	6/6	3/6	4/6	2/6	2/6	6/6	4/6	5/6
MVA-HA-HK/97	0/4	0/4	0/4	0/4	0/4	0/6	1/6	6/6	1/6	1/6	0/6	0/6	6/6	0/6	4/6
MVA-HA-VN/04	2/6	0/6	2/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	2/6	0/6	0/6
NIBRG-14	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
PBS	0/5	0/5	5/5	3/5	4/5	3/5	2/5	5/5	3/5	5/5	0/6	3/6	6/6	4/6	6/6

NOTE. Data are no. of mice with characteristic/total no. of mice in group. Group nos. <6 are the result of fatalities due to causes not related to the experiment. MVA, modified vaccinia virus Ankara; WL, weight loss; wtMVA, wild-type MVA.

no cross-reaction was observed with the other H5N1 strains. The MVA-HA-VN/04 vaccine preparation was less immunogenic, because, after the first vaccination, none of the mice developed HI antibodies against the homologous strain, and only 1 mouse developed VN antibodies. After a second dose, all mice responded, and the GMT increased to 20 and 64 as measured by the HI and VN assays, respectively. The antibodies induced by MVA-HA-VN/04 vaccination cross-reacted with the H5N1 strain A/HK/156/97 and to a limited extent with the strain A/IND/5/05. The adjuvanted NIBRG-14 vaccine preparation, which was included in the experiments as a positive control, induced robust antibody responses against the homologous A/VN/1194/04, which cross-reacted with the strain A/HK/156/97 and A/IND/5/05 both in the HI and VN assays.

Clinical signs. From day 2 after infection onward, mice immunized with PBS or wtMVA developed clinical signs including hunched posture, rapid breathing, ruffled fur, and decreased muscle strength irrespective of the influenza H5N1 virus that was used for infection. These clinical signs were not observed in mice infected with influenza virus A/HK/156/97 or A/VN/1194/04 after vaccination with MVA-HA-HK/97 or MVA-HA-VN/04. MVA-HA-VN/04 vaccination also prevented the development of clinical signs caused by infection with influenza virus A/IND/5/05. The observed protection against clinical signs correlated with reduced loss of body weight after infection (figure 2). In PBS- and wtMVA-immunized mice, an average loss of body weight of 16.2% and 11.5% was observed after infection with influenza virus A/HK/156/97 (figure 2*A*) or 16.9% and 10.4% after infection with influenza virus A/VN/1194/04 (figure 2*B*), respectively. This was largely prevented by vaccination with MVA-HA-HK/97 or MVA-HA-VN/04 (figure 2). Also, infection with influenza virus A/IND/5/05 (figure 2*C*) caused severe loss of body weight in PBS- or wtMVA-immunized control mice (16.9% and 18.6%, respectively), which was significantly reduced by vaccination with MVA-HA-VN/04 but not by vaccination with MVA-HA-HK/97.

Virus replication in organs. Infectious virus titers were determined in brains, intestines, lungs, and spleens on day 4 after infection with influenza viruses A/HK/156/97 (figure 3*A*), A/VN/1194/04 (figure 3*B*), or A/IND/5/05 (figure 3*C*).

After infection, the highest virus replication was observed in the lungs with average lung virus titers of $10^{7.9}$, $10^{7.8}$, and $10^{8.9}$ $TCID_{50}$/g of tissue for PBS control mice infected with influenza viruses A/HK/156/97, A/VN/1194/04, or A/IND/5/05, respectively. Mice vaccinated with wtMVA were not protected, and similar average virus titers were found in the lungs of infected mice. In some mice of both groups, virus replication could be demonstrated in extrarespiratory tissues including brain, intestines, and spleen (table 1). Vaccination with MVA-HA-HK/97 prevented replication of influenza virus A/HK/156/97 in the lungs and other organs completely, whereas, with MVA-HA-VN/04 vaccination, a reduction of virus replication in the lungs was observed in 4 of 6 mice.

After challenge infection with influenza virus A/VN/1194/04, it was the other way around: vaccination with MVA-HA-VN/04 prevented replication completely, whereas vaccination with MVA-HA-HK/97 only partially reduced virus replication. This reduction was statistically significant, compared with that in PBS-inoculated mice ($P < .05$). Vaccination with MVA-HA-VN/04 also prevented replication of influenza virus A/IND/5/05 in the lungs of 4 of 6 mice resulting in reduced average lung titers, compared with those in PBS- and wtMVA-immunized control mice. Vaccination with MVA-HA-HK/97 did not prevent replication of influenza virus A/IND/5/05, and all 6 mice tested positive (table 1). Vaccination with the inactivated whole-virus NIBRG-14 adjuvanted with Stimune not only prevented replication of the homologous influenza virus strain A/VN/1194/04 but also that of A/HK/156/97 and A/IND/5/05.

Pathologic changes and virus replication in the lungs. Four days after infection with each of the 3 HPAI viruses, the mice were killed, and their lungs were inflated with formalin and examined by immunohistochemistry and histological analysis. For all 3 viruses, viral antigen expression and lesions depended on the type of prior immunization. In mice immunized with adjuvanted NIBRG-14, neither viral antigen expression nor lesions were observed after infection with any of the 3 viruses (figure 4*D,* 4*I,* and 4*N*).

After infection with influenza virus A/HK/156/97, PBS- and wtMVA-immunized mice had multifocal expression of viral antigen in bronchiolar and alveolar epithelial cells. This was

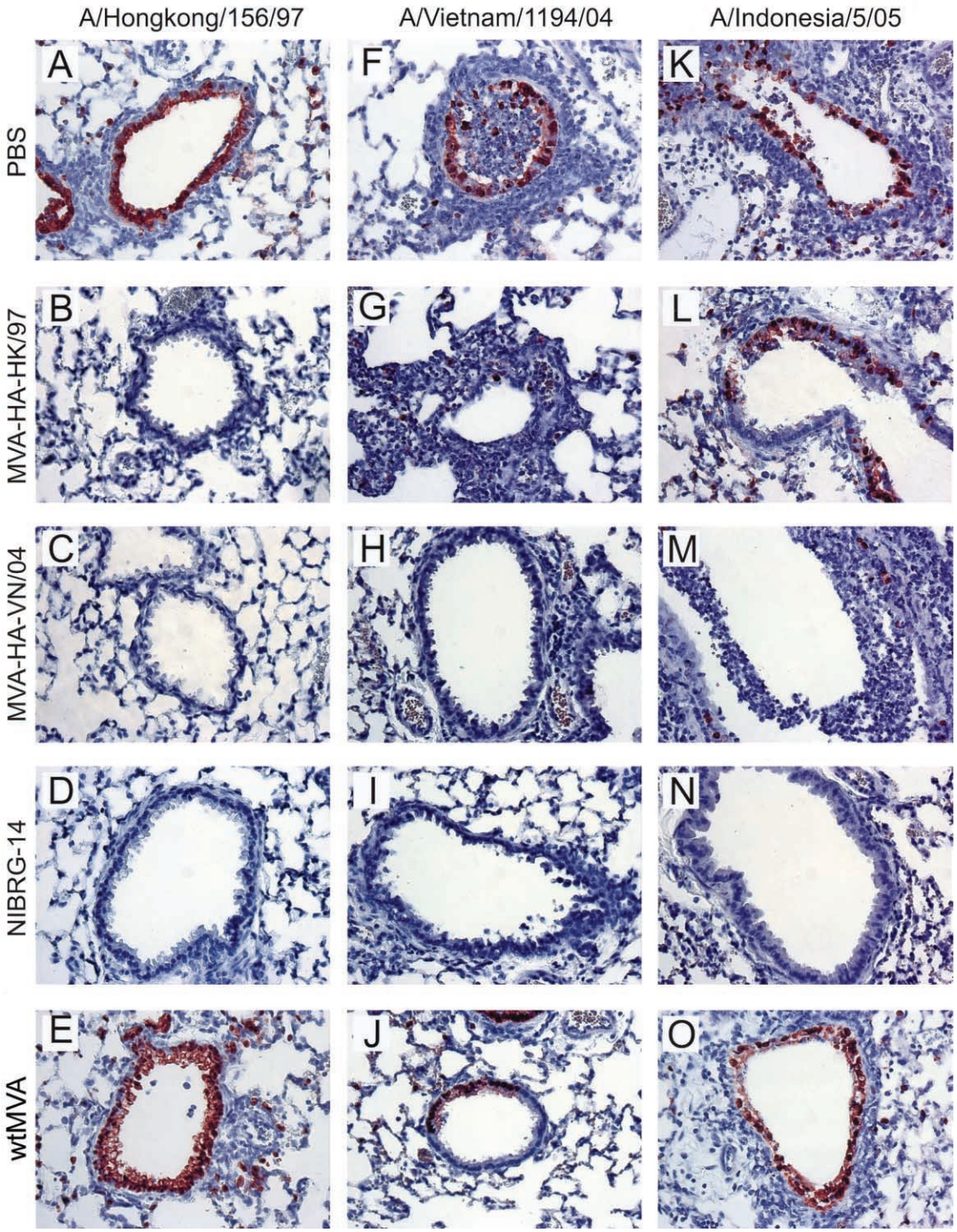

Figure 4. Histopathological analysis and immunohistochemistry of the bronchioles and alveoli in lungs of mice infected with either influenza virus A/HK/157/97, A/VN/1194/04, or A/IND/5/05, as indicated. Influenza virus A/HK/156/97 infection led to viral antigen expression in cells of the bronchiolar wall of PBS- *(A)* and wild-type modified vaccinia virus Ankara (wtMVA)–immunized *(E)* mice, combined with mild peribronchiolar inflammatory infiltrate, whereas, in the lungs of MVA-HA-HK/97– *(B),* MVA-HA-VN/04– *(C),* and Stimune-adjuvanted NIBRG-14–immunized *(D)* mice, no viral antigen was detected. Infection with influenza virus A/VN/1194/04 resulted in expression of viral antigen in cells of the bronchiolar walls of PBS- *(F),* MVA-HA-HK/97– *(G),* and wtMVA-immunized *(J)* mice, also combined with moderate peribronchiolar infiltrate (except for the wtMVA-immunized mice). No viral antigen expression or morphological changes were detected in MVA-HA-VN/04– *(H)* and Stimune-adjuvanted NIBRG-14–immunized *(I)* mice. Infection with influenza virus A/IND/5/05 resulted in abundant viral antigen expression in the bronchioles of PBS- *(K),* MVA-HA-HK/97– *(L),* and wtMVA-immunized *(O)* mice, combined with moderate peribronchiolar infiltrate. Only minimal viral antigen expression was detected in the bronchiolar wall of MVA-HA-VN/04–immunized *(M)* mice, combined with moderate inflammatory infiltrate. No viral antigen was detected in the lungs of Stimune-adjuvanted NIBRG-14–immunized *(N)* mice after infection with influenza virus A/IND/5/05.

associated with mild necrotizing bronchiolitis, characterized by necrosis of bronchiolar epithelial cells and peribronchiolar infiltration by inflammatory cells, mainly lymphocytes (figure 4*A* and 4*E*). In contrast, MVA-HA-HK/97– and MVA-HA-VN/04–immunized mice showed neither viral antigen expression nor lesions.

After infection with influenza virus A/VN/1194/04, PBS- and wtMVA-immunized mice had more widespread expression of viral antigen in bronchiolar and alveolar epithelium than in A/HK/156/97-inoculated mice (figure 4*F* and 4*J*). This was associated with moderate bronchiolitis and mild interstitial pneumonia, characterized by loss of alveolar epithelium and the presence of edema fluid and inflammatory cells (mainly neutrophils) in alveolar lumina. MVA-HA-HK/97–immunized mice appeared to have less viral antigen expression in alveolar epithelium than wtMVA- and PBS-immunized mice, but the extent of interstitial pneumonia was comparable (figure 4*G*). Again, MVA-HA-VN/04–immunized mice showed neither viral antigen expression nor lesions (figure 4*H*).

After infection with influenza virus A/IND/5/05, not only PBS- and wtMVA-immunized mice but also MVA-HA-HK/97–immunized mice had widespread expression of viral antigen in bronchiolar and alveolar epithelium (figure 4*K,* 4*O,* and 4*L*), associated with moderate bronchiolitis and moderate interstitial pneumonia. In contrast, MVA-HA-VN/04–immunized mice only had viral antigen expression in a few bronchiolar epithelial cells, associated with moderate bronchiolitis (figure 4*M*).

DISCUSSION

In the light of the pandemic threat caused by influenza H5N1 viruses, the availability of sufficient doses of safe and effective vaccines is considered a priority [1, 8]. In the present study, we have evaluated recombinant MVA expressing the HA genes of 2 different influenza H5N1 viruses for the induction of protective immunity against 3 different influenza H5N1 viruses belonging to 2 different clades [37] in a mouse model. Vaccination with MVA expressing the HA of influenza H5N1 viruses induced potent antibody responses, which correlated with protection against homologous and heterologous challenge infection.

For the generation of the MVA recombinants, the HA genes were derived from influenza viruses A/HK/156/97 and A/VN/1194/04. The cocirculation of antigenically different influenza virus strains complicates the development of effective vaccines considerably. Usually protective immunity is only induced with vaccines that closely match the circulating strains. The viruses used in the present study belong to distinct clades of H5N1 viruses [37, 38] and are antigenically different [37]. This allowed the assessment of the level of cross-protective immunity induced by vaccination against these 2 viruses. Furthermore, a third H5N1 variant strain was used for challenge infection of the mice: A/IND/5/05, which was antigenically distinct from the other 2 viruses [8].

The recombinant MVA-HA-HK/97 was highly immunogenic. A single immunization already induced antibody responses against influenza virus A/HK/156/97, which were further boosted by a second immunization. These antibodies were not cross-reactive in HI and VN assays with A/VN/1194/04 or A/IND/5/05. MVA-HA-VN/04 was less immunogenic, but, after 2 immunizations, good antibody responses were observed, not only against the homologous virus but also to A/HK/156/97 and to a lesser extent to A/IND/5/05. The observed antibody reactivity pattern is similar to that observed with postinfection ferret serum samples [8]. Thus, this asymmetry in antibody recognition pattern observed with antibodies induced by MVA-HA vaccination resembled that observed with antibodies induced after infection with the original influenza viruses [8].

The NIBRG-14 vaccine preparation was included in the experiments as a positive control and was highly immunogenic in combination with the Stimune adjuvant. This combination not only induced strong antibody responses to the homologous influenza virus A/VN/1194/04 but also to the other 2 H5N1 strains.

The HI and VN antibody titers measured against the 3 H5N1 strains correlated with protection against challenge infection. The MVA-HA-HK/97–immunized mice were only protected against a homologous challenge infection. Vaccination prevented virus replication completely, and, as a result, neither histopathological changes nor clinical signs were observed in these mice. Although the MVA-HA-HK/97–induced antibodies did not cross-react with influenza virus A/VN/1194/04, replication of this virus was reduced, and the immunized mice were protected from clinical signs (table 1). In contrast, no protective effects were seen on challenge infection with influenza virus A/IND/5/05. Although it is known that MVA vaccination can induce strong cytotoxic T lymphocyte (CTL) responses that could have contributed to protection [39], it is unknown at present whether H-2b–restricted cross-reactive CTL epitopes exist on the HA molecule of influenza H5N1 viruses.

Immunization with MVA-HA-VN/04 induced sterilizing immunity against the homologous strain. In addition, strong protective effects were observed against the antigenically distinct influenza viruses A/HK/156/97 and A/IND/5/05. The replication of these viruses was largely reduced in most immunized mice, which correlated with the absence of infected cells in the respiratory tract and the lack of clinical signs. The protection is most likely based on virus-neutralizing HA-specific serum antibodies that pass from the circulation into the alveolar epithelium [40].

Thus, the use of MVA-HA as a candidate vaccine against emerging pandemic H5N1 strains has the potential to induce a broad immune response that protects individuals from severe

clinical signs and histopathological changes in the respiratory tract even when the strains causing the infections do not fully match the vaccine antigen. In addition, MVA-based vaccines have a number of properties that make them favorable vaccine candidates for use in humans. First, recombinant MVA can be considered to be extremely safe viral vectors because of their distinct replication deficiency in mammalian cells and their well-established avirulence in vivo [22–24, 41–43] including the safety of MVA in immune-suppressed macaques [44] or the innocuous application of high doses of recombinant MVA to HIV-infected individuals [27, 45, 46]. Second, industrial scale manufacturing of MVA vaccines appears feasible in recognition of the efforts undertaken to develop MVA as a third-generation vaccine against orthopoxvirus-related biothreats [47]. Third, MVA vector vaccines can deliver multiple heterologous antigens and allow for simultaneous induction of high-level humoral and cellular immunity [20, 24, 48], providing the possibility to develop multivalent vaccines.

Because the production of these MVA-based vaccines is independent of existing production capacity for conventional influenza vaccines, it may help to reduce the envisaged shortage of vaccine doses in the time of an emerging pandemic. Another advantage is that the excellent immunogenicity of these vaccines is independent of the use of adjuvants. We acknowledge that the use of a safe and effective adjuvant could improve the immunogenicity of conventional vaccines and may reduce the antigen quantity required to induce adequate antibody responses (dose sparing). Our results with the Stimune-adjuvanted NIBRG-14 WIV underscores this possibility. However, at present, such potent adjuvant formulations are not considered suitable for use in humans. We conclude that MVA-based H5N1 vaccines are promising vaccine candidates with favorable properties regarding safety, effectiveness, and the potential of rapid large-scale production, which are important in the face of an emerging pandemic.

Acknowledgments

We thank C. Baas, T. Bestebroer, N. Nieuwkoop, and J. Verhagen for excellent technical assistance.

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