

Recombinant Modified Vaccinia Virus Ankara Expressing the Hemagglutinin Gene Confers Protection against Homologous and Heterologous H5N1 Influenza Virus Infections in Macaques

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Background. Highly pathogenic avian influenza viruses of the H5N1 subtype have been responsible for an increasing number of infections in humans since 2003. More than 60% of infected individuals die, and new infections are reported frequently. In light of the pandemic threat caused by these events, the rapid availability of safe and effective vaccines is desirable. Modified vaccinia virus Ankara (MVA) expressing the hemagglutinin (HA) gene of H5N1 viruses is a promising candidate vaccine that induced protective immunity against infection with homologous and heterologous H5N1 influenza virus in mice.

Methods. In the present study, we evaluated a recombinant MVA vector expressing the HA gene of H5N1 influenza virus A/Vietnam/1194/04 (MVA-HA-VN/04) in nonhuman primates. *Cynomolgus* macaques were immunized twice and then were 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.

Results. Immunization with MVA-HA-VN/04 induced (cross-reactive) antibodies and prevented virus replication in the upper and lower respiratory tract and the development of severe necrotizing bronchointerstitial pneumonia.

Conclusion. Therefore, MVA-HA-VN/04 is a promising vaccine candidate for the induction of protective immunity against highly pathogenic H5N1 avian influenza viruses in humans.

Since 2003, the number of bird-to-human transmissions of H5N1 influenza viruses has been increasing, and, as of 28 February 2008, a total of 369 cases in humans (234 of which proved to be fatal) had been reported to the World Health Organization [1]. Thus, there is a risk for the emergence of a pandemic H5N1 strain, either through adaptation of the avian viruses to replication in humans or through the exchange of gene segments with

seasonal influenza A viruses. To limit the influence of a pandemic outbreak caused by these viruses, expediting the availability of safe and effective H5N1 vaccines is highly desirable [2].

However, the development of such vaccines and the production of sufficient vaccine doses for a global vaccination campaign pose a challenge; the combined vaccine production capacity of all manufacturers of seasonal influenza vaccine is limited and is not sufficient to provide, in a timely fashion, the number of doses sufficient for a worldwide vaccination campaign. Therefore, there is considerable interest in dose-sparing vaccination strategies. For example, the use of potent adjuvants may result in a reduction in the amount of hemagglutinin (HA) antigen required for the induction of protective antibody responses. At present, various adjuvants are being evaluated in combination with conventional inactivated vaccine preparations, and these are in various stages of development [3–7].

Alternatively, novel vaccine production technologies are under development to overcome the shortage of vac-

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cines in the case of an H5N1 influenza pandemic. Cell lines have become available for the production of vaccines that are an alternative to vaccines conventionally produced in embryonated chicken eggs [8–13]. The use of reverse genetics for the generation of vaccine strains will further contribute to faster availability of vaccines after the onset of an influenza pandemic [14, 15]. Other novel production technologies include the use of recombinant baculoviruses for the production of influenza virus H5 in insect cells [16–18]. In general, these protein-based vaccines are poorly immunogenic in immunologically naive individuals, and appreciable antibody responses were induced only when a high dose or a combination with an adjuvant was used [19–21].

Since 1997, H5N1 viruses have diverged considerably and are now classified into clades and subclades to reflect their phylogenetic and antigenic differences [2]. These differences complicate vaccine strain selection, and, ideally, vaccines induce cross-clade protective immunity.

New, promising influenza vaccine candidates that may fulfill the described requirements include DNA vaccines and viral vectors that express the HA gene of H5N1 influenza viruses [22–24]. It was recently shown that the replication-deficient poxvirus modified vaccinia virus Ankara (MVA) expressing the HA gene of influenza virus A/Vietnam/1194/04 (H5N1) is highly immunogenic in mice [25]. Two immunizations without an adjuvant induced strong antibody responses, and immunized mice were protected from infection with the homologous virus and the heterologous H5N1 virus A/Indonesia/5/05 [25]. MVA was originally tested in >120,000 individuals and was proved to be a safe and effective vaccine against smallpox [26]. The use of recombinant MVA expressing foreign genes as vaccine candidates induced protective immunity against diseases caused by viruses, bacteria, parasites, or tumors from which the antigens were derived. Thus, MVA has an excellent safety profile in humans, can be used for the delivery of foreign antigens, and can be produced at large scale under the requirements of good manufacturing practices [27, 28]. Other properties are extreme host-range restriction; easy production, under biosafety level (BSL) 1 conditions, in chicken embryo fibroblasts (CEF) and baby hamster kidney cells; and the possibility of long-term storage (i.e., stockpiling) [27, 29–32].

For the reasons outlined above, MVA expressing the HA gene of an H5N1 influenza virus is an attractive and promising pandemic vaccine candidate. However, the immunogenicity and protective efficacy of such vaccines have been demonstrated only in mice and chickens [25, 33, 34]. Because the predictive value of these models for immunogenicity in humans is limited [34, 35], we wished to evaluate a recombinant MVA-H5 vaccine candidate in a nonhuman primate model, to assess the induction of protective immunity against H5N1 viruses from 2 different clades. Therefore, we used cynomolgus macaques that, after infection with H5N1 influenza virus, develop severe interstitial necrotizing pneumonia with a pathogenesis comparable to that

observed in humans [36, 37]. To this end, recombinant MVA expressing the HA gene of influenza virus A/Vietnam/1194/04 was used to immunize cynomolgus macaques twice, which resulted in strong influenza H5 virus-specific, virus neutralizing antibody responses. Thus, induced immunity provided protection against challenge infection with the homologous influenza H5N1 strain from clade 1 and the heterologous strain A/Indonesia/5/05 from clade 2.1. It was concluded that recombinant MVA is a safe and effective vaccine candidate for the induction of protective immunity against H5N1 influenza viruses and warrants further clinical development.

MATERIAL AND METHODS

Vaccine preparation. Recombinant MVA expressing the HA gene of influenza virus A/Vietnam/1194/04 (MVA-HA-VN/04) was prepared as described elsewhere [25]. MVA clonal isolate F6 was used as the parent MVA strain. To generate final vaccine preparations, the virus was amplified in CEF, purified by ultracentrifugation through sucrose, reconstituted in 1 mmol/L Tris-HCL [pH 9.0], and diluted in PBS.

Influenza viruses. Influenza viruses A/Vietnam/1194/04 (A/VN/1194/04) and A/Indonesia/5/05 (A/IND/5/05) were cultured in Madin-Darby canine kidney (MDCK) cells. Infectious virus titers were determined in MDCK cells, as described elsewhere [38].

Macaques. Colony-bred seronegative cynomolgus macaques (*Macaca fascicularis*; age, 3 years) were obtained from Grand Forest Scientific Primate.

Two weeks before the start of the experiment, animals were anesthetized using a cocktail of ketamin (Nimatek; Eurovet Animal Health BV) and domitor (Orion Pharma), and a temperature logger (DST micro-T ultra small temperature logger; Star-Oddi) was placed in the peritoneal cavity. This device recorded the body temperature of the animals every 15 min. Changes in body temperature were calculated by subtracting the mean day (4-h) and night (4-h) temperatures measured on 4 successive days during the period before the challenge from the mean day (4-h) and night (4-h) temperatures measured after infection. This was done for each individual animal.

The macaques were immunized twice, with a 4-week interval between immunizations, and they received $10^{8.5}$ pfu of MVA-HA-VN/04 ($n = 12$), $10^{8.5}$ pfu of wild-type MVA (wtMVA; empty vector control) ($n = 12$), or PBS ($n = 10$) intramuscularly in a volume of 1 mL divided over both legs. Blood samples were collected before immunization and again at 4 weeks after the first and second immunizations. Four weeks after the second immunization, each vaccine group was divided into 2 groups of 6 animals (except for the PBS group, which was divided into groups of 4 and 6 animals) and was placed into BSL-3 isolator units. The animals were anesthetized and inoculated intratra-

cheally with influenza virus A/VN/1194/04 or A/IND/5/05 at a dose of 1.0×10^6 TCID₅₀ in 3 mL of PBS.

After infection, the animals were monitored for the development of clinical signs. Before infection and on days 2 and 4 after infection, throat and nose swab specimens were collected while the animals were anesthetized. Four days after infection, the animals were killed by exsanguination while anesthetized with ketamin and dormitor, and necropsies were performed according to standard procedures. This time point for euthanasia was chosen because it allowed the assessment of gross pathologic and histopathologic changes as well as the extent of virus replication. It was also chosen for ethical reasons, because the development of severe disease in the nonprotected animals was avoided. The experimental protocol was approved by an independent animal ethics committee before the start of the experiments and was performed in compliance with Dutch and European legislation.

Serology. After treatment with cholera filtrate and heat inactivation at 56°C, serum samples were tested for the presence of anti-HA antibodies. For this purpose, a hemagglutination inhibition (HI) assay was used according to a standard protocol using 1% turkey erythrocytes and 4 HA units of either influenza virus A/VN/1194/04 or A/IND/5/05 [39]. For this purpose, viruses were produced from which the basic cleavage site in HA, associated with high virulence, was deleted by reverse genetics. The use of these reverse genetics viruses in the HI assay was validated, and the antibody titers that were obtained were comparable to those against wild-type strains (data not shown). Serum samples were also tested for the presence of virus neutralizing antibodies specific for the 2 influenza viruses by use of a micro virus-neutralization (VN) assay performed using the viruses that were produced by reverse genetics, as described above [40].

In brief, 50- μ L volumes of serial diluted serum samples were incubated with 100 TCID₅₀ of the viruses at 37°C for 1 h, and the mixture then was added to MDCK cells. After 1 h, the cells were washed and subsequently cultured in Eagle minimal essential medium containing bovine serum albumin (BSA; fraction V, 0.3%), 4 μ g/mL trypsin, 2 mmol/L L-glutamin, 100 U/mL penicillin; 100 μ g/mL streptomycin; 0.15% NaHCO₃; 20 mmol/L N-2 hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES), and 0.1 mmol/L nonessential amino acids. After 5 days, residual virus replication was assessed by measuring HA activity in the culture supernatants.

Hyperimmune serum samples obtained from a swan immunized twice with inactivated H5N2 influenza virus A/Duck/Potsdam/1402/86 (Nobilis influenza H5N2 Intervet International) was used as a positive control against the 2 influenza viruses. For calculation purposes, serum samples with an antibody titer of <10 were arbitrarily assigned a titer of 5.

Virus titers in organ tissues. Tissue samples were snap-frozen using a dry ice/ethanol bath and were stored at -70°C. The tissues were homogenized with a Polytron homogenizer (Kinematica AG) in transport medium (Hanks minimal essential medium) contain-

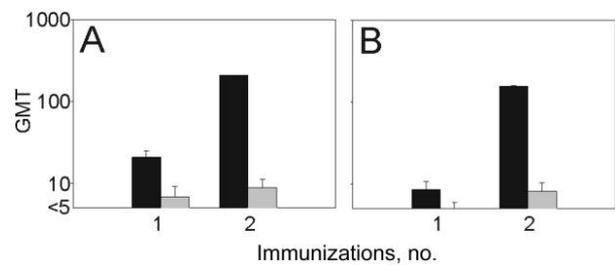


Figure 1. Antibody responses induced by immunization with modified vaccinia virus Ankara expressing the hemagglutinin gene from H5N1 virus A/Vietnam/1194/04. Antibody titers against 2 H5N1 influenza viruses, A/Vietnam/1194/04 (black bars) and A/Indonesia/5/05 (gray bars), were measured in a hemagglutination inhibition assay (A) and a virus neutralization assay (B) at 28 days after the first and second immunization. Data are expressed as geometric mean titers (GMTs).

ing 10% glycerol; 100 U/mL penicillin; 100 μ g/mL streptomycin, polymyxin B, nystatin, and gentamicin; 7.5% NaHCO₃; and 1 mol/L HEPES). Quintuplicate 10-fold serial dilutions of these samples were used to determine the virus titers in confluent layers of MDCK cells.

Histopathologic evaluation and immunohistochemical analysis. Formalin-inflated lungs were fixed in 10% neutral buffered formalin, and cross-sections then were made and embedded in paraffin, sectioned at 4 μ m, and stained with hematoxylin-eosin for histologic evaluation. Sequential slides were stained using an immunoperoxidase method with a monoclonal antibody (Clone HB65 IgG2; American Type Culture Collection) directed against the nucleoprotein of influenza A virus. A horseradish peroxidase-labeled goat anti-mouse IgG2a (Southern Biotech) was used as secondary antibody. The peroxidase was revealed using diaminobenzidine as a substrate, resulting in a deep red precipitate in the nuclei of influenza virus-infected cells and in staining the cytoplasm a less intense red. The sections were counterstained with hematoxylin.

RESULTS

Vaccine induced antibody responses. To assess the ability of the MVA-HA-VN/04 vaccine to induce antibody responses, HI and VN serum antibody titers were measured after 1 and 2 immunizations.

After the first immunization, the animals immunized with MVA-HA-VN/04 developed antibody responses against influenza virus A/VN/1194/04, with geometric mean titers of 20.9 (7 of 12 animals) and 8.5 (4 of 12 animals) measured in the HI and VN assay, respectively (figure 1A and 1B). All animals ($n = 12$) developed an antibody response after the second immunization; the mean HI titer against this virus increased to 207.6 and the mean VN titer to 156.2 (figure 1A and 1B).

Antibody responses to influenza virus A/IND/5/05 HA were detected in 2 of 12 animals with a geometric mean titer of 6.9 in

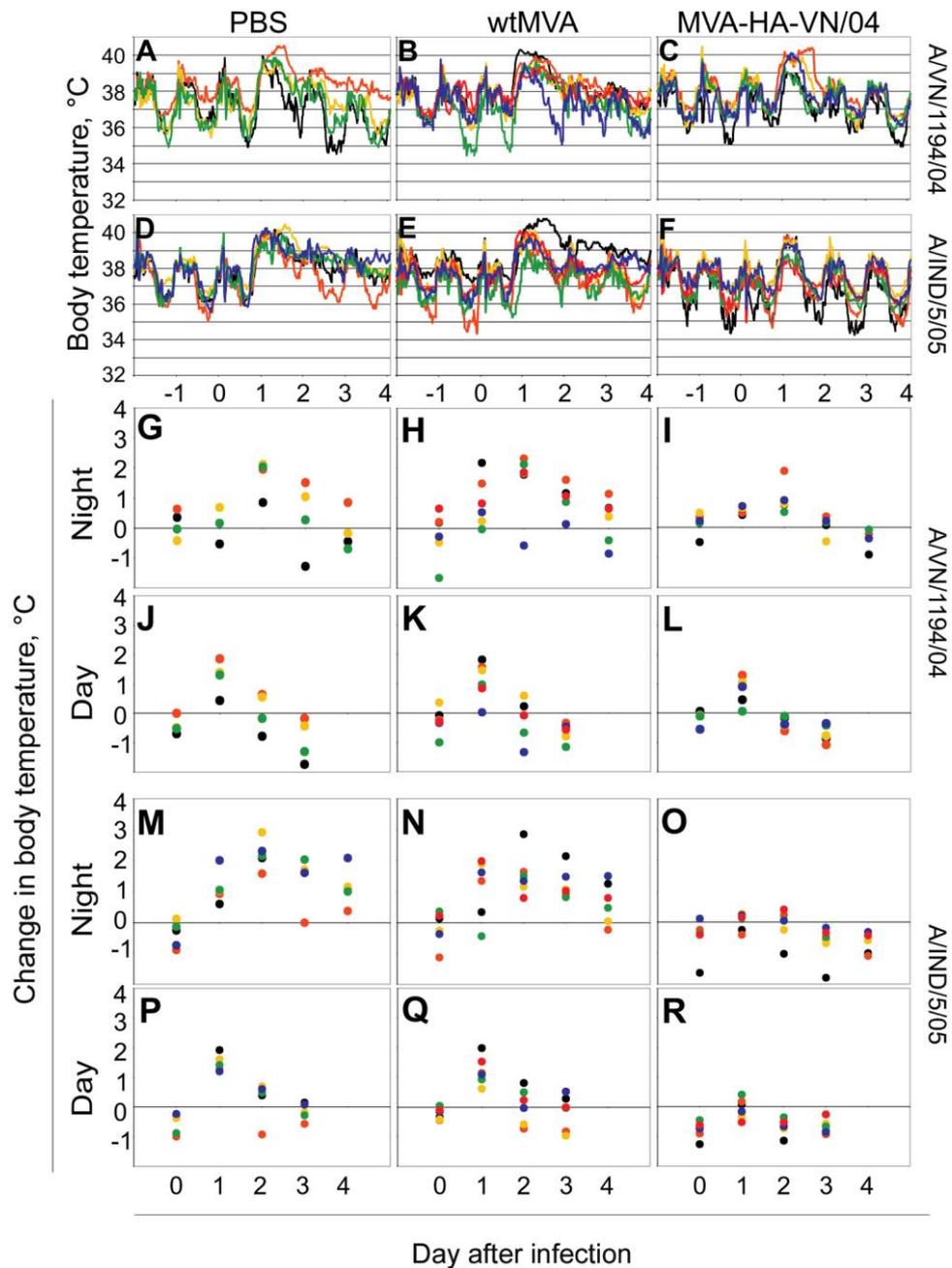


Figure 2. Body temperature recorded before and after infection with influenza virus A/Vietnam/1194/04 (A/VN/1194/04) (A–C) or A/Indonesia/5/05 (A/IND/5/05) (D–F). The animals were immunized with PBS, wild-type (wt) modified vaccinia virus Ankara (MVA), or MVA expressing the hemagglutinin (HA) gene from H5N1 influenza virus A/VN/1194/04 (MVA-HA-VN/04), as indicated. For each animal, changes in body temperature after infection with influenza virus A/VN/1194/04 (G–L) or A/IND/5/05 (M–R) were calculated. Each dot denotes an individual animal. Colors of lines in panels A–C correspond to colors of dots in panels G–L. Colors of lines in panels D–F correspond to colors of dots in panels M–R.

the HI assay, 4 weeks after the first immunization (figure 1A). After the boost immunization, 4 animals had detectable antibody responses with geometric mean HI and VN titers of 8.9 and 8.2, respectively (figure 1A and 1B). None of the animals immunized with PBS or wtMVA developed antibody responses against influenza virus A/VN/1194/04 or A/IND/5/05.

Clinical signs after infection. Before the start of the experiment, a telemetric transponder was implanted in the abdominal

cavity of every animal, to record body temperature during the course of the experiment.

The body temperature of every animal followed a strict day-night cycle during the experiment. Administration of the MVA-HA-VN/04 vaccine preparation did not affect the cycle and the mean day and night body temperatures (data not shown).

After infection with influenza virus A/VN/1194/04, all animals in the PBS group and 5 of 6 animals in the wtMVA group developed a

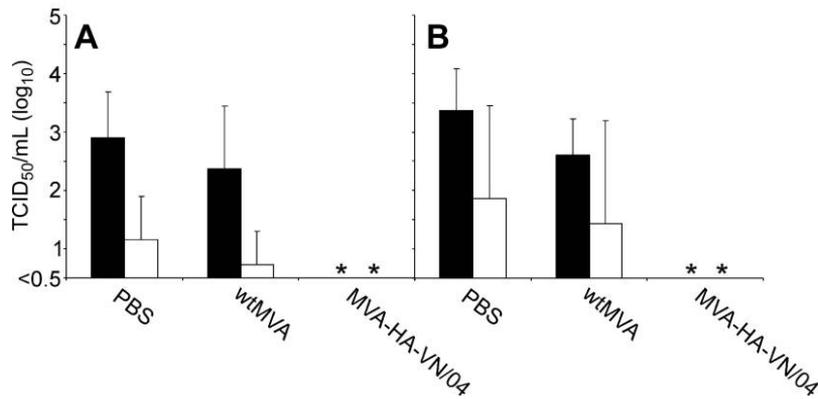


Figure 3. Virus titers in the throat after infection with an H5N1 influenza virus. Virus titers were determined for throat swab specimens obtained on day 2 (black bars) and day 4 (white bars) after infection with influenza virus A/Vietnam/1194/04 (A) or A/Indonesia/5/05 (B). Titers are expressed as the TCID₅₀ of the influenza virus per milliliter (log₁₀). *From none of the animals was virus isolated, resulting in a mean virus titer below the cutoff value. wtMVA, wild-type modified vaccinia virus Ankara; MVA-HA-VN/04, modified vaccinia virus Ankara expressing the hemagglutinin gene from H5N1 influenza virus A/Vietnam/1194/04.

fever within the first day (figure 2A, 2B, 2G, 2H, 2J, and 2K), with mean body temperatures of 38.6°C and 38.8°C for the animals immunized with PBS and wtMVA, respectively, during the subsequent 24 h. The mean body temperature did not decrease to the baseline level on days 3 and 4 in the groups immunized with PBS and wtMVA, respectively (figure 2G and 2H). After infection with influenza virus A/IND/5/05, the animals in the groups immunized with PBS and wtMVA developed mean body temperatures of 39.1°C and 38.9°C, respectively (figure 2D, 2E, 2M, 2N, 2P, and 2Q). The mean body temperature of these animals remained elevated, mainly during the night, in the 4-day period after infection (figure 2M and 2N). The body temperature of the animals immunized with MVA-HA-VN/04 maintained its day-night cycle and remained normal after infection with influenza virus A/VN/1194/04 (figure 2C), with the exception of a small increase noted during day 1 and the following night (figure 2I and 2L). After infection with influenza virus A/IND/5/05, body temperature and the day-night cycle were normal (figure 2F, 2O, and 2R)

Virus detection in the upper respiratory tract. To determine the virus titers in the upper respiratory tract, nose and throat swab specimens were collected on days 0, 2, and 4 after infection.

On day 2 after infection with influenza virus A/VN/1194/04, the results of examination of throat swab specimens were positive for all animals in the PBS group and for 5 of 6 animals in the wtMVA group, with mean titers (±SD) of $10^{2.9} \pm 10^{0.8}$ and $10^{2.4} \pm 10^{1.1}$, respectively (figure 3A). The titers were lower on day 4 in both the PBS group (2 of 4 animals were positive) and in the wtMVA group (1 animal was positive), with geometric mean titers (±SD) of $10^{1.2} \pm 10^{0.8}$ and $10^{0.7} \pm 10^{0.6}$, respectively. After infection with influenza virus A/IND/5/05, the titers were slightly higher for all animals in the PBS and wtMVA group with mean titers (±SD) on day 2 of $10^{3.4} \pm 10^{0.7}$ and $10^{2.6} \pm 10^{0.2}$, respectively (figure 3B). On day 4, the titers had decreased to $10^{1.9} \pm 10^{1.6}$ (3 animals were positive) in the PBS group and $10^{1.4} \pm 10^{1.8}$ (2 animals were positive) in the wtMVA group.

Virus was not detectable in the throat of the MVA-HA-VN/04-immunized animals either on day 2 or 4 after infection with either the homologous or heterologous virus (figure 3A and 3B). The nose swab specimens were negative for all animals in both challenge groups and on both days (data not shown).

Virus detection in organs. The lungs, brain, and spleen were tested for the presence of infectious virus on day 4 after infection. Virus was detectable in the lungs of all animals in the PBS and wtMVA groups that were infected with influenza virus A/VN/1194/04 (figure 4), with mean titers of $10^{5.0} \pm 10^{0.5}$ and $10^{4.9} \pm 10^{0.7}$ TCID₅₀/g of tissue, respectively. After infection

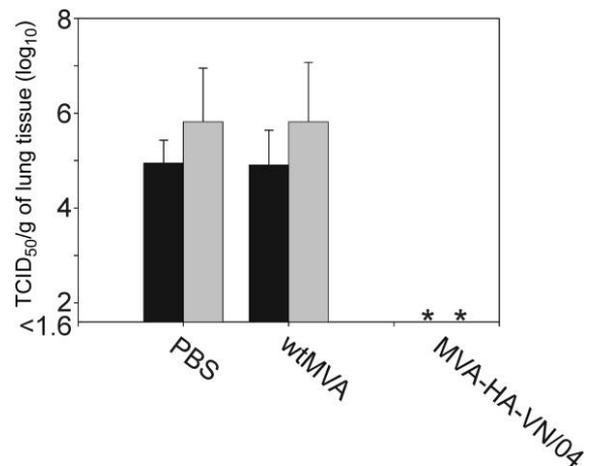


Figure 4. Virus titers in the lungs after infection with H5N1 influenza viruses. Virus titers were determined on day 4 after infection in the lungs of animals that were infected with either A/Vietnam/1194/04 (black bars) or A/Indonesia/5/05 (gray bars). Virus titers are expressed as the TCID₅₀ of the influenza virus per gram of lung tissue (log₁₀). *All animals had negative results of virus isolation, resulting in a mean virus titer below the cutoff value. MVA-HA-VN/04, modified vaccinia virus Ankara (MVA) expressing the hemagglutinin gene from H5N1 influenza virus A/Vietnam/1194/04; wtMVA, wild-type MVA.

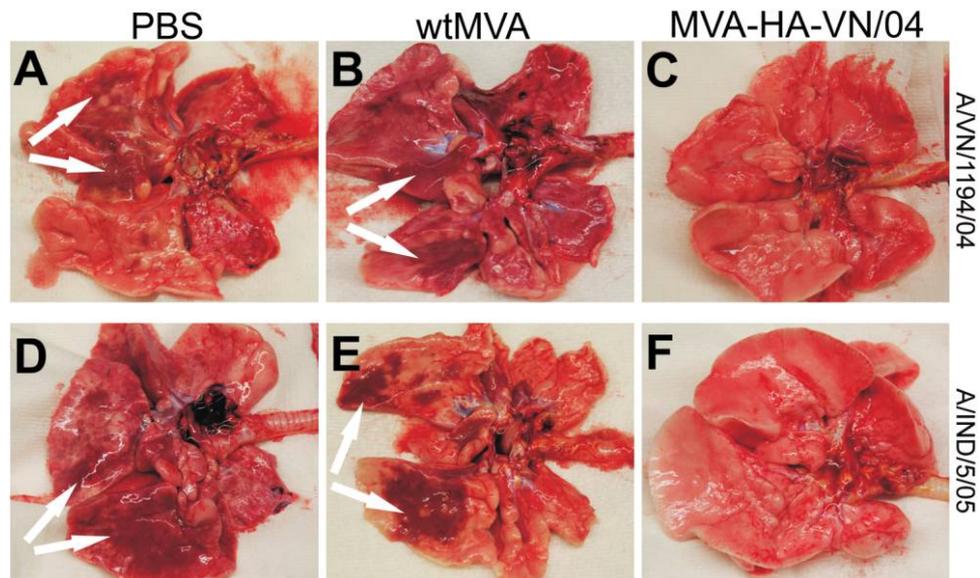


Figure 5. Macroscopic lesions of the lungs after infection with H5N1 influenza virus. The lungs of animals immunized with PBS (A, D), wild-type (wt) modified vaccinia virus Ankara (MVA) (B, E) or MVA-HA-VN/04 (MVA expressing the hemagglutinin gene from H5N1 influenza virus A/Vietnam/1194/04 [A/VN/1194/04]) (C, F) were fixed in formalin on day 4 after infection with influenza virus A/VN/1194/04 (A–C) or A/IND/5/05 (D–F). The arrows denote consolidated areas present in the lungs of animals immunized with PBS or wtMVA after infection (A, B, D, E). The lungs of animals immunized with MVA-HA-VN/04 demonstrated no macroscopic lesions (C, F).

with influenza virus A/IND/5/05, higher infectious virus titers were detected in animals that received PBS or wtMVA, with mean titers of $10^{5.8} \pm 10^{1.1}$ and $10^{5.8} \pm 10^{1.2}$ TCID₅₀/g of tissue noted, respectively. In the lungs of animals immunized with MVA-HA-VN/04, no virus was detected, regardless of the virus that was used for infection (figure 4). No virus was detected in the brain or spleen specimens obtained from any of the animals.

Pathologic findings in the lungs. Lungs were dissected and inflated with formalin on day 4 after infection, to examine pathologic findings. Macroscopically multifocal to coalescing consolidation characterized by depressed, dark red, and firm areas was seen in the animals immunized with PBS or wtMVA after infection with either of the 2 H5N1 influenza viruses (figure 5A, 5B, 5D, and 5E), with a range of 45%–90% of tissue affected. These lesions were far less extensive ($\pm 5\%$) or were even absent in the groups immunized with MVA-HA-VN/04 (figure 5C and 5F).

To examine the lungs in more detail, cross-sections were made and used for histologic analysis. On histopathologic examination, the main lesions were seen in the alveoli and bronchioli. The animals immunized with PBS and wtMVA and infected with influenza virus A/VN/1194/04 had a multifocal, moderate-to-severe, necrotizing bronchiointerstitial pneumonia. In animals immunized with PBS and wtMVA, infection with the A/IND/5/05 strain resulted in pathologic changes that were, overall, more severe than that noted in association with the first virus. The pneumonia was characterized by variable intra-alveolar amounts of proteinaceous fluid (edema) and eosinophilic fibrillar material (fibrin), cellular debris, moderate numbers of alveolar macrophages, and

few neutrophils and eosinophils (figure 6A, 6B, 6D, and 6E). In the alveolar septa, there was multifocal karyorrhexis, karyolysis, and loss of detail of epithelial cells (necrosis), infiltration with few neutrophils and eosinophils, and mild hypertrophy and hyperplasia of type II pneumocytes. In the bronchioles, there was multifocal loss of epithelial cells and intraluminal edema fluid and cellular debris. There was perivascular and peribronchiolar infiltration with many lymphocytes and plasma cells and with few macrophages, neutrophils, and eosinophils (figure 6A, 6B, 6D, and 6E).

In the animals immunized with MVA-HA-VN/04 after infection with influenza virus A/VN/1194/04 or A/IND/5/05, there was multifocal, mild bronchiointerstitial pneumonia characterized by few intra-alveolar macrophages, neutrophils, and eosinophils; few lymphocytes and plasma cells in the alveolar septa; and perivascular and peribronchiolar infiltration (figure 6C and 6F). Bronchus-associated lymphoid tissue was hyperplastic and appeared to be activated in these animals, compared with animals inoculated with PBS and wtMVA.

Detection of virus-infected cells by immunohistochemical analysis. After infection with influenza virus A/VN/1194/04, the lungs of the animals immunized with PBS or wtMVA demonstrated viral antigen expression (figure 7A and 7B). Alveolar epithelial cells, in particular, showed antigen expression as well as a few alveolar macrophages. The infected cells were predominantly associated with the pulmonary lesions. In the lungs of the animals immunized with PBS or wtMVA after infection with influenza virus A/IND/5/05 (figure 7D and 7E), viral antigen expression involved the same cell types

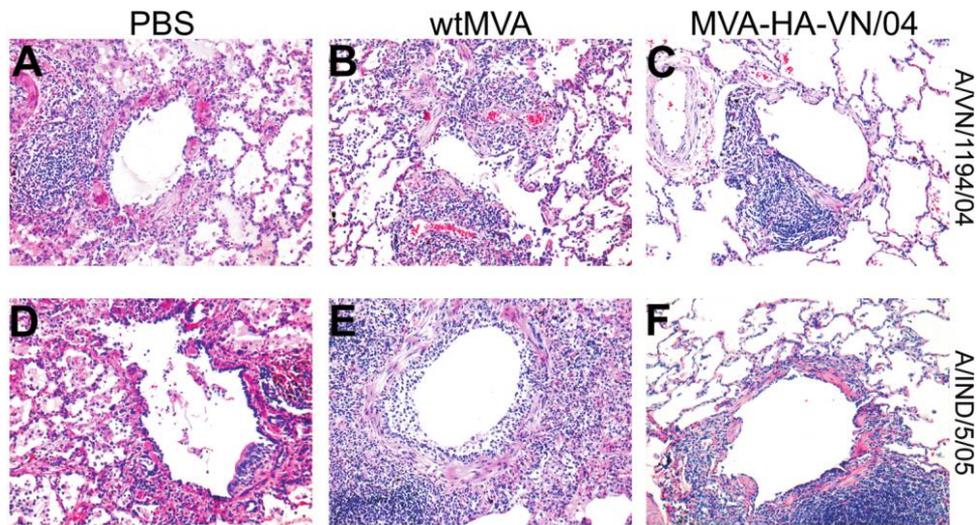


Figure 6. Histopathologic analysis of the lungs on day 4 after infection with influenza virus A/Vietnam/1194/04 (A/VN/1194/04) (A–C) or A/Indonesia/5/05 (A/IND/5/05) (D–F). Histopathologic changes were comparable in PBS- and wild-type (wt) modified vaccinia virus Ankara (MVA)–inoculated animals, with extensive lesions noted in the lungs of these animals. There was mild necrosis, edema, hypertrophy, and hyperplasia of type II pneumocytes combined with peribronchiolar and vascular infiltration. The epithelium of some bronchioles is denuded because of necrosis of the epithelial cells. (A, B, D, E). In the lungs of the animals immunized with MVA expressing the hemagglutinin gene from H5N1 influenza virus A/Vietnam/1194/04 (MVA-HA-VN/04), no major histopathologic changes were observed (C, F).

and location noted after infection with influenza virus A/VN/1194/04 but was more extensive in these animals. No virus-infected cells were detectable in the lungs of animals immunized with MVA-HA-VN/04 that were infected with influenza virus A/VN/1194/04 or A/IND/5/05 (figure 7C and 7F).

DISCUSSION

The aim of the present study was to evaluate the capacity of the MVA-HA-VN/04 vaccine to induce protective immunity against highly pathogenic H5N1 influenza viruses in a nonhuman primate model. *Cynomolgus* macaques were immunized

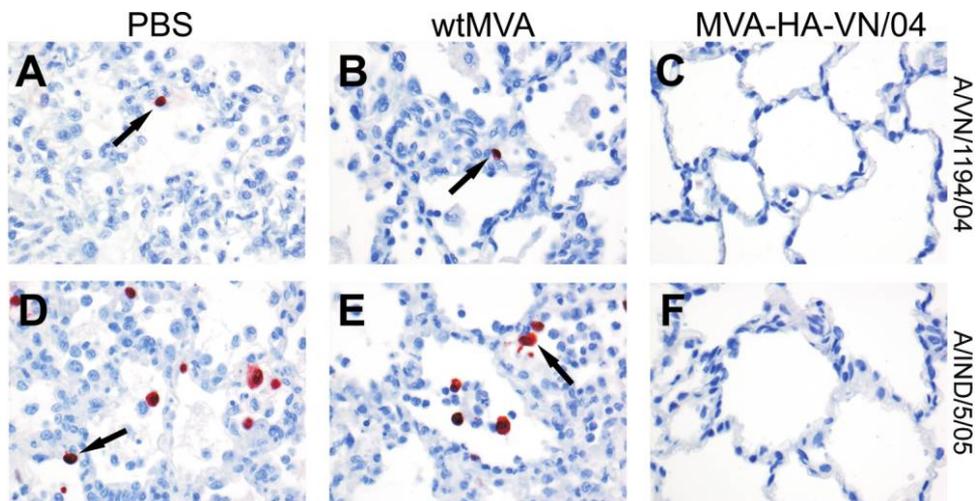


Figure 7. Detection of virus-infected cells in the lungs 4 days after infection with H5N1 influenza viruses. Immunohistochemical analysis was used to stain cells that are positive for the presence of viral antigen showing a deep red staining in the nucleus. Antigen expression by influenza viruses A/Vietnam/1194/04 (A/VN/1194/04) (arrows denote single infected cells) or A/Indonesia/5/05 (A/IND/5/05) was seen in alveolar epithelial cells and in some alveolar macrophages of animals inoculated with PBS (A, D) and wild-type (wt) modified vaccinia virus Ankara (MVA) (B, E). No viral antigen was observed in the lungs of animals immunized with MVA expressing the hemagglutinin gene from H5N1 influenza virus A/Vietnam/1194/04 (MVA-HA-VN/04) (C, F).

twice with MVA-HA-VN/04 and then were challenged with influenza virus A/VN/1194/04 or A/IND/5/05, strains from clade 1 and 2.1, respectively [2].

Immunization of cynomolgus macaques with MVA-HA-VN/04 induced virus-specific HI and VN antibodies that, in the majority of the animals, cross-reacted with the heterologous strain A/IND/5/05 from clade 2.1. Despite the absence of cross-reactive antibodies in some of the animals, they were all protected against infection with the homologous and the heterologous strain. A similar result was obtained in the mouse model [25]. Apparently, the induction of cross-reactive antibody responses that are below the limit of detection was sufficient for protection against infection with the heterologous strain. This observation is in concordance with results obtained in other H5N1 vaccination challenge experiments performed in mice and ferrets [4, 25].

Our data show that vaccination with MVA-HA-VN/04 prevented virus replication and the development of fever and severe interstitial pneumonia after challenge infection. The pathogenesis of infection with influenza viruses A/VN/1194/04 and A/IND/5/05 in cynomolgus macaques was characterized by infection predominantly of pneumocytes, resulting in a necrotizing bronchointerstitial pneumonia. This pathogenesis resembled that observed after infection of macaques with influenza virus A/HK/156/97 [36, 37] or that seen after infection of humans with H5N1 highly pathogenic avian influenza viruses [41, 42]. Apparently, the infection of pneumocytes is sufficient for the development of interstitial pneumonia in primates, regardless of the type of pneumocytes (type I or type II) that are infected [41, 43, 44]. H5N1 viruses bound to epithelial cells of the lower respiratory tract of cynomolgus macaques, most likely through the preferential usage of the (SA)- α 2,3-Gal receptor.

Collectively, the MVA-HA-VN/04 vaccine preparation proved to be highly effective in inducing in primates protective immunity against homologous and heterologous H5N1 influenza viruses. The reduction in viral shedding from the lower and upper respiratory tract may reduce the risk of human-to-human transmission and may, therefore, limit viral spread in the population if this vaccine is used in humans to reduce the influence of the pandemic [45, 46].

The MVA-HA-VN/04 immunization was very well tolerated. Not only was measurement of the body temperature of the animals used as a clinical outcome of the challenge infection, it also allowed monitoring of systemic reactions after vaccination. After MVA-HA-VN/04 or wtMVA vaccination, no increase in body temperature was observed in any of the vaccinated animals. This finding is in concordance with the excellent safety profile of (recombinant) MVA in humans [26]. This safety profile also extends to immunocompromised subjects, because it has been shown that MVA does not replicate in severely immunosuppressed macaques [47].

The presence of preexisting antivector immunity may not be a major concern for the efficacy of MVA-HA-VN/04 vaccine, although this remains to be demonstrated. In mice, efficacy of MVA vaccine was hardly affected by preexisting immunity [48]. More importantly, repeated administrations of a recombinant MVA vaccine to humans boosted specific immune responses directed against the recombinant antigen 5T4 [49]. Therefore, it can be envisaged that immunization of individuals who received smallpox vaccination in the past and repeated applications of MVA-HA-VN/04 should be feasible.

In combination with other favorable properties, such as good stability, which would allow stockpiling of the vaccine, rapid and easy production at large scale under BSL-1 conditions makes recombinant MVA-HA an attractive and promising candidate as a pandemic influenza virus vaccine. On the basis of the data presented here, further clinical development of MVA-HA vaccines seems to be warranted.

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