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Protection of Mice against Lethal Infection with Highly Pathogenic H7N7 Influenza A Virus by Using a Recombinant Low-Pathogenicity Vaccine Strain

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In 2003, an outbreak of highly pathogenic avian influenza occurred in the Netherlands. The avian H7N7 virus causing the outbreak was also detected in 88 humans suffering from conjunctivitis or mild respiratory symptoms and one person who died of pneumonia and acute respiratory distress syndrome. Here we describe a mouse model for lethal infection with A/Netherlands/219/03 isolated from the fatal case. Because of the zoonotic and pathogenic potential of the H7N7 virus, a candidate vaccine carrying the avian hemagglutinin and neuraminidase proteins produced in the context of the high-throughput vaccine strain A/PR/8/34 was generated by reverse genetics and tested in the mouse model. The hemagglutinin gene of the recombinant vaccine strain was derived from a low-pathogenicity virus obtained prior to the outbreak from a wild mallard. The efficacy of a classical nonadjuvanted subunit vaccine and an immune stimulatory complex-adjuvanted vaccine was compared. Mice receiving the nonadjuvanted vaccine revealed low antibody titers, lack of clinical protection, high virus titers in the lungs, and presence of virus in the spleen, liver, kidneys, and brain. In contrast, mice receiving two doses of the immune stimulatory complex-adjuvanted vaccine revealed high antibody titers, clinical protection, ~1,000-fold reduction of virus titers in the lungs, and rare detection of the virus in other organs. This is the first report of an H7 vaccine candidate tested in a mammalian model. The data presented suggest that vaccine candidates based on low-pathogenicity avian influenza A viruses, which can be prepared ahead of pandemic threats, can be efficacious if an effective adjuvant is used.

In 2003, an outbreak of highly pathogenic avian influenza occurred in the Netherlands, caused by an influenza A virus of subtype H7N7 (6, 8). The hemagglutinin (HA) and neuraminidase (NA) genes of the highly pathogenic avian H7N7 influenza viruses displayed a high sequence identity to the HA and NA genes of low-pathogenicity viruses isolated from Dutch mallards in the framework of our ongoing surveillance studies in wild birds (5, 6). During the 2003 outbreak, 89 cases of human infection were detected, including 3 probable cases of human-to-human transmission. Most of these individuals suffered from conjunctivitis or mild respiratory disease, but a fatal case of acute respiratory distress syndrome also occurred. The virus that was isolated from the fatal case, A/Netherlands/219/03, was found to differ from a virus isolated from a case of conjunctivitis, A/Netherlands/33/03, and the chicken isolate A/Chicken/Netherlands/1/03, in 14 amino acid positions scattered throughout the genome (6).

Because of their zoonotic potential and pathogenicity, and because close relatives of the H7N7 viruses that caused the outbreak may still circulate in wild birds, the generation of a vaccine strain based on the H7N7 virus was considered desirable. Because the H7N7 viruses isolated from poultry and humans during the outbreak are highly pathogenic, they cannot be used directly for generating a vaccine strain. Recently, vaccine strains for H5 influenza A virus have been generated by removal of the basic cleavage site from the HA genes of highly pathogenic strains. The resulting genetically modified virus strains, lacking this important determinant of high pathogenicity, were used as seed viruses for vaccine production (9, 11, 17, 25, 26). Since the HA gene of the virus that caused the H7N7 outbreak in the Netherlands was closely related to the HA gene of influenza virus A/Mallard/Netherlands/12/00, we decided to produce a subunit vaccine containing the HA of this low-pathogenicity H7 virus.

Previously, it was shown that vaccination of naive animals with the traditional, nonadjuvanted subunit vaccine did not induce sufficient antibody titers to protect against challenge with influenza A virus (19, 20). Therefore, we tested the use of immune stimulatory complexes (ISCOMs) as an adjuvant in parallel with the traditional nonadjuvanted subunit vaccine. ISCOMs are particles of approximately 40 nm in diameter with incorporated antigens (13). ISCOMs are known to induce both antibody and cell-mediated immunity and have been tested as an adjuvant for influenza vaccines in a number of animal models and in humans (14, 19–21).

Instead of using laborious classical reassortment techniques to produce the vaccine seed virus strain, we used the recently developed reverse genetics technology (2, 3, 7, 15). Reverse genetics techniques enable the generation of a reassortant

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virus with a backbone of influenza virus A/PR/8/34 and H7 and N7 envelope proteins obtained from avian influenza viruses, that can be used as seed virus for a vaccine, within a relatively short period of time (17, 25, 26).

Here, we describe the development and evaluation of a heterologous vaccine against highly pathogenic avian influenza virus of the H7N7 subtype. First, a mouse model for lethal infection with highly pathogenic H7N7 was developed. Subsequently, the protective efficacy of a classical influenza subunit vaccine preparation was compared to that of an ISCOM-adjuvanted vaccine preparation. The classical preparation did not elicit a sufficient immune response to protect mice from a lethal challenge with influenza virus A/Netherlands/219/03 (H7N7) even after two doses. In contrast, two doses of an ISCOM-adjuvanted vaccine preparation were sufficient to protect mice against the lethal challenge, although at day 4 after challenge infection virus could be detected in their lungs.

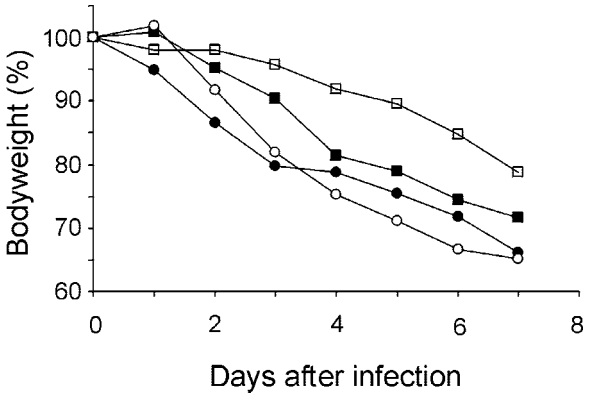

FIG. 1. Loss of body weight as the result of infection with influenza virus A/Netherlands/219/03. Mice were infected intranasally with four different infectious doses: 10^2 (□), 3×10^3 (○), 10^5 (■), and 3×10^6 (●) EID_{50}. Following infection, mice were weighed daily. Percent body weight was calculated compared to body weight at the time of infection.

MATERIALS AND METHODS

Viruses. The molecular clone version of influenza virus A/PR/8/34 has been described previously (2). Influenza virus A/Mallard/Netherlands/12/00 (H7N3) was isolated from a cloacal swab collected from a mallard in the Netherlands in 2000 and subsequently passaged twice in embryonated chicken eggs. Influenza virus A/Netherlands/33/03 (H7N7) and A/Netherlands/219/03 (H7N7) were isolated from patients during the H7N7 outbreak in the Netherlands in 2003 (6) and passaged twice in embryonated chicken eggs. Segment 4 (HA) of influenza virus A/Mallard/Netherlands/12/00 (H7N3) and segment 6 (NA) of influenza virus A/Netherlands/33/03 (H7N7) were amplified by reverse transcription-PCR and cloned in the BsmBI site of a modified version of plasmid pHW2000 (2, 7).

Recombinant influenza virus A/PR/8/34 containing subtype H7 and N7 surface glycoproteins (rPR8-H7N7) was generated by reverse genetics as described previously (2). To this end, transient calcium phosphate-mediated transfections of 293T cells were performed as described previously (2) with plasmids encoding gene segments 1, 2, 3, 5, 7, and 8 of influenza virus A/PR/8/34, segment 4 of influenza virus A/Mallard/Netherlands/12/00, and segment 6 of influenza virus A/Netherlands/33/03. The supernatant of the transfected cells was harvested 48 h after transfection and was used to inoculate embryonated chicken eggs.

Influenza virus rPR8-H7N7 was passaged six times in the allantoic cavity of 11-day-old embryonated chicken eggs. The allantoic fluid was harvested and cleared by low-speed centrifugation. The cleared supernatant was subsequently centrifuged 2 h at 85,000 x *g* in a SW28 rotor at 4°C. The virus pellet was resuspended in 2 ml phosphate-buffered saline (PBS) and loaded on a 20 to 60% (wt/wt) sucrose gradient and centrifuged overnight at 300,000 x g in an SW41 rotor at 4°C.

Vaccine preparation. Decanoyl-*N*-methylglucamide (MEGA-10, Sigma, Zwijndrecht, The Netherlands) was added to the concentrated and purified virus to a final concentration of 2% and ribonucleoprotein complexes were removed by centrifugation through a layer of 20% (wt/wt) sucrose at 150,000 x g for 2 h in an SW41 rotor at 20°C. The resulting HA/NA preparation on top of the sucrose layer was harvested and tested for purity by performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Western blotting using a monoclonal antibody directed against the influenza A virus nucleoprotein was performed to confirm the absence of nucleoprotein in the HA/NA preparations. The amount of protein in the HA/NA preparation was determined using a BCA protein assay kit (Pierce). A part of the preparation was dialyzed against PBS and used without further modification as a traditional HA/NA subunit vaccine preparation. The other part was dialyzed against PBS after addition of cholesterol (1 mg/mg protein, Sigma), phosphatidylethanolamine (1 mg/mg protein, Sigma), and Quillaja glucosides (5 mg/mg protein ISCOPREP 703, Iscotec, Lulea, Sweden). The ISCOM preparation was analyzed by negative contrast electron microscopy, revealing the typical particles with a diameter of approximately 40 nm. An ISCOM-measles virus control vaccine was kindly provided by K. Stittelaar (23).

Immunization and challenge of animals. To generate postinfection antisera, ferrets were inoculated intranasally with approximately 10^6 50% egg infectious doses (EID_{50}) of influenza virus A/Mallard/Netherlands/12/00, A/Netherlands/33/03, or A/Netherlands/219/03. Antisera were collected from these animals after 14 days for use in hemagglutination inhibition (HI) assays. A hyperimmune rabbit antiserum raised against A/Seal/Massachusetts/1/80 (H7N7), that has been described previously (4), was used in parallel.

We used 6- to 8-week-old female BALB/c mice for vaccination-challenge experiments. First, four groups of six mice were infected intranasally with 1×10^2, 3×10^3, 1×10^5 and 3×10^6 EID_{50} to determine the optimal challenge dose for mice. Six groups of six animals each were vaccinated once or twice intramuscularly at 3-week intervals. The following groups were tested: two doses of PBS; two doses of 1 μg of a control ISCOM-measles vaccine; two doses of 5 μg of the HA/NA preparation; a single dose of 5 μg of the ISCOM-H7N7 preparation; two doses of 1 μg of the ISCOM-H7N7 preparation; and two doses of 5 μg of the ISCOM-H7N7 preparation. A blood sample was collected from the animals prior to the second vaccination and prior to virus challenge by orbital puncture.

Three weeks after the second vaccination, all animals were challenged intranasally with 3×10^3 EID_{50} of influenza virus A/Netherlands/219/03. Although we did not determine the 50% lethal dose (LD_{50}) for mice, 3×10^3 EID_{50} corresponds to >30 LD_{50} (Fig. 1). After challenge, the animals were observed for clinical signs and weighed twice daily as an indicator of disease. At day 4 after infection, three animals from each group were sacrificed and analyzed for the presence of virus in the lungs, spleen, liver, kidneys, and brain. The other three animals were sacrificed 14 days after challenge or upon development of severe disease or discomfort, in agreement with national animal welfare regulations.

All intranasal infections, orbital punctures, and euthanasia were performed under anesthesia with inhaled isoflurane. All animal studies were approved by the Animal Ethics Committee of Erasmus Medical Center, Rotterdam, The Netherlands. All experiments were performed under biosafety level 3+ conditions.

Serology. Hemagglutination inhibition assays were performed for the comparison of the antigenic properties of influenza A virus strains using postinfection ferret antisera and a hyperimmune rabbit antiserum and for the determination of antibody levels in vaccinated mice essentially as described previously (22). All serum samples were treated overnight with receptor-destroying enzyme and subsequently incubated at 56°C for 1 hour. Twofold serial dilutions of each antiserum, starting at a 1:20 or 1:40 dilution, were tested for their ability to inhibit the agglutination of turkey or horse erythrocytes by 4 hemagglutinating units of influenza A virus. When horse erythrocytes were used instead of turkey erythrocytes, serum dilutions were made in PBS containing 0.5% bovine serum albumin (fraction V, Gibco, Breda, The Netherlands). Horse erythrocytes were stored in PBS containing 0.5% bovine serum albumin. In the HI assay, 50 μl of a 1% horse erythrocyte dilution was added to each well.

Virus titrations. Virus titrations were performed by end-point titration in MDCK cells as described previously (2). Lungs, spleen, liver, kidneys, and brain were collected and homogenized in 3 ml transport medium, consisting of Hanks' balanced salt solution containing 10% glycerol, 200 U/ml penicillin, 200 mg/ml streptomycin, 100 U/ml polymyxin B sulfate, and 250 mg/ml gentamicin (all from MP Biomedicals, Zoetermeer, The Netherlands), using a Polytron homogenizer (Kinematica AG, Littau-Lucerne, Switzerland) and centrifuged briefly. The supernatant was used directly to inoculate MDCK cells. MDCK cells were inoculated with tenfold serial dilutions of tissue homogenates. One hour after inocu-

TABLE 1. HI assay determining cross-reactivity between A/Mallard/Netherlands/12/00 and two H7N7 viruses isolated during the outbreak, A/Netherlands/33/03 and A/Netherlands/219/03

Strain	HI titer of sera raised against:			
	A/Mallard/NL/12/00[a]	A/NL/33/03[a]	A/NL/219/03[a]	A/Seal/Mass/1/80[b]
A/Mallard/NL/12/00 (H7N3)	80	40	30	1,280
A/NL/33/03 (H7N7)	160	160	80	1,920
A/NL/219/03 (H7N7)	40	80	40	2,560

[a] Postinfection serum raised in a ferret.
[b] Hyperimmune serum raised in a rabbit.

lation, cells were washed once with PBS and grown in 200 μl of infection medium, consisting of EMEM (Cambrex, Heerhugowaard, The Netherlands) supplemented with 4% bovine serum albumin, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml sodiumbicarbonate (Cambrex), 10 mM HEPES (Cambrex), nonessential amino acids (MP Biomedicals), and 20 μg/ml trypsin (Cambrex). Three days after inoculation, the supernatants of infected cell cultures were tested for agglutinating activity using turkey erythrocytes as an indicator of infection of the cells. Infectious titers were calculated from five replicates by the method described earlier (18).

RESULTS

Analysis of antigenic properties of H7 influenza A virus isolates. Sequence analyses of the strains circulating during the H7N7 outbreak in the Netherlands in 2003 showed that the HA genes of the viruses isolated from chickens and humans were 98% identical to the HA gene of a low-pathogenicity avian influenza virus A/Mallard/Netherlands/12/00 (H7N3) isolated within the framework of a surveillance study for influenza A virus in wild birds (5, 6). Because the mallard virus lacked the basic cleavage site in the HA gene characteristic of highly pathogenic avian influenza strains, it was considered a candidate vaccine strain, without the need for genetic modification of the HA gene.

We first compared the antigenic properties of the HA genes of influenza viruses A/Mallard/Netherlands/12/00, A/Netherlands/33/03, and A/Netherlands/219/03. As shown in Table 1, the ferret antisera raised against influenza viruses A/Netherlands/33/03 and A/Netherlands/219/03 had similar homologous and heterologous titers against these viruses. A hyperimmune rabbit antiserum raised against A/Seal/Massachussetts/1/80 (H7N7) was included as a positive control and reacted with all H7 viruses with high titers. Of special interest was the reactivity of the ferret serum directed against influenza virus A/Mallard/Netherlands/12/00 with the other two strains. The titers against A/Netherlands/33/03 and A/Netherlands/219/03 were within twofold of the homologous titer, indicating that these three viruses are antigenically similar. Both horse and turkey erythrocytes were used in the HI assays, but the source of erythrocytes did not have a major influence on the outcome of the experiments. Based on these data, it was anticipated that antibodies raised against the HA of the low-pathogenicity A/Mallard/Netherlands/12/00 would provide protection against the highly pathogenic avian influenza virus. Therefore, the HA of the low-pathogenicity H7N3 virus was selected for use in the candidate vaccine against highly pathogenic avian influenza H7N7 strains.

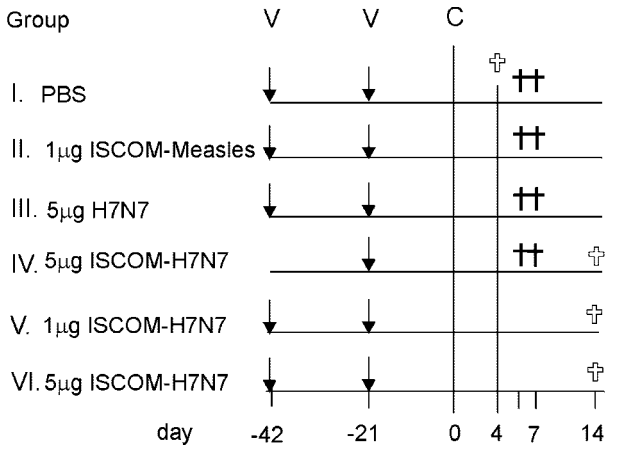

FIG. 2. Overview of the vaccination strategies used to test H7N7 vaccine preparations. Six groups of six mice each were vaccinated once or twice intramuscularly with the different vaccine preparations as indicated (↓). On day 4 after challenge, three animals from each group were sacrificed (open cross). On days 6 and 7 after challenge animals from groups I, II, III, and IV were sacrificed due to severity of signs of disease (cross). On day 14 all remaining animals were sacrificed (open crosses). V: vaccination; C: challenge.

Lethal challenge mouse model for H7N7 influenza A virus. We wished to develop an animal model for lethal infection with the virus isolated from the fatal human case, A/Netherlands/219/03. To this end, groups of six BALB/C mice were infected with 30-fold serial dilutions of infectious virus stocks, ranging in dose from 1×10^2 to 3×10^6 EID_{50}. All mice became severely ill, indicated by loss of body weight (Fig. 1), ruffled fur, and lethargy. Three animals out of each group were sacrificed at day 4 after infection to analyze virus infection of the lungs of these mice. The three remaining mice in each group were sacrificed because of the severity of the disease signs at day 7 after infection, in accordance with national ethical guidelines for experiments with laboratory animals.

In mice infected with 3×10^3, 10^5, and 3×10^6 EID_{50} the same rate of loss of body weight was observed. The mice infected with 10^2 EID_{50} lost body weight slightly more slowly than mice in the other groups, but also became severely ill, necessitating euthanasia at day 7 after infection. In accordance with the relatively low rate of body weight loss, mice infected with a virus dose of 10^2 EID_{50} had ~10-fold lower virus load in the lungs at 4 and 7 days after inoculation compared to the mice infected with 3×10^3, 10^5, and 3×10^6 EID_{50} (data not shown). We decided to use 3×10^3 EID_{50} as a reproducible minimal challenge dose that resulted in lethal infection of mice for our vaccination experiments.

HI antibody titers in vaccinated mice. Mice were immunized according to the schedule depicted in Fig. 2. Three weeks after the first vaccination and 3 weeks after the booster vaccination, blood samples were collected from the vaccinated mice. The sera were used in HI assays to determine the antibody titers against the heterologous challenge virus A/Netherlands/219/03. After the first vaccination with 1 or 5 μg of ISCOM-H7N7, mice developed antibody titers ranging from 160 to 960. In contrast, only three out of six mice vaccinated with the classical subunit influenza vaccine developed detectable antibody titers up to 40 (Fig. 3). After the second vaccination, antibody titers

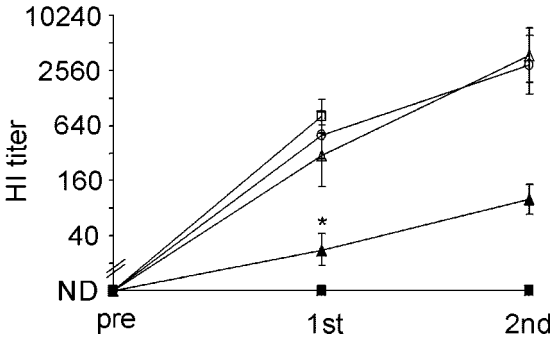

FIG. 3. Serum HI antibody titers against A/Netherlands/219/03 after the first and second vaccinations. Blood samples were taken before the start of the experiment and at 3 weeks after the first and second vaccinations. Serum was used in an HI test against A/Netherlands/219/03. Indicated are geometric mean titers and 95% confidence intervals. The asterisk indicates that in this group three mice had an HI titer below the detection limit and three mice had a titer of 40. ND, not detected. Groups: PBS and ISCOM-measles controls (■); two 5-μg H7N7 vaccinations (▲); one 5-μg ISCOM-H7N7 vaccination (□); two 1-μg ISCOM-H7N7 vaccinations (○); two 5-μg ISCOM-H7N7 vaccinations (△).

in the mice vaccinated with the classical H7N7 subunit vaccine increased, but were still significantly lower than the antibody levels in mice vaccinated with either a single dose or two doses of the ISCOM-H7N7 vaccine. Mice receiving two doses of ISCOM-H7N7 vaccine developed high antibody responses after two vaccinations, ranging from 960 to 7,680 (Fig. 3). There was no significant difference in antibody production between the mice vaccinated with either 1 or 5 μg of the ISCOM-H7N7 vaccine. None of the mice that received PBS or ISCOM-measles vaccine produced detectable HI antibodies.

Protection from lethal infection. Three weeks after the booster vaccination, all animals were challenged with 3×10^3 EID_{50} of influenza virus A/Netherlands/219/03. Following challenge, the body weight of the animals was determined twice daily as an indicator of disease. From 3 days after the challenge onwards, the mice vaccinated once with the ISCOM-H7N7 preparation or twice with the classical influenza vaccine preparation, the control ISCOM-measles, or PBS started to develop signs of disease such as loss of body weight, ruffled fur, and lethargy. At days 6 and 7 postinfection, all the mice from these groups, except for one mouse in the group vaccinated once with 5 μg ISCOM-H7N7 vaccine, were sacrificed for ethical reasons, since they all became very ill and lost 20% of their original body weight (Fig. 4A).

One mouse in the group that was vaccinated once with ISCOM-H7N7 became only slightly ill and recovered completely. The mice vaccinated twice with either 1 or 5 μg of ISCOM-H7N7 vaccine appeared to remain healthy after the H7N7 influenza A virus challenge despite a temporary small loss of body weight. Figure 4B summarizes the survival of the mice upon the lethal challenge with A/Netherlands/219/03.

Virus titers in the organs of vaccinated animals. On day 4 after challenge, three mice from each vaccinated group were sacrificed and their lungs, spleens, livers, kidneys, and brains were collected for analysis of virus replication (Fig. 5). High virus titers were detected in the lungs of all mice on day 4 after challenge. Virus titers in the lungs of mice vaccinated twice

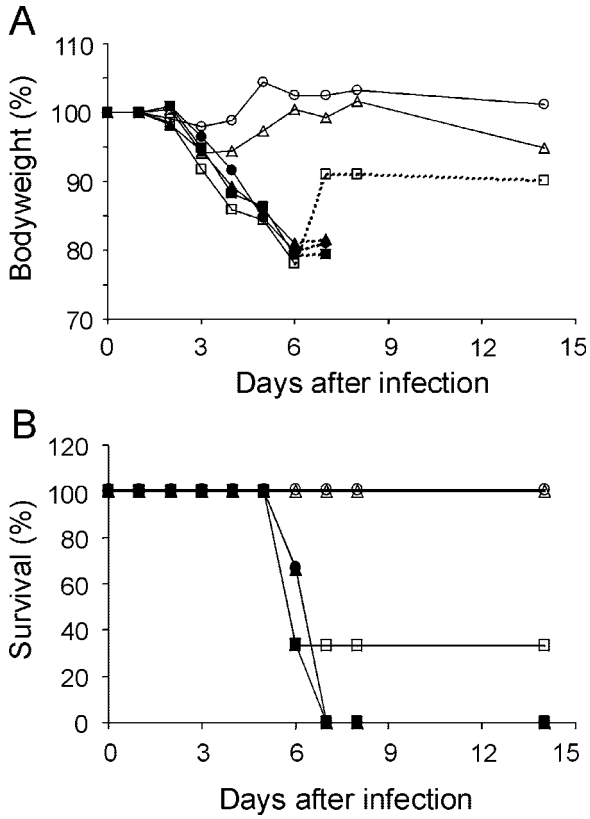

FIG. 4. Loss of body weight and survival of vaccinated mice after challenge with influenza virus A/Netherlands/219/03. Vaccinated mice were challenged intranasally with 3×10^3 EID_{50} of influenza A virus A/Netherlands/219/03. After challenge, mice were weighed daily. Percent body weight per group was calculated compared to body weight at the time of challenge (A). Mice were sacrificed either due to the severity of their disease signs at day 6 or 7 or at the end of the experiment on day 14 after challenge. The dotted line indicates that one or two mice out of the group were already sacrificed and data are thus based on the body weights of the remaining mice. The percentage of mice surviving the lethal challenge as a function of time is also shown (B). Since three mice out of each group were sacrificed at day 4 after infection, this graph is based on the survival of the remaining three mice from day 5 after infection onwards. Groups: PBS (■); ISCOM-measles (●); two 5-μg H7N7 vaccinations (▲); one 5-μg ISCOM-H7N7 vaccination (□); two 1-μg ISCOM-H7N7 vaccinations (○); two 5-μg ISCOM-H7N7 vaccinations (△).

with ISCOM-H7N7 had a geometric mean titer of $10^{4.44}$ 50% tissue culture infectious doses ($TCID_{50}$)/gram tissue (95% confidence interval: $10^{2.33}$ to $10^{6.54}$), approximately 1,000-fold lower than those of the mice in the other groups (geometric mean titer, $10^{7.41}$ $TCID_{50}$/gram tissue, 95% confidence interval: $10^{6.94}$ to $10^{7.87}$, $P < 0.05$, one-way analysis of variance).

In the two groups of mice vaccinated twice with ISCOM-H7N7, virus was not detectable outside the lungs except for marginal virus titers in the spleen and kidney of one mouse vaccinated twice with 1 μg of ISCOM-H7N7 and in the brains of one mouse in each of the groups vaccinated with 1 and 5 μg of ISCOM-H7N7. In contrast, in all other groups of mice virus titers were detected in the spleen, liver, kidney, and brain at 4 days after infection (Fig. 5). Because the number of animals with detectable virus titers in organs other than the lung varied

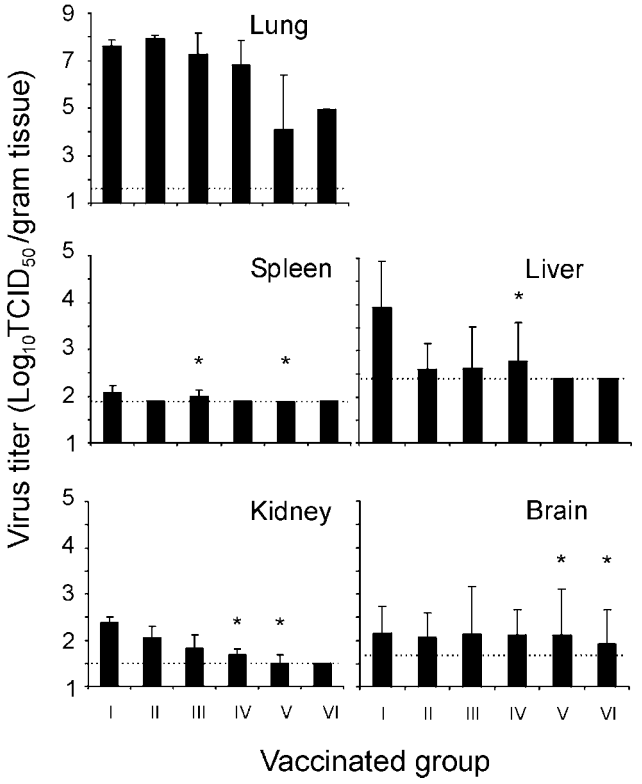

FIG. 5. Virus titers in mice after challenge with influenza virus A/Netherlands/219/03. Vaccinated mice were challenged intranasally with 3×10^3 EID_{50} of influenza A virus A/Netherlands/219/03. On day 4 after challenge three mice from each group were sacrificed, tissues were collected, and virus titers in lungs, spleen, liver, kidney, and brain were determined in MDCK cells. The geometric mean virus titer per group was calculated. To calculate the geometric mean, the cutoff value was used for negatives. Error bars indicate the standard deviation. The dotted line indicates the cutoff value of the assay for each of the organs. Asterisks indicate that only one of the tested animals in the group was positive. Roman numbers refer to the vaccination status as shown in Fig. 2.

between groups, no statistical differences could be calculated for these organs.

DISCUSSION

In 2003 there was an outbreak of highly pathogenic avian influenza (H7N7) in the Netherlands. Because of the zoonotic and pandemic potential of the viruses that caused the outbreak, the availability of an effective vaccine was highly desirable. The HA of a virus isolated from a mallard in 2000 had a high sequence identity to the HA gene of the viruses that caused the outbreak. This offered the opportunity to use this low-pathogenicity virus obtained through routine influenza surveillance in wild birds as the basis of a vaccine directed against highly pathogenic avian influenza H7N7 virus. Using reverse genetics, we generated a reassortant seed strain that was used for vaccine preparation. We compared a classical, nonadjuvanted influenza vaccine preparation containing HA and NA with an ISCOM-adjuvanted preparation.

Our experiments clearly showed that, although an antibody response was mounted, the classical influenza vaccine did not protect against lethal infection with influenza virus A/Netherlands/219/03, not even when two doses were administered. One dose of the ISCOM-adjuvanted vaccine preparation did not protect either. Two doses of the ISCOM-adjuvanted vaccine were required for clinical protection against influenza virus A/Netherlands/219/03. Although two doses of the ISCOM-adjuvanted preparation did not lead to sterile immunity against highly pathogenic avian influenza H7N7, virus titers in the lungs of vaccinated mice were reduced considerably. We speculate that this ~1,000-fold reduction of pulmonary virus titers was responsible for the survival of the animals receiving two doses of the ISCOM vaccine. In the event of a pandemic outbreak, a vaccine offering clinical protection could be sufficient to reduce the clinical impact of infection with the pandemic virus.

Our experiments also showed that it was possible to use a heterologous low-pathogenicity strain as the donor of the HA protein for vaccines directed against highly pathogenic avian influenza virus strains. It should be noted that the HA amino acid sequence of the low-pathogenicity mallard virus and the highly pathogenic avian influenza viruses differed very little; only the HA cleavage site and single-amino-acid substitutions in the signal peptide, HA1, and HA2 were different. Low-pathogenicity strains with such high amino acid sequence identity and such small antigenic differences from highly pathogenic avian influenza strains may not always be available.

An important question that remains is why infected animals were not protected from infection or death despite high HI antibody titers upon repeated vaccination with adjuvanted or nonadjuvanted vaccines, respectively. We performed virus neutralization assays with the sera from vaccinated mice and influenza viruses A/Mallard/Netherlands/12/00 (H7N3) and A/Netherlands/219/03 (H7N7). These experiments revealed that despite the high titers of HI antibodies in these sera (HI titers ranging up to 7,680), virus-neutralizing antibodies were not detectable (titers <40). In contrast, rabbits hyperimmunized with HA and NA of A/Seal/Massachusetts/1/80 (H7N7), which had HI antibody titers of 1,280 and 2,560 against A/Mallard/Netherlands/12/00 (H7N3) and A/Netherlands/219/03 (H7N7), respectively (Table 1), had virus-neutralizing antibody titers against these viruses of 1,280 and 5,120, respectively.

Since the Dutch H7 viruses are thus not resistant to neutralization, the lack of virus neutralization with the mouse sera must be related to the poor induction of neutralizing antibodies upon vaccination. It is of interest to note that the postinfection ferret sera raised in this study also lacked detectable neutralizing antibody titers despite the presence of HI antibodies (Table 1 and data not shown). Furthermore, the H7N7-infected humans in the Netherlands who developed HI antibodies during the outbreak in 2003 did not develop detectable virus-neutralizing antibodies (unpublished). Thus, it may be that the Dutch H7 viruses are poor inducers of neutralizing antibodies. Hence it is possible that cell-mediated immunity or antibodies with poor virus-neutralizing capacity were responsible for the clinical protection of mice vaccinated with the ISCOM-based vaccine.

Experiments like the one described here have been conducted with highly pathogenic strains of the H5N1 subtype, although it is difficult to compare these studies to our own because of the use of different virus subtypes, animal models,

adjuvants, vaccine types, timing, and dosage. Using the low-pathogenicity A/Duck/Singapore/Q/F119-3/97 (H5N3) (12, 26), the low-pathogenicity A/Duck/Hokkaido/67/96 (H5N4), or an H5N1 reassortant thereof (24), inactivated virus preparations were tested in mice for their possible use in a pandemic situation. In these studies, vaccinated mice survived challenge with the highly pathogenic avian influenza H5 strains A/Hong Kong/483/97 and A/Hong Kong/156/97, even when no adjuvant was used in the vaccine preparations. When alum was used as an adjuvant, mice were even protected from infection (12).

However promising these studies may seem compared to ours, our results are more in agreement with vaccination studies in a chicken (20) and monkey (19) model and with experimental vaccination in humans (16). When surface antigen of the same A/Duck/Singapore/Q/F119-3/97 strain used in the mouse studies was used as a vaccine in a phase I randomized trial in human volunteers, two doses of a preparation with the adjuvant MF59 were required to reach antibody levels that are considered protective (16).

Although ISCOMs are not registered for use in humans, the immune response to an ISCOM-adjuvanted influenza vaccine has been tested in humans. When ISCOMs were tested in humans in a randomized, double-blind study, antibody responses to the ISCOM-adjuvanted preparation were improved compared to antibody responses to the conventional influenza vaccine preparation (21). Although in this study individuals were vaccinated with an ISCOM preparation containing influenza A virus antigens against which there was preexisting immunity, the results in a range of animal models suggest that the ISCOM adjuvant could also work very well to induce immune responses in naive individuals. In addition, ISCOMs are used as an adjuvant in registered equine influenza vaccines (1, 14).

In case of a pandemic, a two-dose vaccine will be impractical. Therefore, there is an urgent need for new adjuvants or improved vaccine delivery approaches, yielding better immune responses and improved protection against lethal infection, preferably after administration of a single dose.

Although vaccination experiments using highly pathogenic H5 viruses with a deleted basic cleavage site have been successful in animals (9–11), these vaccine strains can only be produced as soon as a pandemic threat arises. Furthermore, removal of the basic cleavage site requires an extra modification step and thus more time in the production of a reassortant seed virus. Since we show here that a heterologous vaccine could work well, prototypic envelope proteins of potentially pandemic viruses could be cloned in advance to speed up seed virus production further. Alternatively, vaccine seed viruses could be generated by classical reassortment, eliminating the need for reverse genetics technology. This would not only bypass the patent-related costs of influenza vaccines, but would also enable vaccine manufacturers to produce vaccines without the need for facilities equipped for working with genetically modified organisms.

An important conclusion that can be drawn from the experiments described above is that prototypic low-pathogenicity strains obtained through routine surveillance of wild birds could be used effectively to generate vaccines directed against highly pathogenic avian influenza viruses long before outbreaks in poultry or pandemic threats emerge. A repository of seed virus containing the HAs of viruses with known zoonotic or pandemic potential (H1, H2, H3, H5, H7, and H9) could be prepared in advance. High-growth strains could be generated in order to respond quickly to outbreaks and pandemic threats. Based on the antigenic properties of the virus strains, the most suitable vaccine candidate could then be selected from the repository using new methods for antigenic characterization (22).

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