Recombinant Soluble, Multimeric HA and NA Exhibit Distinctive Types of Protection against Pandemic Swine-Origin 2009 A(H1N1) Influenza Virus Infection in Ferrets[∇]

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The emergence and subsequent swift and global spread of the swine-origin influenza virus A(H1N1) in 2009 once again emphasizes the strong need for effective vaccines that can be developed rapidly and applied safely. With this aim, we produced soluble, multimeric forms of the 2009 A(H1N1) HA (sHA₃) and NA (sNA₄) surface glycoproteins using a virus-free mammalian expression system and evaluated their efficacy as vaccines in ferrets. Immunization twice with 3.75-μg doses of these antigens elicited strong antibody responses, which were adjuvant dependent. Interestingly, coadministration of both antigens strongly enhanced the HA-specific but not the NA-specific responses. Distinct patterns of protection were observed upon challenge inoculation with the homologous H1N1 virus. Whereas vaccination with sHA₃ dramatically reduced virus replication (e.g., by lowering pulmonary titers by about 5 log₁₀ units), immunization with sNA₄ markedly decreased the clinical effects of infection, such as body weight loss and lung pathology. Clearly, optimal protection was achieved by the combination of the two antigens. Our observations demonstrate the great vaccine potential of multimeric HA and NA ectodomains, as these can be easily, rapidly, flexibly, and safely produced in high quantities. In particular, our study underscores the underrated importance of NA in influenza vaccination, which we found to profoundly and specifically contribute to protection by HA. Its inclusion in a vaccine is likely to reduce the HA dose required and to broaden the protective immunity.

The recent emergence of the pandemic swine-origin 2009 A(H1N1) influenza virus strongly emphasizes the potential of influenza viruses to cause morbidity and mortality in the human population on a global scale. Worldwide, over 200 countries and overseas territories or communities have reported laboratory-confirmed cases of the pandemic virus, including more than 16,000 deaths (http://www.who.int/csr/don/2010_03_26/en/index.html). Vaccination is the primary method to prevent or lower the burden of influenza disease. However, as illustrated again by the 2009 pandemic, a rapid response during the early phase of an outbreak is hampered by the time-consuming vaccine strain preparation and vaccine manufacturing process currently used. This, combined with the notorious capacity of influenza viruses to escape from existing immunity by antigenic drift and shift, stresses the need for novel, safe, and preferably broadly effective vaccines that can be produced rapidly and in flexible response to newly emerging antigenic variants.

The currently licensed influenza virus vaccines are com-

posed of the viral envelope glycoproteins, the hemagglutinin (HA) and neuraminidase (NA). Antibodies elicited by these two large glycoproteins have distinct properties in immunity against influenza virus. Antibodies to HA generally neutralize viral infectivity by interference with virus binding to sialic acid receptors on the target cells or, subsequently, by preventing the fusion of the viral and cellular membranes through which the viral genome gains access to the target cell. Antibodies to NA disable release of progeny virus from infected cells by inhibiting the NA-associated receptor-destroying enzymatic activity. The HA-mediated humoral immunity has been characterized most extensively and has been shown to prevent virus infection. The contribution of NA antibodies to preventing disease has been less well studied. They appeared to produce a kind of permissive immunity (15) characterized by a decrease in infectious virus release from apical surfaces of infected epithelia (3, 8, 16, 17, 37, 38, 40), reducing the probability of virus shedding and spread into the environment.

Immunization with the combination of HA and NA provides enhanced protection against influenza (3, 14, 18). Although HA and NA are equivalently immunogenic (16), the humoral immune response toward conventional inactivated vaccines or virus infection is naturally skewed toward HA since HA and NA occur on the viral surface at an approximately 4:1 ratio (44). In addition, in intact virions, HA immunologically outcompetes NA in B and T cell priming as shown in mice (20). This antigenic competition is not seen in vaccinated animals

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when HA and NA are administered separately (18, 34). The currently licensed pandemic vaccines as well as the seasonal trivalent vaccines are generally prepared from whole viruses and are hence biased to contain more HA than NA antigen. Adapting the HA/NA ratio in vaccine formulations in favor of NA may provide a more balanced humoral immune response, resulting in higher NA antibody levels and increased protection against disease (21, 40).

Recombinantly produced HA and NA antigens allow the development of vaccines in which the relative amounts of both antigens can be easily controlled. Eukaryotic expression systems, both mammalian and insect, are the preferred platforms for production of such glycoproteins in view of their better preservation of the proteins' natural antigenic structure. We have addressed the efficacy of recombinantly produced HA and NA subunits of the 2009 A(H1N1) influenza virus as vaccines against homotypic influenza virus in a ferret model, with particular emphasis on the contribution of the NA antigen. Thus, we expressed soluble, multimeric forms of the HA and NA antigens of the pandemic H1N1 virus in a mammalian expression system, purified the glycoproteins by single-step affinity chromatography, and subsequently immunized ferrets either with one or with both antigens and with or without ISCOM Matrix M (IMM) as an adjuvant. The animals responded serologically to both antigens, but only when administered the adjuvant. Interestingly, inclusion of NA in the vaccine enhanced the levels of HA antibodies and of virus-neutralizing activity. Significant protection, as judged particularly from the dramatically ($5\text{-log}_{10}\text{-unit}$) reduced viral lung titers, was observed upon homologous challenge in the animals immunized with HA-containing vaccines in combination with ISCOM Matrix M. Interestingly, vaccine formulations containing NA clearly reduced the clinical effects of infection.

MATERIALS AND METHODS

Influenza A challenge virus. Influenza virus A/Netherlands/602/2009 was isolated from the first case of a laboratory-confirmed 2009 A(H1N1) infection in the Netherlands by inoculation of 11-day-old embryonated chicken eggs (32). Virus stocks of influenza virus A/Netherlands/602/2009(H1N1) were prepared by infecting confluent Madin-Darby canine kidney (MDCK) cells. After cytopathological changes were complete, culture supernatants were cleared by low-speed centrifugation and stored at -70°C . Infectious virus titers were determined in MDCK cells as described previously (36). All experiments with these viruses were performed under biosafety level 3 (BSL-3) conditions.

Preparation of HA and NA antigens. Human codon-optimized sequences encoding the soluble hemagglutinin ectodomain (sHA, amino acids [aa]17 to 522) and the neuraminidase head domain (sNA, aa 75 to 469) of influenza virus A/California/04/2009(H1N1) were synthesized (GenScript) and cloned into a derivative of expression plasmid pSI-Ig (30) for expression in HEK293T cells. The HA gene was preceded by a sequence encoding an N-terminal CD5 signal peptide and followed by sequences encoding a C-terminal artificial GCN4 trimerization domain (GCN4-pII) (12) and a Strep tag for affinity purification (IBA GmbH) as described recently (4, 9). The NA gene was preceded by sequences successively coding for an N-terminal CD5 signal peptide, a double Strep tag (One-STrEP; IBA GmbH), and an artificial GCN4 tetramerization domain (GCN4-pLI) (12).

Protein expression and purification. HEK293T cells were transfected with the sHA and sNA expression plasmids using polyethyleneimine (PEI) in a 1:5 ratio (μg DNA to μg PEI). After 6 h of incubation, the transfection medium was replaced by 293 SFM II expression medium (Invitrogen) supplemented with sodium bicarbonate (3.7 g/liter), glucose (2.0 g/liter), Primatone RL-UF (3.0 g/liter), penicillin (100 units/ml), streptomycin (100 μg /ml), glutaMAX (Gibco), and 1.5% dimethyl sulfoxide (DMSO). Tissue culture supernatants were harvested at 5 to 6 days posttransfection, and sHA and sNA proteins were purified from the culture medium using Strep-Tactin affinity chromatography (IBA

GmbH). sHA and sNA protein expression and purification were confirmed by Western blotting using a Strep-Tactin-horseradish peroxidase (HRP) conjugate (IBA GmbH) (data not shown) and SDS-PAGE analysis. Oligomerization of the proteins was determined by gel filtration chromatography and by blue-native PAGE analysis. Quantification of protein amounts was done using bovine serum albumin (BSA) as a reference.

Ferrets. Healthy young adult outbred female ferrets (*Mustela putorius furo*; between 6 and 12 months old) were purchased from a commercial breeder. The animals were checked for the absence of antibodies against circulating seasonal A/H1N1 and A/H3N2 influenza viruses and against the swine-origin influenza A/NL/602/09 virus by hemagglutination inhibition (HI) assay. An independent animal ethics committee approved the experimental protocol before the start of the experiment.

Immunizations and infections. Thirty-six seronegative ferrets were divided into six groups of six ferrets each and vaccinated twice with the following formulations: 3.75 μg sHA₃ plus 3.75 μg sNA₄ in phosphate-buffered saline (PBS) (group 1), 3.75 μg sHA₃ in ISCOM Matrix M (IMM) (Isconova, Uppsala, Sweden) (group 2), 3.75 μg sNA₄ in IMM (group 3), 3.75 μg sHA₃ plus 3.75 μg sNA₄ in IMM (group 4), PBS (group 5), and IMM (group 6). Vaccinations were performed with an interval of 20 days under anesthesia with ketamine in the quadriceps muscles of the hind leg in a total volume of 1 ml. Ferrets were housed in groups and received food and water *ad libitum*. At 32 days after the last vaccination, the animals were anesthetized with ketamine-medetomidine (reversed with atipamezole), weighed, and subsequently challenged intratracheally with 1×10^6 50% tissue culture infective doses (TCID₅₀) of influenza A/NL/602/09(H1N1) in a volume of 3 ml PBS (7, 42). Ferrets were subsequently monitored three times daily for the development of clinical signs. Before infection and 2 and 4 days after infection, nose and throat swabs of each ferret were collected while ferrets were anesthetized with ketamine. Four days after inoculation, animals were weighed and subsequently euthanized by exsanguination while under anesthesia with ketamine and medetomidine. Necropsies were performed according to standard procedures. One ferret in group 1 died between the first and second vaccinations due to reasons unrelated to the experiment.

Serology. Serum samples were collected before vaccination, at the day of the second vaccination (day 20), and at the day of challenge (day 52). Sera were stored at -20°C until use. Sera were tested for the presence of anti-HA antibodies using a hemagglutination inhibition (HI) assay with 1% turkey erythrocytes and for the presence of virus-neutralizing antibodies using a micro-virus neutralization (VN) assay as described previously (11, 33). Sera were tested for the presence of antibodies reactive with influenza A/NL/602/09(H1N1) virus. For this purpose, reverse genetics viruses were produced. The titers obtained with these viruses were comparable to those against the wild-type strains (data not shown). Positive control serum specific for influenza A/NL/602/09(H1N1) virus was obtained from a ferret infected with this virus (32). Other H1N1 influenza viruses used in the HI assay were A/Netherlands/386/86 (NL/86), A/Netherlands/25/80 (NL/80), A/New Jersey/8/76 (NJ/76), A/Swine/shope/1/56 (Sw/56), A/Italy/1443/76 (It/76), A/Iowa/15/30 (Io/30), A/Puerto Rico/8/34 (Pr/34), and A/Brisbane/59/07 (IVR-148 vaccine strain; IVR/148). Serum samples from ferrets infected with these viruses were used as a positive control in this assay (6).

Sera were also tested for the presence of neuraminidase-inhibiting (NI) antibodies using a previously described fetuin-based assay (29). Briefly, 96-well Nunc MaxiSorp plates were coated overnight at 4°C with 100 μl of 5- $\mu\text{g}/\text{ml}$ fetuin. Sixty-microliter volumes of serially diluted serum samples were incubated for 30 min at 37°C with an equal volume of sNA₄-containing culture supernatant (prediluted in PBS with Ca [0.901 mM]-Mg [0.493 mM] to give a half-maximum optical density at 450 nm [OD₄₅₀] of 1.5), after which 100 μl of the mixture was added to the fetuin-coated wells. After 1 h of incubation at 37°C , the plates were washed and neuraminidase activity was subsequently measured by adding peroxidase-labeled peanut agglutinin (2.5 $\mu\text{g}/\text{ml}$; Sigma), incubating for 1 h at room temperature, washing the plates, and adding 100 μl of peroxidase substrate (TMB) to each well. After 5 min, the reaction was stopped by the addition of 100 μl of 0.3 M phosphoric acid, and OD values were measured at 450 nm using an enzyme-linked immunosorbent assay (ELISA) reader (EL-808 [BioTEK]). To test the sera for cross-reactive NI antibodies, sNA₄ expression constructs similar to the ones described above for A/California/04/2009(H1N1) were also made for the head domains of A/Kentucky/UR06-0258/2007(H1N1) (aa 75 to 470) and A/turkey/Turkey/1/2005(H5N1) influenza virus (aa 55 to 449). Sera specific for influenza A/NL/602/09(H1N1) and A/turkey/Turkey/1/2005(H5N1) viruses obtained from a ferret infected with these viruses were used as a positive control.

Virus replication in the upper and lower respiratory tracts. Samples of all lobes of the right lung and of the accessory lobe were collected from the infected ferrets, snap-frozen on dry ice with ethanol, and stored at -70°C until further processing. Lung samples were weighed and subsequently homogenized with a

FIG. 1. Design and expression of soluble, multimeric HA (sHA) and NA (sNA) proteins of 2009 A(H1N1) influenza virus. (A) Schematic representation of the recombinantly expressed sHA and sNA protein constructs. For sHA, the HA ectodomain (aa 17 to 522) is expressed with an N-terminal CD5 signal peptide and a C-terminal trimerization (GCN4-pII) GCN4 domain and Strep-Tag (ST). For sNA, the NA head domain (aa 75 to 469) is expressed with an N-terminal CD5 signal peptide, a OneSTrEP (OS) peptide, and a tetramerization (GCN4-pLI) GCN4 domain. (B) Coomassie blue stained reducing SDS-PAGE of affinity-purified sHA and sNA proteins.

FastPrep-24 (MP Biomedicals, Eindhoven, Netherlands) in Hanks' balanced salt solution containing 0.5% lactalbumin, 10% glycerol, 200 U/ml penicillin, 200 µg/ml streptomycin, 100 U/ml polymyxin B sulfate, 250 µg/ml gentamicin, and 50 U/ml nystatin (ICN Pharmaceuticals, Zoetermeer, Netherlands) and centrifuged briefly. Nose and throat swabs were stored directly at -70°C in the same medium used to homogenize the lung samples. Quadruplicate 10-fold serial dilutions of throat, nose, and lung samples were used to infect MDCK cells as described previously (36). The HA activity of the culture supernatants collected at 5 days postinoculation (dpi) was used as an indicator of infection. The titers were calculated according to the Spearman-Kärber method and expressed as log TCID₅₀ per gram for lung tissue or per ml for swabs (22).

Histopathology. At 4 postinoculation (dpi) with influenza A/NL/602/09 virus, ferrets were euthanized and lungs were observed macroscopically and weighed before samples from the right lungs were collected to determine the virus titers. Subsequently, left lung lobes were inflated with 10% neutral buffered formalin. After fixation and embedding in paraffin, lungs were sectioned at 4 µm and tissue sections were examined by staining with hematoxylin and eosin (H&E).

Statistical analysis. Significance among animal groups was analyzed by one-way analysis of variance (ANOVA) and Tukey test after ANOVA. Differences were considered significant at a P value of <0.05 .

RESULTS

Production of sHA₃ and sNA₄ antigens. Constructs were designed to express the trimeric HA ectodomain (aa 17 to 522) and the tetrameric NA head domain (aa 75 to 469) of the 2009 A(H1N1) influenza virus, as pictured in Fig. 1A. The sHA₃ and sNA₄ proteins were produced by expression in HEK293T cells and purified from the culture medium by affinity chromatography, yielding glycoproteins of the expected size (Fig. 1B). Gel filtration analysis indicated the trimeric and tetrameric oligomeric natures of the HA and NA subunits, respectively (data not shown). The multimeric complexes were also biologically active, further confirming their native state, as judged by their sialic acid binding (sHA₃) (unpublished data) and neuraminidase activity (sNA₄) (see below).

Antibody responses induced by immunization with sHA₃ and sNA₄. The glycoproteins were tested for their ability to induce protective immunity against homologous virus challenge. Ferrets were immunized at days 0 and 20 with sHA₃ plus sNA₄ without adjuvant (sHA+sNA), with sHA₃ plus sNA₄ adjuvanted with ISCOM Matrix M (IMM) (sHA+sNA+IMM), or with similarly adjuvanted sHA₃ (sHA+IMM) or sNA₄ (sNA+IMM). Sera were collected at the day of the second immunization and prechallenge (days 20 and 52), and antibody responses were measured by HI and VN assays against the homologous virus and by NI assay (Fig. 2). No

responses with any of these assays were observed in control animals vaccinated with PBS or with adjuvant only but also not in the animals immunized with the nonadjuvanted mixture of sHA₃ and sNA₄. In contrast, the immunizations with adjuvanted sHA₃ (sHA+IMM) induced high HI titers, with a geometric mean titer of 91 at the day of challenge. Interestingly, the additional inclusion of sNA₄ (sHA+sNA+IMM) significantly increased the average HI titers to 468 ($P < 0.05$ by one-way ANOVA and Tukey test). Also, the NI titrations revealed the adjuvant-dependent induction of NA antibodies, which were low after one immunization (Fig. 2) but strongly boosted after two immunizations (Fig. 2). However, in this case no clear augmentation of these titers due to the coadministration of sHA₃ was observed. Consistent with the observed HI titers, high VN titers were found both in the sHA+IMM- and in the sHA+sNA+IMM-vaccinated animals (Fig. 2). Also here, the coadministration of the sNA₄ antigen with the sHA+sNA+IMM vaccine resulted in an increase in the mean VN titer, with average values ranging from 1:202 to 1:468 in the sHA+IMM and sHA+sNA+IMM groups, respectively.

Protection against clinical signs after infection with 2009 A(H1N1) influenza virus. Vaccinated ferrets were challenged with 10^6 TCID₅₀ 2009 A(H1N1) at 5 weeks after the second vaccination. From day 2 after inoculation onwards, clinical signs were observed in inoculated ferrets, which included breathing difficulties, lethargy, decreased appetite, and weight loss. In general, only mild clinical signs were observed in ferrets of groups 2, 3, and 4, while more severe symptoms were observed in ferrets of groups 1, 5, and 6. Loss of body weight became obvious in the PBS- and IMM-vaccinated control groups as well as in the nonadjuvanted sHA+sNA vaccine group (Fig. 3). Interestingly, the animals immunized with sHA+IMM showed nearly similar weight losses, while body weights were not significantly affected after vaccination with both the sNA₄-containing formulations (groups sNA+IMM and sHA+sNA+IMM). More or less consistently, the lung weights of the ferrets determined postmortem showed a corresponding tendency, with adjuvanted sNA₄-vaccinated animals having the least disease-related increase due to lung consolidation (Fig. 3).

Gross pathological and histopathological findings in the lungs of ferrets. Four days after inoculation with influenza 2009 A(H1N1) virus, the lungs of the ferrets were examined

FIG. 2. Antibody response to vaccination with multimeric 2009 A(H1N1) influenza virus HA and NA antigens. Ferrets were immunized on day 0 and day 20 with 3.75 μ g sHA₃ + 3.75 μ g sNA₄ (sHA+sNA), 3.75 μ g sHA₃ in adjuvant (ISCOM Matrix M [IMM]) (sHA+IMM), 3.75 μ g sNA₄ in adjuvant (sNA+IMM), 3.75 μ g sHA₃ + 3.75 μ g sNA₄ in adjuvant (sHA+sNA+IMM), PBS, or IMM, as indicated. The antibody response to the 2009 A(H1N1) influenza virus was evaluated by hemagglutination inhibition (HI) (upper panel), virus neutralization (VN) (second panel), and neuraminidase inhibition (NI) assays (lower two panels). Each dot represents the result for one ferret. Horizontal lines represent means. The horizontal gray bar indicates the detection limit of the assay.

macroscopically and weighed before samples were taken for assessing virus replication and histopathological changes. Dark red and firm consolidated areas were observed macroscopically in lungs of inoculated ferrets. The percentage of affected lung

FIG. 3. Clinical effects after challenge inoculation with 2009 A(H1N1) influenza virus. Ferrets immunized as described in the legend to Fig. 2 were inoculated intratracheally on day 52 with 10^6 TCID₅₀ of virus. Body weight losses are expressed as percentage of body weight before infection (upper panel). Lung weights are expressed as percentage of body weight, as an indicator of lung consolidation (middle panel). Lungs were observed macroscopically and scored for lung area percentage displaying consolidated areas (bottom panel). Mean values are displayed; error bars indicate standard deviations. The horizontal gray bar indicates the detection limit of the assay.

tissue was estimated and was found to vary between groups. Mean percentages of affected areas in the lungs of about 50% were observed in ferrets of groups 1, 5, and 6, while the extent of consolidation was less pronounced in ferrets of groups 2, 3, and 4, which showed less than 25% of the lung area being affected (Fig. 3). The relative lung weight also was lower in these groups than in ferrets of groups 1, 5, and 6 (Fig. 3).

The histopathological changes observed at day 4 postinoculation in the lungs of ferrets mock vaccinated with PBS or adjuvant (IMM) only or vaccinated with the nonadjuvanted sHA₃+sNA₄ were characteristic for a moderate to severe necrotizing broncho-interstitial pneumonia. Multifocally, many neutrophils and macrophages and variable numbers of erythrocytes, edema fluid, and fibrin were present in the alveoli of the lungs of these ferrets. In addition, inflammatory infiltrates

FIG. 4. Examples of histopathological findings in lungs of ferrets after inoculation. (A) Inflammatory infiltrates and loss of epithelial cells in the bronchiolar walls and cellular debris in the bronchiolar lumen observed in the lungs of unprotected ferrets mock vaccinated with PBS or adjuvant only (IMM) or vaccinated with the nonadjuvanted sHA₃+sNA₄. (B) Proteinaceous fluid (edema) and infiltrate of inflammatory cells in the alveoli of lungs of ferrets mock vaccinated with PBS or adjuvant only (IMM) or vaccinated with the nonadjuvanted sHA₃+sNA₄. (C) Peribronchiolar infiltrate and cellular debris in the bronchiole of a ferret vaccinated with sHA+IMM. (D) Inflammatory infiltrate in the alveolar septa and hypertrophy and hyperplasia of type II pneumocytes in lungs of ferrets vaccinated with sHA+IMM. (E) Peribronchiolar infiltrate observed in lungs of ferrets of the sNA+IMM and sHA+sNA+IMM groups. (F) Absence of inflammatory cells and hyperplasia of type II pneumocytes in alveoli of lungs of ferrets of the sNA+IMM and sHA+sNA+IMM groups. H&E staining was used. Magnifications, $\times 20$ (bronchioles) and $\times 40$ (alveoli).

were present in the alveolar septa, in the bronchioles, in the bronchi, and in the walls of bronchi and bronchioles. A dramatic reduction in histopathological changes was observed in the adjuvanted sNA₄-vaccinated animals (sNA+IMM and sHA+sNA+IMM groups), while ferrets immunized with sHA+IMM were partially protected from developing pathology (Fig. 4).

Protection against virus replication in the upper and lower respiratory tracts. To measure the effect of vaccination on the virus replication in the respiratory tract, virus titers in lungs, throat, and nose were determined at 4 days after inoculation. As shown in Fig. 5 the challenge virus replicated efficiently in the lungs of the control ferrets (PBS and IMM groups) and in the animals immunized with the nonadjuvanted mixture of sHA₃ and sNA₄ (sHA+sNA group), with mean viral titers of approximately 10^7 to 10^8 TCID₅₀/gram tissue. These viral loads were reduced by about 5 log₁₀ units in the animals immunized with the sHA₃ protein in adjuvant (sHA+IMM group) and in

FIG. 5. Viral titers in lungs, noses, and throats of challenge-inoculated animals. Virus replication in the ferrets immunized and challenged as described in the legend to Fig. 3 was analyzed at 4 days after inoculation. Virus titers were determined in lung homogenates (upper panel), nose swaps (middle panel), and throat swaps (bottom panel). Titers were assayed by means of endpoint titration in MDCK cells. Each dot represents the result for one ferret. Horizontal lines represent means. The horizontal gray bar indicates the detection limit of the assay.

animals coimmunized with sHA₃ and sNA₄ in adjuvant (sHA+sNA+IMM group). Mean viral loads were reduced by 2 to 3 log₁₀ units in animals immunized with adjuvanted sNA₄ antigen (sNA+IMM group).

High viral loads in the nose were observed in the control animals (PBS and IMM groups) (Fig. 5) at day 4 after the challenge. Though not statistically significant due to the large variations in titers within groups, these viral loads appeared to be somewhat lower in the animals immunized with adjuvanted sHA₃ or adjuvanted sNA₄ or with the nonadjuvanted sHA₃+sNA₄ combination. The highest reduction of nose viral titers was found in animals immunized with the adjuvanted combination of sHA₃ and sNA₄ antigens. Viral titers in the throat were generally high and were not significantly affected by vaccination, except in the animals vaccinated with the ad-

FIG. 6. Induction of cross-neutralizing antibodies by vaccination with multimeric 2009 A(H1N1) influenza virus sHA₃ and sNA₄ antigens. (A) Sera of ferrets immunized twice with sHA₃ or sHA₃+sNA₄, both in adjuvant, as described in the legend to Fig. 2 were tested in an HI assay for activity toward different influenza viruses, including A/Swine/shope/1/56, A/Italy/1443/76, A/NL/386/86, A/Iowa/15/30, A/NL/25/80, A/New Jersey/8/76, A/PR/8/34, and IVR/148 influenza H1N1. Mean titers are displayed; error bars indicate standard deviations. (B) Sera of ferrets immunized once or twice with sNA₄ or sHA₃+sNA₄, both in adjuvant, were pooled and tested in a NI assay for activity against the sNA₄ of A/Kentucky/UR06-0258/2007(H1N1) and A/turkey/Turkey/1/2005(H5N1) influenza viruses. The NA of A/California/04/2009(H1N1) was used as a positive control. Positive-control sera specific for A/NL/602/09(H1N1) or A/turkey/Turkey/1/2005(H5N1) influenza virus were obtained from a ferret infected with these viruses. Average titers from two replicates are displayed; error bars indicate standard deviations. The horizontal gray bar indicates the detection limit of the assay.

juvanted combination of sHA₃ and sNA₄. These ferrets did not have detectable titers in the throat.

Cross-reacting antibody responses induced by immunization with sHA₃ and sNA₄. To investigate whether the antibodies induced by the sHA₃ and sNA₄ antigens could cross-react with other H1N1 influenza viruses, we performed additional HI and NI assays with the postvaccination sera. As expected, the highest HI titers were measured against the homologous virus, while various extents of cross-reactivity were observed with a range of other H1 strains (Fig. 6A). Thus, no cross-reactivity was detected for A/Swine/shope/1/56, A/Italy/1443/76, A/Iowa/15/30, A/PR/8/34, and IVR/148, whereas significant cross-reactivity was measured against A/NL/25/80 and A/New Jersey/8/76 and, particularly, against A/NL/386/86, more or less consistent with the sequence similarities of their antigenic do-

main (Table 1). This was the case with the sera from both the sHA₃+IMM- and the sHA₃+sNA₄+IMM-vaccinated animals. Consistent with the earlier observed differences in HI activity against the homologous virus (Fig. 2), the levels of cross-reactivity were markedly higher with the sera from ferrets immunized with sHA₃+sNA₄+IMM than with those from sHA₃+IMM-immunized animals, confirming again the enhancing effect of the sNA₄ antigen. HI titers against each strain were detected in control sera of ferrets infected with the homologous influenza A/H1N1 virus (data not shown).

To investigate the cross-reactivity of the NA antibodies, we produced sNA₄ glycoprotein complexes of two other N1 influenza viruses, the human H1N1 strain A/Kentucky/UR06-0258/2007 and the avian H5N1 strain A/turkey/Turkey/1/2005. When tested in our NI assay, there was a strong neuramini-

TABLE 1. Sequence homology of the antigenic regions within HAs of different H1N1 strains

H1N1 strain	GenBank protein accession no.	% Sequence homology of the HA antigenic domains to that of A/California/04/09 ^a
A/California/04/09	ACQ76318.1	100
A/Netherlands/602/09	ACQ45338.1	98.6
A/Swine/shope/1/56	— ^b	NA ^c
A/Italy/1443/76	—	NA
A/Netherlands/386/86	AAK51350.1	66.2
A/Iowa/15/30	AAD25303.1	76.1
A/Netherlands/25/80	AAK51352.1	67.6
A/New Jersey/8/76	AAA43210.1	78.9
A/Puerto Rico/8/34	ACV89502.1	64.8
A/Brisbane/59/07 (IVR-148 vaccine strain)	ADI99532.1	56.3

^a Antigenic domains as defined in reference 39.^b —, sequence not available.^c NA, not applicable.

dase-inhibiting activity with the pooled sera of the sNA₄+IMM- and sHA₃+sNA₄+IMM-immunized animals against the avian H5N1 virus sNA₄ protein, while some inhibition of the seasonal H1N1 virus sNA₄ protein was observed (Fig. 6B). Of note, a control serum derived from an H5N1 virus-infected chicken tested negative against both human H1N1 virus sNA₄ proteins.

DISCUSSION

For the induction of protective immune responses against influenza viruses, the externally exposed parts of the virion glycoproteins are considered to be the key targets. As these antigens naturally occur in virions as multimeric structures, we have prepared these ectodomain complexes of the 2009 A(H1N1) influenza virus in the form of soluble HA trimers (sHA₃) and soluble NA tetramers (sNA₄). When these antigens were administered to ferrets in combination with the adjuvant ISCOM Matrix M either alone or in combination, protective immunity against a homologous challenge was induced. While the effect of immunization with sHA₃ reduced pulmonary virus titers significantly, immune responses to sNA₄ reduced the severity of disease. Although the differences per parameter were not significant, the sNA₄-vaccinated animals displayed an overall lower morbidity with respect to weight loss, lung consolidation, lung lesions, and lung histopathology than the sHA₃-vaccinated animals. The strongest reduction in virus titers in the upper and lower respiratory tracts was seen in animals vaccinated with the adjuvanted sHA₃+sNA₄ mixture, indicating that the more optimal protection against influenza is conferred by immunity elicited by the combination of these antigens. This is the first study showing the distinctive effects of immunization with soluble forms of HA and NA antigens and, particularly, the added value of combining these recombinant soluble antigens for eliciting protective immunity against influenza viruses, in particular toward the pandemic 2009 A(H1N1) influenza virus.

Correlates of protection against infection have so far not been extensively investigated for the pandemic 2009 A(H1N1) influenza virus. In our study the strong reduction in mean

pulmonary virus titers after challenge infection correlated with the presence of HI and VN titers, whereas the marked reduction in lung pathogenicity was associated with the presence of NI titers, indicating that optimal protection against influenza is achieved by immunity against both HA and NA antigens. The level of serum antibodies inhibiting hemagglutination as determined by an HI assay is generally accepted as a measure of influenza immunity. Antibodies neutralizing NA activity are also likely to play a direct role in decreasing virus replication by preventing virus release from infected cells (26, 40).

An interesting finding of our study was the differential effect of combining the HA and NA antigens on the antibody levels induced against each of these antigens. Compared with the NI titer observed after immunization with (adjuvanted) sNA₄ alone, the additional inclusion of sHA₃ in the vaccine was without much effect, confirming the lack of antigenic competition between the two antigens when coadministered other than in the context of a virion. In contrast, strongly increased VN titers and statistically significantly higher HI titers were observed upon vaccination with the (adjuvanted) antigen combination than after vaccination with sHA₃ only. These enhanced antibody levels correlated with the increased reduction in virus titers in the upper respiratory tract, while the enhanced reduction in pathogenic effects correlated with NA-specific immune responses. The mechanism by which the NA antigen affects the immune reaction to the HA antigen is quite intriguing and currently not understood, and it hence warrants further investigation.

Typical for influenza A viruses, antigenic variants of HA and NA within a certain virus subtype that are able to escape from existing immunity are gradually selected in the human population. This process of antigenic drift calls for the almost annual adjustment of the seasonal vaccine composition in response to newly arising variants. Also, in view of the threat of future influenza pandemics caused, for instance, by an avian H5N1 virus, future vaccine development should be directed to inducing broadly protective immunity. Studies in mice have suggested that inclusion of NA in the vaccine increases the level of protection against drifted strains within the same subtype (2). This type of cross-protective immunity may be engendered, at least in part, by humoral immunity against NA, since NA antibodies against seasonal human H1N1 have been shown in mice to have intrasubtypic protectivity against H5N1 (37). Our *in vitro* NI assays also clearly demonstrated that the 2009 A(H1N1) NA-specific humoral immunity induced in ferrets exhibited cross-neutralizing activity against sNA₄ of a human seasonal H1N1 and, more potently, against sNA₄ of an avian H5N1 strain. In the approximately 50 amino acids that constitute the putative antigenic regions (39), the NA sequence of pandemic A/California/04/2009(H1N1) has 64% identity to that of avian A/turkey/Turkey/1/2005(H5N1) NA but only 36% identity to that of human A/Kentucky/UR06-0258/2007(H1N1), which is consistent with the observed cross-reactivity. The 2009 A(H1N1) HA-specific cross-neutralizing activity, as we measured using the standard HI assay, was restricted to the swine H1N1 virus strains A/NL/386/86, A/NL/25/80, and A/New Jersey/8/76, while no cross-reactivity was observed against the vaccine strain (IVR/148), which is quite consistent with the sequence homology of the relevant antigenic regions within HA. Essentially the same results were obtained with

ferret sera obtained from ferrets infected with the influenza A/NL/602/09 virus (data not shown). NA has been found to have a lower rate of mutations, perhaps as a result of the lower immunoselective pressure on this antigen during natural infection (1, 25). It has been proposed that influenza vaccines including the more slowly evolving NA may hence be less vulnerable to antigenic changes in newly emerging viruses and thereby provide longer-lasting immunity (15). Prevalent NA-specific cross-reactive humoral immunity elicited by seasonal H1N1 infection or vaccination may be a tentative explanation for the moderate severity and mortality rates of 2009 A(H1N1) influenza virus in humans and may provide a dampening effect on the severity of a possible future H5N1 pandemic.

Interestingly, only in ferrets vaccinated with one or both antigens in combination with the adjuvant ISCOM Matrix M were antibody responses detected and ferrets protected against challenge infection. These observations further highlight the importance of the use of a proper adjuvant in influenza vaccine formulations. The adjuvant used in the current study, ISCOM Matrix M, is a formulation based on proprietary state-of-the-art immune-stimulating complex technology. Compared to earlier generations of ISCOM adjuvants, ISCOM Matrix M lacks toxicity and is considered safe while retaining its strong adjuvant activity. In other preclinical studies with influenza A/H5N1 virus vaccines it also has been demonstrated that this adjuvant potentiates specific antibody responses (31).

The recombinant subunit approach that we applied in our studies provides several advantages for the production of influenza virus vaccines compared to more traditional virus-dependent methods and may hence be extremely valuable in combating seasonal epidemics and future pandemics. The HA-to-NA ratio of the vaccine can easily be adjusted due to the independent expression of HA and NA. The reactogenicity to the vaccine may be decreased, since the recombinant proteins are highly purified and free of egg-derived proteins. The response time for vaccine preparation can be reduced, since the genes encoding the antigens can be rapidly cloned into the expression vectors, bypassing the necessity for reverse genetics or adaptation of viruses to growth in culture systems. The antigenicity of the glycoproteins reflects that of the natural strains, since direct gene cloning excludes the risk of immunogenicity changes caused by mutations acquired during virus propagation in culture systems. Furthermore, the recombinant subunit approach obviates the deleterious effects on antigenicity often associated with classical virus inactivation. Lastly, the approach avoids the need for high biocontainment facilities necessary to grow virus-dependent vaccines. The advances in production quality and quantity of recombinant pharmaceuticals in mammalian cell lines make these expression systems increasingly feasible for clinical application (10). Recombinant full-length HA (5, 13, 28, 35, 41) and NA (27) and soluble forms of HA (4, 43) (but not of NA) have been previously described to function as effective vaccine antigens against influenza virus. A major advantage of expressing soluble, multimeric forms of the HA and NA antigens rather than their full-length counterparts is their highly efficient, single-step affinity purification from the culture supernatant, hence not requiring the solubilization of membranes using detergents.

This study particularly underscores the underrated importance of NA in protective vaccination against influenza. Since

the current inactivated influenza virus vaccines are standardized only for the amount of HA, the NA content is variable, as is, consequently, the frequency and level of seroconversion to NA, which are often rather poor (23, 24). Our results are in line with previous results (3, 14, 19, 21, 37) supporting the notion that future influenza vaccines including sufficient immunogenic amounts of neuraminidase may provide a better and possibly more broadly effective immune response toward influenza viruses. In addition, inclusion of NA in the vaccine may also reduce the dose of HA antigen required for the induction of protective immunity. Further studies into these aspects are certainly warranted.

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