Oligomeric Recombinant H5 HA1 Vaccine Produced in Bacteria Protects Ferrets from Homologous and Heterologous Wild-Type H5N1 Influenza Challenge and Controls Viral Loads Better than Subunit H5N1 Vaccine by Eliciting High-Affinity Antibodies

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Recombinant hemagglutinin from influenza viruses with pandemic potential can be produced rapidly in various cell substrates. In this study, we compared the functionality and immunogenicity of bacterially produced oligomeric or monomeric HA1 proteins from H5N1 (A/Vietnam/1203/04) with those of the egg-based licensed subunit H5N1 (SU-H5N1) vaccine in ferrets challenged with homologous or heterologous H5N1 highly pathogenic influenza strains. Ferrets were vaccinated twice with the oligomeric or monomeric rHA1 or with SU-H5N1 (Sanofi Pasteur) emulsified with Titermax adjuvant and were challenged with wild-type homologous (A/Vietnam/1203/04; clade 1) or heterologous (A/Whooperswan/Mongolia/244/2005; clade 2.2) virus. Only the oligomeric rHA1 (not the monomeric rHA1) immunogen and the SU-H5N1 vaccine provided protection against the lethality and morbidity of homologous and heterologous highly pathogenic H5N1. Oligomeric rHA1 generated more cross-neutralizing antibodies and higher levels of serum antibody binding to HA1, with stronger avidity and a better IgG/IgM ratio, than monomeric HA1 and SU-H5N1 vaccines, as determined by surface plasmon resonance (SPR). Importantly, viral loads after heterologous H5N1 challenge were more efficiently controlled in ferrets vaccinated with the oligomeric rHA1 immunogen than in SU-H5N1-vaccinated ferrets. The reduction of viral loads in the nasal washes correlated strongly with higher-avidity antibodies to oligomeric rHA1 derived from H5N1 clade 1 and clade 2.2 viruses, as measured by SPR. This is the first study to show the role of antibody avidity for the HA1 globular head domain in reduction of viral loads in the upper respiratory tract, which could significantly reduce viral transmission.

The most effective way to curtail pandemics is by mass vaccination (33, 41). At the moment there are two types of licensed vaccines against seasonal influenza in the United States: subunit (split) inactivated vaccines (IV) and live cold adapted attenuated influenza vaccine (LAIV) (10, 34). Both vaccines are grown in chicken eggs. The process of constructing a new vaccine strain based on newly circulating viruses is quite lengthy. It involves *in vivo* (in chicken eggs) or *in vitro* (in cell culture using reverse genetics techniques) reassortment between the internal genes of a donor virus, such as A/PR/8/34, and the hemagglutinin (HA) and neuraminidase (NA) genes of the new influenza virus strain. The candidate vaccine strains must be further selected on the basis of high growth capability in eggs and high yield of HA content before they can be used for production of vaccines. This process is used for the production of seasonal influenza vaccines every year, but it may pose an impediment to the initiation of rapid mass vaccination against spreading pandemic influenza, as was evident for the 2009 H1N1 virus.

Recombinant HA-based vaccines provide an alternative that could save several months of manufacturing time, since the HA gene of the newly circulating strain is available shortly after virus isolation. Vaccines utilizing the expression of recombinant HA in insect, yeast, plant, and mammalian cells are under development and/or in clinical trials (13, 23, 40, 47, 51). The main challenge to the recombinant technology is to ensure that the HA products resemble the native virion-associated trimeric spike proteins and

can elicit robust immune responses targeting protective conformational epitopes in the globular domain of HA (39, 44).

Expression of recombinant HA proteins in bacterial systems could provide a rapid and economical approach for early response to impending influenza pandemic. However, it was not known if nonglycosylated proteins would present antigenically relevant epitopes. Recently, we demonstrated that bacterially produced influenza HA1 domains (amino acids [aa] 1 to 320) from several pandemic strains are properly folded, form functional oligomers that can agglutinate red blood cells (RBC), and elicit broadly neutralizing antibodies upon immunization (16, 20, 21). The oligomerization signal was mapped to the first five amino acids in the N terminus of HA1 (20).

In the present study, to better understand the importance of oligomerization of the recombinant HA1 globular head domain

Received 22 June 2012 Accepted 28 August 2012

Published ahead of print 5 September 2012

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doi:10.1128/JVI.01596-12

for immunogenicity, cross-protection, and control of viral loads, we compared the functionality and immunogenicity of bacterially produced oligomeric or monomeric HA1 proteins from H5N1 (A/Vietnam/1203/04) with the egg-based licensed subunit H5N1 (SU-H5N1) vaccine in a ferret challenge model using two different clades of highly pathogenic (HP) H5N1 influenza virus. We further investigated the correlates of protection by comparing the immune sera derived from ferrets immunized with oligomeric or monomeric H5N1 HA1 proteins (from A/Vietnam/1203/2004) produced in bacteria compared with sera from ferrets that received the licensed H5N1 inactivated subunit vaccine (Sanofi Pasteur). All immunogens were mixed with Titermax adjuvant and were administered to ferrets twice at 15 µg HA per dose prior to challenge with wild-type homologous (A/Vietnam/1203/2004) or heterologous (A/Whooperswan/Mongolia/244/2005 [WS]) H5N1 viruses. Surface plasmon resonance (SPR)-based real-time kinetics was used to measure antibody titers, antibody isotype (fraction of bound antibodies, whether IgG, IgM, or IgA), and antibody-antigen dissociation rates against the HA1 domains derived from both homologous and heterologous H5N1 strains. SPR technology-based real-time kinetics provides important information about the quantity and quality of the vaccine-induced antibodies to different domains within HA that is not captured by traditional neutralization assays. We found that the oligomeric rHA1 vaccine was superior to the monomeric rHA1 and to the inactivated H5N1 vaccine in generating high-avidity antibodies against the HA1 domain. A strong correlation was found between the off-rates of HA1-bound antibodies and the viral loads in the upper respiratory tract. These data suggest that antibody avidity is likely to play an important role in controlling viral replication *in vivo* and thus may reduce morbidity and interhost transmission even after exposure to heterologous H5N1 strains.

MATERIALS AND METHODS

Expression vector and cloning of H5N1 HA1 derivatives. cDNA corresponding to the HA gene segment of H5N1 A/Vietnam/1203/2004 (clade 1) and A/Whooperswan/Mongolia/244/2005 (clade 2.2) was generated from RNA isolated from an egg-grown wild-type virus strain and was used for cloning. pSK is a T7 promoter-based expression vector where the desired polypeptide can be expressed as a fusion protein with a His₆ tag at the C terminus. DNA encoding HA1 (aa 1 to 320) of A/Vietnam/1203/2004 or A/Whooperswan/Mongolia/244/2005 was cloned as NotI-PacI inserts in the pSK expression vector (17).

Protein expression, refolding, and purification. *Escherichia coli* Rosetta Gami cells (Novagen) were used for expression and production of HA1 from different H5N1 strains (A/Vietnam/1203/2004 and A/Whooperswan/Mongolia/244/2005) and purified by HisTrap fast-flow chromatography as previously described (20, 21).

Gel filtration chromatography. Proteins at a concentration of 5 mg/ml were analyzed on a Superdex S200 XK 16/60 column (GE Healthcare) pre-equilibrated with phosphate-buffered saline (PBS), and protein elution was monitored at 280 nm as previously described (20). Protein molecular weight marker standards (GE Healthcare) were used for column calibration and generation of standard curves, to identify the molecular weights of the test protein samples. Endotoxin levels in the purified proteins were <10 endotoxin units (EU)/ml, as determined with the *Limulus* amoebocyte lysate (LAL) assay (Lonza Cologne GmbH).

Hemagglutination assay. Human erythrocytes were separated from whole blood (Lampire Biologicals). After isolation and washing, 30 µl of a 1% (vol/vol) human RBC suspension in 1% bovine serum albumin (BSA)-PBS was added to 30-µl serial dilutions of purified HA1 proteins or

influenza virus in 1% BSA-PBS in a U-bottom 96-well plate (total volume, 60 µl). Agglutination was read after incubation for 30 min at room temperature.

Transmission electron microscopy. Electron microscopy analysis was essentially performed as previously described (16). Briefly, after negative staining of the sample for 2 min with 1% uranyl acetate, sample-bearing grids were analyzed in a CM120 transmission electron microscope (FEI, Hillsboro, OR) operating at 120 kV. Defocus values between −1.5 and −2.5 µm and a nominal magnification of ×45,000 were used for recording images on SO-163 film (Kodak). A Super Coolscan 9000 ED (Nikon) was used for digitalization. After binning the scanned images four times, the final pixel size used for the analysis of the different H5N1 A/Vietnam/1203/2004 (clade 1) HA1 oligomeric conformations was 6.2 Å/pixel. Bsoft (12), EMAN (28), and SPIDER (38) were used for the analysis. Particles showing rosette-like shapes, which we took to represent HA oligomers of trimers, were selected ($n = 1,066$) and extracted using a box size of 64 pixels. A selection of 12 oligomers was used as an initial reference, and iterative refinement was performed using principal component analysis as implemented in SPIDER.

Ferret immunization and challenge studies. (i) Vaccination of ferrets and blood collection. All ferrets (Marshall Farms) used in the study were confirmed as seronegative for circulating seasonal influenza A (H1N1 and H3N2) and influenza B viruses by HI. Female Fitch ferrets ($n = 6$ in each group) were vaccinated intramuscularly in the quadriceps muscle on day 0, given a booster on day 21, and challenged with virus on day 35. Control animals ($n = 6$) were mock vaccinated with phosphate-buffered saline (PBS; pH 7.2). Each animal was vaccinated twice with 15 µg of recombinant HA1 (oligomeric or monomeric HA1 fractions) in sterile 0.9% saline or with licensed inactivated H5N1 subunit vaccine (15 µg of HA antigen as indicated on the vaccine vial that was confirmed using single radial immunodiffusion assay [SRID] in our lab) (lot number UD08916; Sanofi Pasteur). All three immunogens were mixed with the adjuvant formulation, Titermax (Titermax Inc., Norcross, GA), at a 1:1 ratio. The volume for all intramuscular vaccinations was 0.5 ml. The first and second vaccinations were given in the left and right hind legs, respectively. Blood was collected from anesthetized ferrets via the anterior vena cava. The collected blood was transferred to a tube containing a serum separator and clot activator and allowed to clot at room temperature. Tubes were centrifuged at 6,000 rpm for 10 min; serum was separated, aliquoted, and stored at $-80 \pm 5^\circ\text{C}$. All procedures were performed in accordance with the National Research Council (NRC) Guidelines for the Care and Use of Laboratory Animals, the Animal Welfare Act, and the Centers for Disease Control (CDC)/National Institutes of Health (NIH) Bio-Safety Guidelines in Microbiological and Biomedical Laboratories and approved by the Institutional Animal Care and Use Committee (IACUC).

(ii) Infection and monitoring of ferrets. Animal experiments with H5N1 influenza virus were performed in an AAALAC-accredited animal biosafety level 3 (ABSL-3) enhanced facility. Animals were infected and monitored as previously described (55), except that 5% isoflurane anesthesia was used. Briefly, ferrets were anesthetized with isoflurane and infected intranasally with 1×10^6 50% egg infectious doses (EID₅₀) ($\sim 1 \times 10^{5.75}$ 50% tissue culture infective doses [TCID₅₀]/ml), which is equivalent to 1,000 50% lethal doses (LD₅₀) of A/Vietnam/1203/2004 (clade 1) or A/Whooperswan/Mongolia/244/2005 (clade 2.2) in a volume of 1 ml. Animals were monitored daily for temperature, weight loss, loss of activity, nasal discharge, sneezing, and diarrhea following viral challenge. To determine viral load in nasal washes, 1.5 ml of 0.9% saline was administered to each naris, and the washes were collected each day postchallenge from each ferret. Temperatures were measured using an implantable temperature transponder (BMDS, Sayre, PA) and were recorded at approximately the same time each day. Preinfection values were averaged to obtain a baseline temperature for each ferret. Clinical signs, including sneezing and nasal discharge, inappetence, dyspnea, neurological signs, respiratory distress, and level of activity, were assessed daily. The scoring

system used to assess activity level was as follows: 0 = alert and playful; 1 = alert but playful only when stimulated; 2 = alert but not playful when stimulated; 3 = neither alert nor playful when stimulated. Based on the daily scores for each animal in a group, a relative inactivity index was calculated.

(iii) Determination of viral loads. Viral loads in nasal washes were determined by the plaque assay. Briefly, MDCK cells plated in six-well tissue culture plates were inoculated with 0.1 ml of virus-containing sample serially diluted in Dulbecco's modified Eagle's medium (DMEM). Virus was adsorbed to cells for 1 h with shaking every 15 min. Wells were overlaid with 1.6% (wt/vol) Bacto agar (Difco, BD Diagnostic Systems, Palo Alto, CA) mixed 1:1 with L-15 medium (Cambrex, East Rutherford, NJ) containing antibiotics and 0.6 mg/ml trypsin (Sigma, St. Louis, MO). Plates were incubated for 5 days. Cells were fixed for 10 min using 70% (vol/vol) ethanol and then overlaid with 1% (wt/vol) crystal violet. Cells were then washed with deionized water to visualize plaques. Plaques were counted and compared to uninfected cells.

(iv) Hemagglutination inhibition (HI) assay. Receptor-destroying enzyme (RDE)-treated ferret sera were serially diluted in V-bottom 96-well microtiter plates followed by the addition of 4 hemagglutination units (HAU) of influenza virus. Following incubation for approximately 30 min, a 1% suspension of human RBC in PBS (pH 7.2) was added and mixed by agitation. The human RBCs were allowed to settle for 30 min at room temperature, and HI titers were determined by the reciprocal value of the last dilution of sera that completely inhibited hemagglutination of human RBCs. A negative titer was defined as 1:10.

(v) Ethics statement. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh (permit number A-3187-01). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Postvaccination serum antibody binding to H5N1 A/Vietnam/1203/2004 (clade 1) and A/Whooperswan/Mongolia/244/2005 (clade 2.2) HA proteins and off-rate measurements by surface plasmon resonance. Steady-state equilibrium binding of postimmunization ferret sera was monitored at 25°C using a ProteOn surface plasmon resonance biosensor (Bio-Rad) as previously described (19). The H5N1 rHA1 or mammalian cell-derived glycosylated HA0 proteins (Immune Technology Corp) were coupled to a general layer compact (GLC) sensor chip with amine coupling in the test flow cells. Samples of 60 μ l freshly prepared sera at 10-fold dilutions were injected at a flow rate of 30 μ l/min (120-s contact time) for association, and dissociation was performed over a 600-s interval (at a flow rate of 30 μ l/min). rHA1-bound antibodies were isotyped by sequential injection of antibodies specific to ferret IgA, IgG, and IgM. Responses from the protein surface were corrected for the response from a mock surface and for measurements from a separate, buffer-only injection. Monoclonal antibody (MAb) 2D7 (anti-CCR5) was used as a negative control in these experiments. Binding kinetics for the postvaccination ferret sera and data analyses were calculated using Bio-Rad ProteOn manager software (version 2.0.1).

Antibody off-rate constants, which describe the stability of the complex, i.e., the fraction of complexes that decays per second, were determined directly from serum sample interaction with HA proteins using SPR (as described above) and calculated using the Bio-Rad ProteOn manager software for the heterogeneous sample model. For all polyclonal sera, it was important to demonstrate that the dissociation rates were independent of total HA-binding-antibody titers. To that end, parallel dissociation curves for 10-fold and 100-fold dilutions of postvaccination ferret sera were established as previously described (19). To improve the measurements, the off-rate constants were determined from two independent SPR runs.

RESULTS

Oligomeric but not monomeric HA1 forms rosettes and agglutinates human RBC. Recombinant H5N1 HA1 protein (A/Vietnam/1203/2004) expressed in *E. coli* and refolded under controlled conditions contained a high fraction of oligomeric forms in gel filtration chromatography (Fig. 1A). To further characterize the physical structure and functionality of the monomeric and oligomeric forms, we purified the high-molecular-weight (MW) (Fig. 1B) and low-MW (Fig. 1C) fractions by size exclusion chromatography (SEC) and tested their binding to conformation-dependent H5N1 neutralizing human monoclonal antibodies (MAbs) (Fig. 1D), their negative staining in electron microscopy (Fig. 1E), and their hemagglutination of human RBC (Fig. 1F). Both SEC-purified monomeric and oligomeric fractions remain as stable HA1 oligomers (Fig. 1B) and HA1 monomers (Fig. 1C), as determined by gel filtration following purification of the two fractions. Both the rHA1 oligomeric and monomeric proteins were properly folded and presented conformational epitopes, as judged by binding to two neutralizing human monoclonal antibodies, FLA5.10 and FLA3.14, that target different sites in the HA1 globular head domain (15, 17) (Fig. 1D). Electron microscopy showed that the high-MW oligomers formed rosette-like structures (~75%) with an average diameter of ~25 nm (Fig. 1E, panel b). The rosette-like structures were further classified into like sets. Averages of the typical particles from each class were then obtained to enhance the common features (Fig. 1E, panel c), showing rosettes of 3 to 5 spikes, ~10 nm long and ~5 nm wide. In order to validate the classification analysis, particles from each class were analyzed, and individual rosettes showing the features of the correspondent average are shown (Fig. 1E, panel d). On the other hand, while most monomers presented a globular shape ~5 to 7 nm in diameter, some exhibited an elongated pattern, ~10 nm long and ~3 nm wide at the base of the globular head (Fig. 1E, panel a, inset), compatible with the expected dimensions of monomeric HA1. In the hemagglutination assay, oligomeric rHA1 agglutinated human RBC with endpoint titers of 4 ng, similar to the egg-derived H5N1 subunit vaccine (Sanofi Pasteur), which showed hemagglutination until 15 ng HA, while the monomeric HA1 did not agglutinate RBC (Fig. 1F). These findings confirm the requirement of oligomeric HA1 for RBC lattice formation and further demonstrate that even in the absence of the HA2 stalk, bacterially produced HA1 (aa 1 to 320) forms functional oligomers resembling the rosettes of virus-derived HA.

Oligomeric HA1 elicits significantly higher-avidity homologous anti-HA1 antibodies than monomeric HA1 and licensed subunit H5N1 vaccine in ferrets. The immunogenicity of the oligomeric and monomeric HA1 fractions as well as the licensed inactivated H5N1 subunit vaccine was evaluated in ferrets. Ferrets (12 per group) were vaccinated twice with either subunit H5N1 vaccine or rHA1 (15 μ g) mixed with the Titermax adjuvant (1:1 ratio) on days 0 and 21. Sera were collected 2 weeks after the second immunization and evaluated by SPR using rHA1-coated chips. As seen in Fig. 2A, most of the ferrets vaccinated with the oligomeric HA1 generated 2- to 4-fold-higher titers of anti-HA1 binding antibodies against H5N1 A/Vietnam/1203/2004 than the ferrets immunized with either monomeric rHA1 or subunit H5N1 vaccine, as verified by maximum resonance units (RU). Moreover, calculation of antigen-antibody complex dissociation rates revealed that oligomeric-HA1-vaccinated ferrets generated anti-

FIG 1 Biochemical and functional characterization of bacterially expressed and purified H5N1 HA proteins from *E. coli*. (A to C) Superdex S-200 gel filtration chromatography of bacterial H5N1 HA1 (aa 1 to 320) protein. The HA1 domain (aa 1 to 320) of A/Vietnam/1203/2004 (H5N1) was expressed in *E. coli* as a fusion protein with a His₆ tag at the C terminus. The purified protein ran as a single band at the expected molecular weight in reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified H5N1 HA1 protein with an intact N terminus (aa 1 to 320) (A) was subjected to gel filtration, and two peaks (45 to 60 ml and 81.2 to 86 ml), representing the oligomeric (B) and monomeric (C) forms, respectively, were collected. The collected fractions were concentrated to 5 mg/ml and subjected to gel filtration. The panels show superimposed elution profiles of purified HA proteins (red and blue lines) overlaid with calibration standards (gray line). The elution volumes of protein species are shown above the peaks. (D) Steady-state binding equilibrium analysis of human H5N1 neutralizing MAbs FLA3.14 and FLA5.10 (10 μ g/ml) to purified oligomeric and monomeric fractions of the H5N1 HA1 domain immobilized on a sensor chip through the free amine group in SPR. (E) Negative-stain electron micrographs of H5N1 A/Vietnam/1203/2004 monomeric (a) and oligomeric (b to d) forms. The area marked with a square in panel a is enlarged in the inset. In panel b, rosette-like structures used for analysis are circled. The middle and lower panels show class average images and representative rosettes from each class, respectively. Bars: 100 nm (upper panels) and 20 nm (inset in upper left panel and middle and lower panels). (F) Agglutination of human RBC by oligomeric and monomeric fractions of H5N1 HA1 (aa 1 to 320) protein (starting concentration of 5 μ g/ml) along with subunit H5N1 vaccine (starting concentration of 1 μ g/ml). Serial dilutions of purified HA1 proteins were mixed with washed RBC, and hemagglutination was read after 30 min at room temperature.

HA1 antibodies with significantly higher avidity (up to 2 log₁₀ lower off-rates) than ferrets immunized with monomeric HA1 or subunit H5N1 vaccine (Fig. 2B). For calculation of the dissociation kinetics (off-rates), the SPR assay was performed with a low

density of the antigen on the chip surface to provide only one epitope-paratope interaction for each intact antibody and was optimized using a MAb (bivalent IgG) and a Fab counterpart to obtain comparable kinetics (18, 19, 22, 50). Similar results were

FIG 2 Oligomeric H5N1 HA1 (aa 1 to 320) elicits higher-titer and higher-avidity HA1-binding antibodies than the monomeric HA1 and subunit H5N1 vaccines in ferrets. Ferret sera (diluted 1:10) obtained following two immunizations either with subunit H5N1 vaccine or with monomeric or oligomeric HA1 fractions were injected simultaneously onto H5N1 HA1 (A/Vietnam/1203/2004) protein immobilized on a sensor chip through the free amine group and onto a blank flow cell free of peptide. (A) Total bound antibodies from the polyclonal postvaccination ferret sera to the immobilized HA1 protein, shown in resonance units (RU). (B) Postvaccination serum antibody affinity to the HA1 domain, as measured by antigen-antibody complex off-rate constants in SPR analysis of ferret sera after immunization with either monomeric or oligomeric rHA1 or subunit H5N1 vaccine. Serum antibody off-rate constants were determined as described in Material and Methods. (C) IgM fraction of total antibodies bound to HA1 for individual immune sera in the two recombinant vaccine groups (either monomeric or oligomeric rHA1) or

obtained in SPR studies using mammalian rHA0. Unfortunately, the commercial HA0 was found to consist primarily of nonfunctional forms (that did not bind sialic acid and did not cause hemagglutination of human RBC) and could not be used to distinguish between the binding of antibodies generated by the monomeric and oligomeric functional HA immunogens. Furthermore, we found in several recent studies that high-avidity antibodies against HA2 can interfere with accurate measurements of antibody off-rates against the HA1 globular head (18, 19).

We also measured the isotype distribution of the HA1-bound polyclonal serum antibodies by SPR. The monomeric rHA1 and subunit H5N1 vaccines elicited antibodies with a higher fraction of IgM (i.e., lower IgG/IgM ratio) than the oligomeric rHA1. IgM antibodies are predicted to have lower binding avidity and higher dissociation rates (Fig. 2C).

Homologous and heterologous highly pathogenic H5N1 viral challenge of ferrets vaccinated with oligomeric or monomeric rHA1 or with inactivated subunit H5N1 vaccine. For the wild-type H5N1 challenge study, ferrets were immunized twice with the oligomeric or monomeric fraction of rHA1 (Fig. 1B and C, respectively) or with the licensed inactivated H5N1 subunit vaccine (A/Vietnam/1203/04; Sanofi Pasteur). All immunogens were administered at 15 μ g HA per dose, mixed (1:1) with the Titermax adjuvant. For the challenge, ferrets were anesthetized with isoflurane and infected intranasally with 1×10^6 50% egg infectious doses (EID₅₀) ($\sim 1 \times 10^{5.75}$ TCID₅₀/ml), which is equivalent to 1,000 LD₅₀ of wild-type HP H5N1 A/Vietnam/1203/2004 (clade 1) or A/Whooperswan/Mongolia/244/2005 (clade 2.2) virus in a volume of 1 ml. The same dose for both viruses was used, as they exhibit similar pathogenicity profiles in naïve ferrets. All ferrets succumbed to disease between days 5 to 7 after infection with either of these highly pathogenic wild-type H5N1 viruses. Following viral challenge, ferrets were monitored daily for temperature, weight loss, loss of activity, nasal discharge, sneezing, and diarrhea.

As can be seen in Fig. 3, the oligomeric rHA1 was highly protective against both homologous and heterologous HP H5N1 influenza strains, as judged by ferret survival (Fig. 3A and D), weight loss (Fig. 3B and E), and sickness score (Fig. 3C and F). The same level of protection was observed in the groups vaccinated with the adjuvant-containing inactivated H5N1 subunit vaccine (Fig. 3). In contrast, the monomeric rHA1 immunogen was not very protective: the majority of ferrets vaccinated with this protein died at the same rate as unvaccinated animals (Fig. 3) after homologous (67%) or heterologous (83%) challenge (Fig. 3A and D). Furthermore, all ferrets vaccinated with the monomeric rHA1 lost weight (Fig. 3B and E) and demonstrated high sickness scores (Fig. 3C and F).

Ferrets vaccinated with oligomeric rHA1 generated higher HI titers of antibody against homologous and heterologous H5N1 viruses following vaccination than those receiving monomeric or inactivated subunit H5N1 vaccine. The HI titers after vaccination and prior to challenge were variable. However, ferrets

licensed subunit H5N1 vaccine. Correlation statistics of the total antibody binding, percent bound IgM antibodies, and off-rate constants of the postvaccination ferret sera between the oligomeric versus monomeric HA1 and oligomeric versus licensed subunit H5N1 vaccine groups were highly significant, with P values of <0.05 (t test).

FIG 3 Challenge of vaccinated (oligomeric HA1, monomeric HA1, and SU-H5N1 vaccines) and unvaccinated ferrets with wild-type HP H5N1 influenza viruses. Six ferrets in each group were immunized with monomeric HA1 (mono-HA1; open green circles), oligomeric HA1 (oligo-HA1; magenta circles), the licensed subunit H5N1 vaccine (SU-H5N1; blue circles), or PBS (mock; open black circles). Following two immunizations, ferrets (six animals per group) were infected intranasally with 1×10^6 EID₅₀ of A/Vietnam/1203/2004 (clade 1) (A to C) or A/Whooperswan/Mongolia/244/2005 (clade 2.2) (D to F). Animals were scored for percent survival (A and D), percent original body weight (B and E), and clinical sickness scores (C and F).

receiving the oligomeric rHA1 had significantly higher HI titers against A/Vietnam/1203/2004 (Fig. 4A) and heterologous virus strains from H5N1 clade 2.2 (Fig. 4B), H5N1 clade 2.1 (Fig. 4C), and H5N1 clade 2.2 (Fig. 4D) than the monomeric-HA1-vaccinated or inactivated-SU-H5N1-vaccinated animals (Fig. 4A to D). These findings suggested that HI (or virus neutralization titers) may not be fully predictive of the challenge outcome in the ferret model, since some of the ferrets that survived following wild-type HP H5N1 virus challenge (especially in the subunit vaccine group) showed HI titers of $<1:40$. Similar observations were reported before, including those in a study conducted by Lipatov et al. (2, 6, 26, 30).

Oligomeric HA1 reduced viral loads in the nasal washes more effectively than subunit H5N1 vaccine in ferrets challenged with heterologous H5N1 (clade 2.2) virus. In addition to survival and weight retention, the capacity to reduce viral replica-

tion in the upper and lower respiratory tract is an important attribute of successful influenza vaccines, especially against HP H5N1 strains, as they reach very high titers in the upper and lower respiratory tract, which are associated with hypercytokinemia (7, 11). To that end, viral loads in the nasal washes from ferrets challenged with A/Vietnam/1203/2004 and A/Whooperswan/Mongolia/244/2005 were measured on day 3 (peak virus replication). As can be seen in Fig. 5A, following homologous challenge (A/Vietnam/1203/2004), high viral loads were measured in the nasal washes of unvaccinated animals and animals vaccinated with monomeric rHA1. In comparison, the viral loads in nasal washes of animals vaccinated with oligomeric HA1 or inactivated SU-H5N1 were lower (>2 log₁₀ reduction of average viral titers). Interestingly, after heterologous H5N1 virus challenge (A/Whooperswan/Mongolia/244/2005), the lowest viral loads were observed in nasal washes from animals vaccinated with the oligomeric rHA1 relative

FIG 4 Oligomeric H5N1 HA1 (aa 1 to 320) elicits higher HI antibody titers than monomeric HA1 or subunit H5N1 vaccines in ferrets. Animals were immunized with recombinant HA1 proteins (monomeric or oligomeric HA1 fractions) or subunit H5N1 vaccine mixed with Titermax adjuvant every 3 weeks. Sera were collected 2 weeks after the second vaccination and analyzed in a HI assay using human RBC against various virus strains from different H5N1 antigenic clades. The results are representative of three experiments. Correlation statistics of the HI titers of the postvaccination ferret sera between the oligomeric versus monomeric HA1 and oligomeric versus licensed subunit H5N1 vaccine groups were highly significant, with P values of <0.05 (t test).

to the mock-vaccinated, SU-H5N1-vaccinated, and monomeric-rHA1-vaccinated animals (Fig. 5B). This was an unexpected finding and may indicate differences in either the specificity or quality of the antibodies generated by the oligomeric rHA1 versus the subunit vaccine.

Reduction of influenza viral loads in the nasal washes of ferrets challenged with heterologous H5N1 strongly correlates with antibody binding avidity for oligomeric HA1. To better understand the requirement of serum antibody parameters for effective control of viral replication, we used oligomeric rHA1 from clade 2.2 virus (A/Whooperswan/Mongolia/244/2005) to probe the binding avidity of the postvaccination ferret immune sera. Similar to the A/Vietnam/1203/2004 rHA1 protein and other rHA1 proteins generated in our bacterial system, the A/Whooperswan/Mongolia/244/2005 rHA1 protein folded properly into higher-order oligomers, as determined by size exclusion chromatography and binding to conformation-dependent H5N1-neutralizing human monoclonal antibodies. As can be seen in Fig. 6A, immune sera from ferrets vaccinated with the oligomeric rHA1 showed significantly higher avidity for oligomeric rHA1 derived from A/Whooperswan/Mongolia/244/2005, as evidenced by dissociation rates $2 \log_{10}$ units lower than those of sera from monomeric-rHA1-vaccinated and SU-H5N1-vaccinated ferrets. Together with the data in Fig. 3, these findings suggested that reduction in peak viral loads after heterologous H5N1 challenge may correlate with the antibody avidity for the HA1 globular head domain. Indeed, significant negative correlation was found between the day 3 H5N1-A/Whooperswan/Mongolia/244/2005 viral loads and serum antibody off-rates of binding to rHA1 derived from A/Whooperswan/Mongolia/244/2005 (Fig. 6B). Similarly, an inverse correlation was found between the dissociation rates of binding to the rHA1 derived from H5N1 A/Vietnam/1203/2004 (vaccine strain) and day 3 H5N1 A/Vietnam/1203/2004 viral loads

(Fig. 6C). We predict that the high-avidity antibodies also curtailed virus replication in the lungs following lethal challenge, as was previously observed in other successful vaccinations of animals (42). Unfortunately, it was not feasible to measure lung virus loads without premature sacrifice of the animals.

In summary, oligomeric recombinant HA1 (adjuvant) elicited significantly higher-avidity antibodies against both the homologous and heterologous HA1 than the monomeric rHA1 and the licensed inactivated SU-H5N1 vaccine. Such high-avidity antibodies are likely to provide better protection during early stages of viral challenge.

DISCUSSION

The ferret model has been extensively used to evaluate influenza virus pathogenicity and vaccine candidates. It was previously reported that in addition to protection from lethality, weight loss, and clinical symptoms, control of viral replication in the lungs and nasal turbinates is an important parameter of vaccine effectiveness (1, 29, 49). Furthermore, vaccine-induced cross-protective immunity in this model does not always correlate with HI or virus neutralization titers, the traditional measures of vaccine responses (26, 42). It was postulated that other immune parameters play important roles in cross protection, including antibody-dependent cell cytotoxicity (ADCC) and cell-mediated immunity (9, 26).

We showed previously that it is possible to generate a functional oligomeric rHA1 domain in the absence of the HA2 stalk domain (16, 20, 21, 22). We further analyzed the impact of the oligomeric versus monomeric H5N1 HA1 proteins on immunogenicity and protective efficacy of the globular domain and evaluated the quality of antibodies generated in ferrets. For comparison, we vaccinated a group of animals with the licensed H5N1 inactivated subunit (SU-H5N1) vaccine at the same dose (15 μ g

FIG 5 Oligomeric HA1 provides better control of heterologous H5N1 viral load than subunit H5N1 vaccine following wild-type H5N1 virus challenge in ferrets. Viral loads in nasal washes following challenge of vaccinated ferrets with wild-type H5N1 influenza viruses, A/Vietnam/1203/2004 (clade 1) (A) and A/Whooperswan/Mongolia/244/2005 (clade 2.2) (B) on day 3 post-virus challenge are shown. Data points are for individual ferrets. Horizontal lines represent the average viral load (PFU) from the nasal washes of each group (six ferrets per group).

HA) and mixed with the same adjuvant (Titermax). This non-adjuvant-containing licensed SU-H5N1 vaccine was not very immunogenic in humans when administered without adjuvant (48).

The present study confirmed that the oligomeric fraction of rHA1 forms rosette-like structures, as visualized by EM. The rosettes contained four to six trimers, similar to the rosettes formed by purified virion-derived HA (37), and agglutinated human RBC. Upon ferret immunization, it was found that only the oligomeric, not the monomeric, rHA1 generated high levels of antibody binding (maximum RU) and high-avidity antibodies against the oligomeric HA1 protein, as judged by lower serum antigen-antibody dissociation rates ($2\text{-log}_{10}\text{-unit}$ off-rate difference). Furthermore, the IgG/IgM ratios were much higher for immune sera from oligomeric-HA1-vaccinated ferrets than for immune sera from ferrets that received monomeric HA1. Based on the work of Pierce and Liu, it is likely that oligomeric immunogens are more efficient than monomeric forms in initiating the conformation-induced oligomerization of B cell receptor microcluster formation and signal transduction required for antibody class switching and antibody affinity maturation (36).

Following vaccination, ferrets were challenged with homo-

FIG 6 Oligomeric HA1 generates higher-affinity antibodies against both homologous and heterologous HA1 domain than the subunit H5N1 vaccine in immunized ferrets, and these antibodies correlate with lower viral loads in nasal washes following challenge with heterologous wild-type H5N1 virus. (A) Postvaccination ferret serum antibody affinity to rHA1 derived from clade 2.2 H5N1 (A/Whooperswan/Mongolia/244/2005) was measured by antigen-antibody complex off-rate constants. SPR analysis of sera from ferrets vaccinated with either oligomeric (red circles) or monomeric (black circles) H5N1 HA1 or with the subunit H5N1 vaccine (blue circles) was performed with properly folded functional oligomeric H5N1 HA1 (A/Whooperswan/Mongolia/244/2005, clade 2.2). Serum antibody off-rate constants were determined as described in Materials and Methods. Correlation statistics of the off-rate constants for postvaccine ferret sera between different vaccine groups were highly significant for oligomeric HA1 versus subunit H5N1 and oligomeric HA1 versus monomeric HA1 vaccine groups, with P values of <0.005 (t test). (B and C) Correlation between viral loads in nasal washes for 18 ferrets (six per group) following challenge with wild-type H5N1 A/Whooperswan/Mongolia/244/2005 (clade 2.2) and the individual serum antibody dissociation rates in ferrets immunized with H5N1 HA1 proteins (red, oligomers; black, monomers) or subunit H5N1 vaccine (blue) derived from A/Vietnam/1203/2004 (clade 1) virus strain binding to functional oligomeric H5N1 HA1 derived from A/Whooperswan/Mongolia/244/2005 (B) or viral loads in nasal washes for 18 ferrets (six per group) following challenge with wild-type H5N1 A/Vietnam/1203/2004 (clade 1) with rHA1 derived from A/Vietnam/1203/2004 (C) in SPR. The polyclonal serum anti-HA1 antibody off-rates show strong inverse correlation with virus titers of A/Whooperswan/Mongolia/244/2005 (B) or A/Vietnam/1203/2004 (C) on day 3 following virus challenge ($r < -0.45$).

gous HP H5N1 (A/Vietnam/1203/04; clade 1) or heterologous (A/Whooperswan/Mongolia/244/2005; clade 2.2) strains and were monitored for 2 weeks. Both the animals receiving the inactivated SU-H5N1 vaccine and those receiving the oligomeric rHA1 were completely protected from lethality and weight loss and had very low sickness scores after challenge with both homologous and heterologous H5N1 strains. In contrast, the majority of ferrets vaccinated with the monomeric rHA1 succumbed to the virus challenge. Viral load measurements in the nasal washes showed differences between the ferrets challenged with homologous and heterologous H5N1 virus. Both the oligomeric rHA1 and SU-H5N1 vaccines reduced viral loads by more than 2 log units on day 3 after challenge with the H5N1 A/Vietnam/1203/2004 (homologous) virus. But in the case of the heterologous H5N1 (A/Whooperswan/Mongolia/244/2005) virus challenge, oligomeric rHA1 was more effective in reducing viral loads on day 3 than the inactivated SU-H5N1 vaccine (Fig. 5). To better understand this unexpected finding, we evaluated the avidity of antibody binding to HA1 of the heterologous clade 2.2 H5N1 virus (A/Whooperswan/Mongolia/244/2005). Surprisingly, the antibodies elicited by oligomeric HA1 showed antibody-antigen dissociation rates that were 1 to 2 log₁₀ units lower than those of antibodies from the SU-H5N1-vaccinated ferrets or monomeric-rHA1-vaccinated ferrets. With current limitations in SPR technology due to mass transport effects and differences in the concentrations of influenza virus-specific antibodies in the polyclonal sera, it is a challenge to accurately measure the serum on-rates, especially with complex antigens like hemagglutinin and native influenza virus particles.

When we plotted the viral loads of individual animals on day 3 after challenge with the A/Whooperswan/Mongolia/244/2005 virus versus the polyclonal serum antibody off-rates against HA1 derived from A/Whooperswan/Mongolia/244/2005, a strong inverse correlation was found (Fig. 6B). Therefore, high-avidity binding to the HA1 globular head domain in SPR was a good predictor of reduction in viral loads in the upper respiratory tract following challenge with heterologous H5N1 virus.

Protection from lethality in animals vaccinated with the adjuvant-containing subunit vaccine 'may be' possible through antibodies against the glycosylated form of the HA0 (some of which are not measured in our SPR) that can provide some protection through alternative mechanisms, including ADCC or via cell-mediated immunity (CMI). Memory CD4⁺ T cells were recently shown in a mouse model to provide some protection against lethality through a gamma interferon-dependent mechanism (31). Recently, monoclonal antibodies targeting highly conserved epitopes in the stem region of HA were reported to have broad cross neutralization *in vitro* and to some degree *in vivo* (5, 8, 14, 35, 43). However, such stem antibodies are not commonly elicited by traditional vaccination and are not likely to have played a role in vaccine-induced protection in the present study, including in animals vaccinated with the subunit vaccine. We showed previously that human sera from individuals vaccinated multiple times with the licensed H5N1 (A/Vietnam) subunit vaccine (≥ 90 μ g HA/dose) did not contain heterologous cross-clade-neutralizing antibodies (27).

Our finding is in agreement with previous studies demonstrating the importance of trimeric and oligomeric influenza vaccines, particularly for eliciting cross-protective immunity against heterologous challenge (4, 25, 51, 52), but here we define for the first

time the role of antibody avidity in reduction of viral loads in the upper respiratory tract, which will be very important not only for controlling virus replication and prevention of lung infection but also for limiting virus transmission. In previous studies with adjuvant-containing versus non-adjuvant-containing H5N1 vaccine, we demonstrated strong correlation between antibody affinity for functional rHA1 (in SPR) and cross-clade neutralization (15, 19). In a previous study, we evaluated the quality of antibodies elicited by a virus-like-particle (VLP) vaccine against H5N1 (A/Indonesia/5/2005). We found that the VLP vaccine elicited predominantly oligomer-specific antibodies that in turn resulted in neutralization of multiple heterologous H5N1 strains (22).

The present study also underscores the value of measuring antibody avidity against homologous and heterologous viruses. The use of functional HA1 (in place of intact HA) in the SPR assays was essential. Binding the entire HA may not detect differences in avidity because most individuals have pre-existing antibodies against the more conserved HA2 domain, and each vaccination (even with avian influenza virus) triggers a strong recall response against HA2 that precedes and sometime dominates the response against the HA1 domain (15, 18, 19).

Our study underscores earlier findings that HI and virus neutralization assays do not provide complete information about the quality of the antibodies generated in response to different vaccine modalities. Elucidation of these qualitative polyclonal antibody responses (especially antibody affinity) in addition to information provided by traditional assays like HI or microneutralization assay (MN) following different vaccine approaches (including adjuvants) can be very helpful in the development of better vaccines against human diseases. Real-time SPR kinetic assays using appropriate HA antigenic domains are able to provide in-depth information on the quality and quantity of the antibodies generated after vaccination or infection that can be extremely useful in understanding the immune responses to vaccination and infection. In the present study, the SPR data on antibody binding to functional HA1 and antibody avidity, as measured by dissociation off-rates, could provide additional correlates of virus control *in vivo*, especially against heterologous challenge. Our findings in these reports indicate that for early control of virus replication, especially of heterologous strains, antibody affinity may play a more critical role in controlling virus replication than antibody titers. During the recent 2009 H1N1 pandemic, there were several reports of severe disease associated with low-affinity antibodies (32). Furthermore, a delayed clearance of viral load and marked cytokine activation in severe cases of H1N1 2009 infections were reported, but in these reports, antibody avidity was not measured (45, 46).

High-affinity polyclonal antibodies may cross-react with heterologous strains by tolerating some amino acid mismatches, as long as the target conformation is maintained. Alternatively, they may target more conserved sites within the HA1 globular head, which are most stably presented in the oligomeric hemagglutinin, as was recently demonstrated for several MAbs (3, 24, 53, 54).

In summary, bacterially expressed recombinant HA1 immunogens, which contain a high percentage of functional oligomers, may provide an alternative vaccine platform. When combined with the appropriate adjuvant, they are likely to generate high-affinity antibodies with the capacity to neutralize heterologous strains, control virus replication in the upper respiratory tract, and reduce virus transmission.

ACKNOWLEDGMENTS

This work was partly supported by the Intramural Research Program of the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health to J.F. and NIH/NIAID award U01AI077771 to T.M.R.

We thank Alasdair C. Steven (NIAMS, National Institutes of Health) for facilitating the use of an electron microscope. We thank Marina Zaitseva and Vladimir Lugovtsev for a thorough review of the manuscript.

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