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Single- and multiple-clade influenza A H5N1 vaccines induce cross protection in ferrets

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Abstract

The rapid evolution, genetic diversity, broad host range, and increasing human infection with avian influenza A (H5N1) viruses highlight the need for an efficacious cross-clade vaccine. Using the ferret model, we compared induction of cross-reactive immunity and protective efficacy of three single-clade H5N1 vaccines and a novel multiple-clade H5N1 vaccine, with and without MF59 adjuvant. Reverse genetics (rg) was used to generate vaccine viruses containing the hemagglutinin (HA) and neuraminidase genes of wild-type H5N1 viruses. Ferrets received 2 doses of inactivated whole-virus vaccine separated by 3 weeks. Single-clade vaccines (7.5 µg HA per dose) included rg-A/Vietnam/1203/04 (clade 1), rg-A/Hong Kong/213/03 (clade 1), and rg-A/Japanese white eye/Hong Kong/1038/06 (clade 2.3). The multiple-clade vaccine contained 3.75 µg HA per dose of each single-clade vaccine and of rg-A/Whooper Swan/Mongolia/244/05 (clade 2.2). Two doses of vaccine were required to substantially increase anti-HA and virus neutralizing antibody titers to H5N1 viruses. MF59 adjuvant enhanced induction of clade-specific and cross-clade serum antibody responses, reduced frequency of infection (as determined by upper respiratory tract virus shedding and seroconversion data), and eliminated disease signs. The rg-A/Hong Kong/213/03 vaccine induced the highest antibody titers to homologous and heterologous H5N1 viruses, while rg-A/Japanese white eye/Hong Kong/1038/06 vaccine induced the lowest. The multiple-clade vaccine was broadly immunogenic against clade 1 and 2 viruses. The rg-A/Vietnam/1203/04 vaccine (the currently stockpiled H5N1 vaccine) most effectively reduced upper respiratory tract virus shedding after challenge with clade 1 and 2 viruses. Importantly, all vaccines protected against lethal challenge with A/Vietnam/1203/04 virus and provided cross-clade protection.

Keywords

H5N1 influenza; Vaccine; MF59 Adjuvant

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1. Introduction

Influenza A (H5N1) viruses remain a major concern due to their rapid evolution, genetic diversity, broad host range, and ongoing circulation in wild and domestic birds in Eurasia and Africa. Since 2003, H5N1 influenza viruses have swept through poultry flocks across Asia and spread westward through Eastern Europe to India and Africa [1]. To date, these viruses have been isolated from avian species in 63 countries, and the more than 300 human infections documented in 12 countries have had a ~60% mortality rate [2]. H5N1 influenza viruses are currently divisible into 10 unique clades on the basis of phylogenetic analysis of their hemagglutinin (HA) genes [2]. However, most H5N1 viruses belong to clades 1 and 2. These genetically distinct groups also reflect the geographical distribution of their avian hosts [3]. Viruses in clade 1 are found mainly in Southeast Asia, South Korea, and Japan. The more diverse clade 2 viruses comprise three different subclades that spread to Indonesia (subclade 1), to the Middle East and Africa via India and Europe (subclade 2), and to Southeast Asia (subclade 3) [4].

There are greater obstacles to optimal control of H5N1 virus infection in humans than for seasonal influenza, which is currently well controlled by annual vaccination. Antiviral drugs may be useful at early stages of an influenza pandemic [5], but their effectiveness is limited by the emergence of resistance and the narrow time window during which treatment must be administered [6–8]. Therefore, vaccines remain the cornerstone of influenza control. H5N1 viruses circulate primarily among avian species, and therefore no epidemiological model has been established to predict the emergence of predominant antigenic variants [9]. The broad antigenic and genetic diversity and ongoing rapid evolution of the H5N1 influenza viruses is a serious obstacle to the establishment of a strategy for control and prevention. Effective H5N1 pandemic preparedness will require vaccines that offer broad cross-clade protection and are readily available to large population groups [10–12]. They must also provide sufficient amount of antigen to rapidly induce protective immunity [13].

The production of H5N1 influenza vaccines from wild-type virus is not generally accepted because of the attendant risks and limited feasibility [14]. Several technologies being developed for production of H5N1 pandemic vaccines have shown encouraging preclinical results. These include vector vaccines that express influenza virus protein [15], DNA vaccines that encode the HA of A/Hong Kong/156/97 (H5N1) influenza virus [16], and reverse genetics technology [17,18]. The latter approach has produced an effective H5N1 vaccine that protects ferrets against lethal, heterologous challenge with the A/Vietnam/1203/04 (H5N1) virus [19].

In phase I clinical trials of candidate H5N1 (clade 1) vaccines, two 90-μg doses of HA delivered via a rg-A/Vietnam/1203/04 subvirion vaccine induced protective levels of serum antibody titers in humans [20]. Subsequent research has sought to reduce the necessary dose of HA by adding adjuvants to increase the immunogenicity of vaccines. Of the various types of adjuvants used, the oil-in-water emulsion MF59 is one of the most promising [21]. Two doses of an A/Duck/Singapore/97 (H5N3) subunit vaccine with MF59 adjuvant induced protective levels of antibodies that cross-reacted with different H5N1 strains; a boost dose 16 months later restored antibody titers to protective levels [12,22], and induced cross-clade neutralizing antibodies [23]. In recent clinical trials, split-virion H5N1 vaccine formulated with a proprietary adjuvant system induced cross-clade neutralizing antibody responses that paralleled the positive results of preclinical studies [11].

In the present study we used the ferret model, which is currently considered the most suitable small-animal model for preclinical evaluation of influenza vaccines, to compare the immunogenicity, cross reactivity, and protective efficacy of three single-clade and one

multiple-clade inactivated whole-virus H5N1 influenza vaccine derived by reverse genetics and formulated with and without MF59 adjuvant.

2. Materials and Methods

2.1 Viruses and cells

The H5N1 influenza viruses A/Vietnam/1203/04 (A/VN/1203/04), A/WhooperSwan/Mongolia/244/05 (A/WS/MG/244/05), and A/Japanese White Eye/Hong Kong/1038/06 (A/JWE/HK/1038/06) used for challenge were obtained from the World Health Organization influenza collaborating laboratories. Viruses were grown in the allantoic cavities of 10-day-old embryonated chicken eggs (eggs) for 48 h at 37°C. Allantoic fluid containing virus was harvested and stored at -70°C until use. Virus yields, determined as 50% egg infectious dose (EID₅₀) per milliliter of virus stock, ranged from 8.8 to 10.3 log₁₀EID₅₀/ml. All animal challenge experiments using wild-type H5N1 viruses were performed in a BSL3+ containment facility approved by the United States Department of Agriculture. Madin Darby canine kidney (MDCK) cells obtained from the American Type Culture Collection (Manassas, VA) were grown in an atmosphere of 5% CO₂ at 37°C in 1x MEM (Invitrogen, Carlsbad, CA) supplemented with 5% fetal calf serum, 200 mM L-glutamine, 40 µg/ml gentamicin, 1x MEM vitamins solution (Sigma, St. Louis, Missouri), 1x antibiotic-antimycotic solution (Sigma, St. Louis, Missouri), and 0.2% sodium bicarbonate.

2.2 Preparation of whole-virus vaccines

Vaccine strains rg-A/VN/1203/04 (clade 1), rg-A/HK/213/03 (clade 1) [18], rg-A/WS/MG/244/05 (clade 2.2), and rg-A/JWE/HK/1038/06 (clade 2.3) were generated at St. Jude Children's Research Hospital in Good Manufacturing Practice (GMP)-grade facilities. Reverse genetics technology was used to generate viruses containing the HA (from which the polybasic amino acids that are associated with high virulence were removed) and NA genes of the wild-type H5N1 virus in the background of A/Puerto Rico/8/34 (H1N1) virus [18]; the vaccine strains were rescued in WHO-approved Vero cells. The vaccines were propagated in the allantoic cavities of 10-day-old eggs at 37°C for 48 h and were inactivated by adding 37% formaldehyde to the allantoic fluid at a final concentration of 0.025%. Inactivation was confirmed by absence of virus growth in 2 consecutive passages in eggs. Vaccines were concentrated by Amicon ultrafiltration and by ultracentrifugation through a 25% and a 70% sucrose cushion gradient; purified by ultracentrifugation through a continuous sucrose gradient for 2.5 h; then pelleted by centrifugation at 76,000 × g for 1 h at 4°C. The HA protein content of the vaccines was determined by the single radial immunodiffusion (SRID) method [24]. Each dose of single-clade vaccine contained 7.5 µg of rg-A/VN/1203/04, rg-A/HK/213/03, or rg-A/JWE/HK/1038/06 HA. Each dose of multiple-clade vaccine contained 3.75 µg each of rg-A/VN/1203/04, rg-A/HK/213/03, rg-A/JWE/HK/1038/06, and rg-A/WS/MG/244/05 HA (15 µg HA total per dose). The decision to include two clade 1 viruses in the multiple clade vaccine was based on two factors: (1) A/VN/1203/04 is an H5N1 vaccine currently approved for human use; (2) A/HK/213/03 virus vaccine was shown to elicit high serum antibody responses in ferrets [28]. Adjuvant was added by mixing MF59 (Novartis Vaccines and Diagnostics) 1:1 v/v with vaccine diluted in 1x STE (250 µL diluted vaccine with 250 µL adjuvant per dose). For vaccines without adjuvant, 1x STE was used to make a standard dose volume of 0.5 ml of vaccine.

2.3 Vaccination and challenge

Young adult male and female ferrets 3–8 months of age were obtained from either the ferret breeding program at St. Jude Children's Research Hospital or Marshall Farms (North Rose, NY). All animal experiments were approved by the Animal Care and Use Committee of St. Jude Children's Research Hospital and performed in compliance with relevant institutional

policies of the National Institutes of Health and the Animal Welfare Act. Ferrets were screened to ensure seronegativity to A/New Caledonia/20/99 (H1N1) and A/New York/55/04 (H3N2) viruses. All vaccines were given in 2 doses (0.5 ml each) injected intramuscularly at a 3-week interval and performed in a BSL2 facility. Fifteen ferrets were vaccinated with each adjuvanted vaccine and 5 animals with each nonadjuvanted vaccine. Control ferrets were given vehicle only. One week after the second (boost) dose of vaccine, ferrets were anesthetized with isoflurane and inoculated intranasally with 10^6 EID₅₀ of a wild-type H5N1 challenge virus in 1 ml sterile PBS. The groups of ferrets given adjuvanted vaccines were challenged with H5N1 influenza viruses A/JWE/HK/1038/06, A/WS/MG/244/05, and A/VN/1203/04. Those given vaccines without adjuvant were challenged with A/VN/1203/04 (H5N1) wild-type virus. Table 1 delineates the experimental groups. Ferrets were then monitored daily for 14 days for weight change, temperature, clinical disease signs, and relative inactivity index. The latter was calculated by the method of Reuman *et al.* [25]. Body temperature was measured via transponders subcutaneously implanted between the shoulder blades (BioMedic Data Systems, Inc., Seaford, DE).

2.4 Nasal washes

Nasal washes were collected from each ferret on days 3, 5, and 7 post-challenge. Ferrets were anesthetized with ketamine, and 0.5 ml of PBS with antibiotics was slowly introduced into each nostril, recovered, measured, and brought to a volume of 1.0 ml with sterile PBS containing antibiotics. Bovine serum albumin 7.5% was added at a ratio of 1:20 v/v as a stabilizing agent. Virus in the samples was titrated in eggs and expressed as log₁₀EID₅₀/ml as described by Reed and Muench [26]. The lower limit of detection was 0.75 log₁₀EID₅₀/ml, and for computation purposes these values were not included into calculation of mean virus titers. Mean virus titers were compared by non-parametric t-test pairwise comparison (P=0.05) by using the GraphPad Prism 5.0 package.

2.5 Serologic testing

Serum samples were taken 3 weeks after the first vaccination, 1 week after the boost dose, and 21 days after challenge. Reference viruses used for serologic testing were reverse genetics strains, and all serologic testing was performed in the BSL3+ containment facility. Serum was treated with receptor-destroying enzyme (Denka Seiken Co., Japan) overnight at 37°C, heat-inactivated at 56°C for 45 min, diluted 1:10 with sterile PBS, and tested by hemagglutination inhibition (HI) assay with 0.5% packed chicken red blood cells (CRBC). Virus-neutralizing antibody titers were determined in MDCK cells. Briefly, the 50% tissue culture infectious dose (TCID₅₀) was determined for each vaccine virus, and 2-fold serial dilutions of serum were incubated with 100 TCID₅₀ of virus for 1 h at 37°C. The mixture was then added to MDCK cells and incubated for 72 h at 37 °C in 5% CO₂. After 72 hours, the HA activity of the supernatant was assessed by HA assay with 0.5% packed CRBC. Neutralizing titers were expressed as the reciprocal of the serum dilution that inhibited 50% of the HA activity of 100 TCID₅₀ of virus.

3. Results

3.1 Vaccine immunogenicity

After vaccination of ferrets, we analyzed the development of antibodies against homologous virus. Three weeks after the first dose of H5N1 vaccine, serum HI titers to homologous virus were undetectable or low (<10 to 36) for nonadjuvanted vaccine; for adjuvanted vaccine, all vaccine groups had detectable HI titers against homologous virus, with rg-A/HK/213/03 vaccinated animals having the highest titer (128) (Table 2). After the boost, most titers increased substantially from the first dose (32-fold in the case of rg-A/HK/213/03 vaccine) for nonadjuvanted vaccines; the exception was the rg-A/JWE/HK/1038/06 vaccine, where ferrets

had no detectable titers even after the second dose. All animals given the adjuvanted vaccine had detectable serum antibodies (mean titer, 123) to the homologous virus after the boost dose.

The first dose of all vaccines induced detectable homologous virus neutralizing antibodies (Table 3), and addition of MF59 adjuvant increased the mean virus neutralizing titer only slightly. The benefit of MF59 adjuvant was more pronounced after the second dose of vaccine, which increased the titers to homologous virus by as much as 40 times compared to titers following the first dose. In contrast, ferrets receiving nonadjuvanted rg-A/VN/1203/04 or rg-A/JWE/HK/1038/06 vaccines showed no increase or a minor increase in virus neutralizing titers to homologous virus after the boost dose, and animals vaccinated with rg-A/HK/213/03 vaccine showed an increase of ~10-fold. Virus neutralizing titers were higher than HI titers after both the nonadjuvanted and adjuvanted vaccine boosts.

The first dose of all vaccines was relatively poorly immunogenic (Tables 2, 3). After the second dose, rg-A/HK/213/03 vaccine induced the highest antibody titers to homologous virus among the single-clade vaccines. The multiple-clade vaccine induced lower titers to H5N1 viruses than the single-clade vaccines, with two exceptions: (1) HI and virus neutralizing titers against A/JWE/HK/1038/06 virus were lower after the boost dose of nonadjuvanted single-clade rg-A/JWE/HK/1038/06 vaccine than after the boost dose of nonadjuvanted multiple-clade vaccine, and (2) very similar titers to A/HK/213/03 virus were induced by the nonadjuvanted multiple-clade and rg-A/HK/213/03 vaccines. Interestingly, the rg-A/WS/MG/244/05 constituent of the multiple-clade vaccine induced antibody titers to homologous virus that were equivalent to or higher than those induced by rg-A/VN/1203/04 and rg-A/JWE/HK/1038/06 vaccines, at half the dose of HA.

3.2 Induction of cross-reactive immunity

We analyzed the extent of cross-reactive immunity to clade 1 (A/VN/1203/04 and A/HK/213/03) and clade 2 (A/JWE/HK/1038/06 and A/WS/MG/244/05) viruses. Both of the clade 1 vaccines (rg-A/HK/213/03 and rg-A/VN/1203/04) induced antibodies to clade 2 viruses (Tables 2, 3). Following the boost, ferrets vaccinated with adjuvanted rg-A/HK/213/03 (clade 1) had higher virus neutralizing titers to the clade 2 viruses (A/JWE/HK/1038/06 and A/WS/MG/244/05) than to clade 1 virus (A/VN/1203/04). HI and virus neutralizing antibody titers against heterologous virus clades were higher in those ferrets that had received the vaccine adjuvanted with MF59, as compared with ferrets immunized with nonadjuvanted vaccines. All vaccines induced a similar level of antibody response to clade 2 viruses after the first dose, but titers to clade 2 viruses after the boost were highest for ferrets receiving the homologous vaccine. Two doses of rg-A/JWE/HK/1038/06 (clade 2) vaccine induced greater serum reactivity to A/HK/213/03 (clade 1) virus than did two doses of rg-A/VN/1203/04 (clade 1) vaccine. Intriguingly, two doses of rg-A/JWE/HK/1038/06 (clade 2) vaccine induced lower antibody titers against A/WS/MG/244/05 virus (also clade 2) than did two doses of rg-A/VN/1203/04 or rg-A/HK/213/03 (clade 1) vaccine. In summary, all vaccines induced cross-clade antibodies and in some cases, titers were higher to viruses from a different clade than the vaccine strain versus titers to non-homologous viruses from the same clade as the vaccine. These include rg-A/JWE/HK/1038/06 vaccine, where HI titers were several fold higher to rg-A/HK/213/03 than to homologous virus after the boost, and the rg-A/HK/213/03 vaccine (clade 1), where virus neutralizing and HI titers after the boost were higher to rg-A/WS/MG/244/05 (clade 2) than rg-A/VN/1203/04 (clade 1).

3.3 Protection against challenge with lethal A/VN/1203/04 (H5N1) virus

We assessed the protective efficacy of single- and multiple-clade vaccines given with and without adjuvant against lethal H5N1 virus challenge. After challenge with A/VN/1203/04 (H5N1) virus, all unvaccinated control animals became extremely lethargic, lost weight (Fig.

1a), showed neurological signs, and died or were euthanized by day 9 post-challenge. Importantly, all ferrets vaccinated with two doses of either homologous or heterologous nonadjuvanted H5N1 vaccine survived lethal challenge. Increased body temperature, reduced activity (data not shown), and weight loss (10% weight loss on day 6 post-challenge, Fig. 1a) were observed only in animals vaccinated with the rg-A/JWE/HK/1038/06 vaccine. Neurological signs, including hind limb paresis and/or paralysis, and circling, were not seen in any vaccinated animals. Virus was detected in the upper respiratory tract (URT) of almost all vaccinated ferrets on day 3 post-challenge; more than 50% of animals continued to shed on day 5, and only one ferret (vaccinated with rg-A/JWE/HK/1038/06 vaccine) was shedding virus on day 7 post-challenge (Fig. 1b). Virus titers in the URT were significantly lower ($P<0.05$) in all vaccine groups compared to those in the control group except for rg-A/JWE/HK/1038/06 vaccine group on day 3 post challenge (Fig. 1b).

Ferrets vaccinated with two doses of MF59-adjuvanted H5N1 vaccines also survived lethal challenge with A/VN/1203/04 (H5N1) virus. Clinical signs of infection were absent or minor in vaccinated animals and no weight loss was observed (Fig. 1c). Ferrets which received adjuvanted vaccines did not gain weight while those which received vaccine alone gained weight. This observation is likely due to the fact that ferrets in the latter group were in many cases 4 months younger than ferrets in the other group, and thus their weight had not leveled out yet. Ferrets vaccinated with multiple-clade and rg-A/VN/1203/04 vaccines had a minor decrease in activity after challenge. The inactivity indices were similar in all groups vaccinated with adjuvanted and nonadjuvanted vaccines, with the exception of the rg-A/JWE/HK/1038/06 vaccine group, in which the relative inactivity index was 0 in the adjuvanted-vaccine group versus 0.49 in the nonadjuvanted-vaccine group (data not shown). On day 1 after challenge, the rg-A/JWE/HK/1038/06 vaccine group (both with and without adjuvant) had an approximately 1°C increase in body temperature; the other vaccine groups had no temperature increase or a minimal (0.2°C) increase (data not shown). Significantly less virus was shed from the URT on days 3 and 5 post-challenge by vaccinated animals than by the control groups ($P<0.05$), and no vaccinated animals continued to shed virus on day 7 post-challenge (Fig. 1d). Fewer animals shed virus after receiving adjuvanted vs. nonadjuvanted vaccine, but the mean URT virus titers did not differ significantly between these groups ($P>0.05$) (Fig. 1d). Addition of adjuvant to the multiple-clade and rg-A/HK/213/03 vaccines did not noticeably reduce the viral load. In summary, both nonadjuvanted and adjuvanted vaccines protect ferrets against lethal H5N1 virus challenge, and the inclusion of MF59 adjuvant reduces clinical disease signs and the frequency of virus shedding from the URT.

3.4 Protection against challenge with non-lethal H5N1 viruses

Only ferrets immunized with adjuvanted H5N1 vaccines were challenged with non-lethal viruses (A/JWE/HK/1038/06 and A/WS/MG/244/05). All ferrets challenged with A/JWE/HK/1038/06 (H5N1) virus, including controls, remained active, had no fever (data not shown), and gained weight (Fig. 2a). Mild respiratory signs were observed in control ferrets and ferrets vaccinated with the homologous rg-A/JWE/HK/1038/06 vaccine or multiple-clade vaccine (about two ferrets per group sneezed on single occasions during days 4–12 after challenge). All control ferrets shed virus in the URT on days 3 and 5 post-challenge; only two vaccinated ferrets (in the rg-A/HK/213/03 and multiple-clade groups, respectively) continued to shed virus on day 5 post-challenge (Fig. 2b).

A/WS/MG/244/05 virus appeared to be more pathogenic to naïve ferrets than A/JWE/HK/1038/06 virus: we observed an increase of as much as 1.9°C in body temperature (data not shown), as much as 11.3% weight loss (Fig. 2c), and a 0.58 relative inactivity index in control animals challenged with A/WS/MG/244/05 virus (data not shown). All vaccinated ferrets challenged with A/WS/MG/244/05 virus showed no clinical signs of infection (Fig. 2c). In

many cases vaccinated ferrets had significantly lower URT virus titers ($P < 0.05$) than did controls on days 3 and 5 after challenge, and complete virus clearance was observed by day 7 (Fig. 2d). Virus shedding was most inhibited in the group vaccinated with rg-A/VN/1203/04, in which virus was cleared by day 5. The minor respiratory signs and the reduction/absence of URT shedding suggest that rg-A/VN/1203/04 was the vaccine most effective against both of the non-lethal viruses (A/JWE/HK/1038/06 and A/WS/MG/244/05).

3.5 Seroconversion after challenge with lethal and non-lethal H5N1 viruses

A 4-fold increase in the HI titer post-challenge was considered to be serologically suggestive of infection. The rg-A/VN/1203/04 vaccine (adjuvanted and nonadjuvanted formulations) was most effective in preventing a 4-fold antibody titer increase after lethal challenge with A/VN/1203/04 virus (Fig. 3a-b). A greater number of ferrets had a 4-fold increase in HI titer (and a response to a greater number of antigens) when vaccines were given without adjuvant, indicating the MF59-adjuvanted vaccine formulation offered greater protection against infection than the nonadjuvanted formulation.

After challenge with A/JWE/HK/1038/06 virus, a 4-fold increase in HI titer was seen in the control group and the group vaccinated with rg-A/HK/213/03, suggesting that the rg-A/HK/213/03 vaccine was least effective in preventing infection. The rg-A/VN/1203/04 vaccine and the multiple-clade vaccine were 100% effective in preventing a 4-fold increase in HI titer with A/WS/MG/244/05 virus. Groups vaccinated with rg-A/HK/213/03 had the highest frequency of 4-fold increases in HI titer after challenge, and animals vaccinated with rg-A/JWE/HK/1038/06 had an intermediate result. The rg-A/VN/1203/04 vaccine was again the only vaccine that prevented a 4-fold antibody titer increase after challenge with both clade 2 viruses. It is worth mentioning that in some instances 4-fold increases in HI titers did not correspond to URT viral titers, and this may be attributable to the sensitivity of the HI test with CRBC.

4. Discussion

We evaluated a novel multiple-clade reverse genetics–derived inactivated whole-virus H5N1 influenza vaccine and three single-clade vaccines in the ferret model by comparing their immunogenicity, cross reactivity, and protective efficacy. The multiple-clade adjuvanted vaccine could be useful in allowing timely initiation of vaccination against an unknown pandemic virus. All vaccines protected ferrets against lethal challenge with A/VN/1203/04 virus and markedly reduced clinical disease signs and virus replication in the upper respiratory tract after non-lethal challenge with A/JWE/HK/1038/06 or A/WS/MG/244/05 virus. Two doses of vaccine were required to substantially increase HI and virus neutralizing antibody titers to both homologous and heterologous H5N1 viruses. Inclusion of MF59 adjuvant increased antibody titers to both homologous and heterologous viruses. Importantly, we found that two doses of the adjuvanted single-component rg-A/VN/1203/04 vaccine, the H5N1 vaccine that is stockpiled in various parts of the world, was most effective in reducing the upper respiratory tract virus load after both homologous and cross-clade challenge. Our results strongly support the previous observation of greater antibody induction by two doses of inactivated whole-virus rg-A/HK/213/03 (H5N1) vaccine in ferrets [19] and the results of clinical studies of adjuvanted, inactivated subunit, or split-virion vaccines (rg-A/VN/1203/04 or rg-A/VN/1194/04) in adults [11,27].

A feature highly desirable in a pandemic influenza vaccine is the ability to induce cross-reactive immune responses sufficient to protect against variants that have undergone antigenic drift. We assessed the immunogenicity and cross reactivity of H5N1 influenza vaccines representing different HA clades/subclades as well as the immune response induced by a multiple-clade H5N1 vaccine. Interestingly, the highest serum antibody titers induced by the vaccines were those against the A/HK/213/03 HA antigen, suggesting that A/HK/213/03 may be a more

immunogenic antigen than other H5N1 viruses. Alternatively, the sensitivity of the HI assay may be enhanced by the arginine found at residue 223 of the A/HK/213/03 HA1 subunit [28]. The immune correlates of protection remain poorly defined for H5N1 influenza vaccines and may differ from those established for seasonal influenza vaccines. Concerning overall cross reactivity, the multiple-clade vaccine induced serum antibody titers to homologous viruses that were almost as high as those induced by single-clade vaccines (in the case of rg-A/JWE/HK/1038/06 vaccine, higher). Three single-clade vaccines also induced cross-reactive immunity to all four antigens, but antibody titers to heterologous viruses were lower. It is worthy of note that the addition of MF59 adjuvant induced a stronger cross-reactive immunity as compared to nonadjuvanted vaccines. Interestingly, titers against the A/HK/213/03 and A/JWE/HK/1038/06 antigens were not proportional to the lower dose in ferrets that received the multiple-clade vaccine vs. the single-clade vaccines, possibly reflecting a reaction between antigens that increased the vaccine's immunogenicity.

Preclinical animal studies are useful for evaluation of the protective efficacy of vaccines against highly pathogenic influenza A (H5N1) viruses. Importantly, all vaccinated animals in the present study were protected against death after challenge with a high dose (10^6 EID₅₀) of lethal A/VN/1203/04 (H5N1) virus and were protected against morbidity after a similar dose of non-lethal A/WS/MG/244/05 or A/JWE/HK/1038/06 H5N1 virus (ours is the first study to our knowledge to examine A/JWE/HK/1038/06 virus infection in the ferret model). Rg-A/VN/1203/04 vaccine offered the best protective efficacy and provided substantial cross reactivity and immunogenicity, although it induced lower serum antibody titers than the multiple-clade vaccine. Further, nonadjuvanted rg-A/VN/1203/04 vaccine protected against lethal challenge with homologous virus, despite inducing a relatively low antibody titer to that virus; conversely, we must consider that rg-A/VN/1203/04 was the only vaccine evaluated that was homologous to lethal challenge virus.

In some instances, ferrets were protected from morbidity or lethality of challenge despite relatively low serum antibody titers (i.e. ferrets receiving nonadjuvanted vaccines then challenged with A/VN/1203/04), which raises the question of whether HI and VN assays, both of which primarily detect antibodies to influenza virus HA, are adequate measures of a protective antibody response. We must comment that the use of CRBC for the estimation of serum antibody responses against avian H5N1 influenza viruses may be a limitation of the current study, and the use of horse RBC may increase the level of sensitivity. However, the possibility that both humoral and cellular immune responses play a role in protection of ferrets against influenza cannot be ruled out. This possibility is particularly valid in the case of whole-virion vaccines, which (unlike subunit vaccines) contain internal conserved proteins targeted by cell-mediated immune responses. Little is currently known about the cellular immune response in ferrets, as the necessary reagents are unavailable. In the mouse model, in which the role of T-cell immunity to influenza virus is known [29], MF59 adjuvant enhanced T-cell responses to a trivalent influenza vaccine significantly more than numerous other adjuvants [30]. In humans, the level of cellular immune response is positively correlated with protection from influenza [31,32]. Clinical trials have showed that adjuvanted vaccines for hepatitis B and malaria induce cellular immune responses that may contribute to protection [33,34].

Whole-virus H5N1 vaccines can reduce the amount of antigen required to induce an adequate immune response [35], as can adjuvants. These considerations are important in view of the likely gap between vaccine production and demand during an influenza pandemic. Because of safety concerns, MF59 and aluminum salts are the only vaccine adjuvants approved for use in humans [21]. Aluminum salts do not always enhance immunity to split H5N1 influenza vaccines in humans [36], whereas MF59 had an excellent safety profile and enhanced the immunogenicity of split H5 vaccines in clinical trials [23,37,38]. In our study, one dose of whole-virus H5N1 vaccine with MF59 adjuvant increased HI titers to homologous virus, and

two doses increased virus neutralizing titers as much as 30 times more than nonadjuvanted vaccine. Additionally, MF59-adjuvanted vaccines induced cross-reactivity, prevented fever, and reduced URT viral load to a greater extent than nonadjuvanted vaccines. Similarly, in a recent preclinical study by Baras et al [39], two doses of an H5N1 split vaccine with a proprietary oil-in-water emulsion-based adjuvant increased cross-reactivity in ferrets and decreased URT virus shedding. In our study, rg-A/VN/1203/04 was the vaccine that most effectively reduced the URT viral load and URT shedding of all challenge viruses. This information is noteworthy, as URT shedding is the main method of transmission of influenza virus, and a decrease in the URT viral load may suppress transmission. Reduction of virus shedding appeared to depend on the overall immunogenicity of the vaccine and the homology of vaccine to the challenge virus. MF59 adjuvant helped to reduce the severity of clinical signs after non-homologous challenge, but it remains unclear whether adjuvant reduces the URT viral load in ferrets when used with naturally highly immunogenic vaccines. The rg-A/VN/1203/04 vaccine, which is currently the H5N1 vaccine approved for human use, was also the vaccine that most effectively reduced the incidence of infection as determined by seroconversion.

At least 6 months are likely to elapse between the start of an influenza pandemic and the initial availability of a strain-specific vaccine. Stockpiling of a broadly cross-reactive vaccine could help to ensure the interim availability of an alternative vaccine until an antigenically matched vaccine becomes available. Further, after receiving a cross-reactive H5N1 influenza vaccine, individuals may require only a single dose of the strain-specific vaccine, as the result of cross-clade priming [4]. Clinical trials have shown that a protective antibody response to H5N1 viruses can be induced by priming and boosting with A/DK/Singapore/97 (H5N3) vaccine [23] or by boosting with A/VN/1203/04 after priming with a heterologous H5N1 virus [2].

In summary, our results showed that a multiple-clade H5N1 influenza vaccine can provide protective cross-clade immunity. Such a vaccine may elicit protection against a broader range of viruses than a single-clade vaccine. If single vaccines of different clades are stockpiled, it may be possible in the event of a pandemic to mix various single vaccines to create a cross-clade protective vaccine for use until an antigenically matched vaccine is available. Our findings also suggest that a heterologous vaccine can offer protection, mainly if adjuvanted with MF59. Importantly, the rg-A/VN/1203/04 vaccine administered as described provided immunogenicity, cross reactivity, and protective efficacy comparable to that of the multiple-clade vaccine. In the future, it would be beneficial to compare single- and multiple-clade vaccines based on other H5N1 vaccine strains and challenge viruses to determine if multiple-clade H5N1 vaccines repeatedly offer advantages comparable to a single-clade vaccine. Use of the MF59 adjuvant considerably increased clade-specific and cross-clade antibody responses to all vaccines, an effect that may help to spare antigen in the event of a pandemic. The fact that in some instances, such as with the A/VN/1203/04 challenge/nonadjuvanted vaccine, protection from mortality was provided despite relatively low serum antibody titers, suggests that the role of cellular immunity in this model should also be investigated.

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Fig. 1.

Change in weight (A, C) and virus titers in the upper respiratory tract (B, D) of vaccinated and control ferrets after challenge with A/VN/1203/04 (H5N1) virus. Ferrets were vaccinated twice (at 3-week intervals) with inactivated whole virus without MF59 adjuvant (A, B) or with MF59 adjuvant (C, D) and were challenged with 10^6 EID₅₀ of A/VN/1203/04 (H5N1) virus one week after the second vaccination. Weight was monitored daily. Each data point represents mean % weight change (A, C) (n=5). The mean SD of all data points in A was $\pm 4.9\%$; the mean SD of all data points in C was $\pm 4.5\%$. The lower limit of detection of virus in the upper respiratory tract (B, D) was 0.75 log₁₀EID₅₀/ml (dashed line) and for computation purposes values lower than that were not included into calculation of mean virus titers. Results are shown as individual and mean $\pm$ SD virus titers in nasal washes. $^{\infty}P < 0.05$ compared with the unvaccinated control group. X - only 4 ferrets challenged in this group; * - 4 ferrets died by day 7; ** - 2 ferrets died by day 5; *** - all ferrets died by day 7. Abbreviations: rg-VN - rg-A/VN/1203/04 vaccine; rg-HK - rg-A/HK/213/03 vaccine; rg-JWE - rg-A/JWE/HK/1038/06 vaccine; Multi - multiple-clade vaccine.

Fig. 2.

Change in weight (A, C) and virus titers in the upper respiratory tract (B, D) of vaccinated and control ferrets after challenge with A/JWE/HK/1038/06 (A, B) or A/WS/MG/244/05 (C, D) (H5N1) virus. Ferrets were vaccinated twice (at a 3-week interval) with inactivated whole-virus H5N1 vaccine with MF59 adjuvant and were challenged with 10^6 EID₅₀ of H5N1 virus one week after the second vaccination. Weight was monitored daily. Each data point represents mean % weight change (A, C) (n=5). The mean SD of all data points in A was $\pm 3.4\%$; the mean SD of all data points in C was $\pm 4.3\%$. The lower limit of detection of virus in the upper respiratory tract (B, D) was 0.75 log₁₀EID₅₀/mL (dashed line) and for computation purposes values lower than that were not included into calculation of mean virus titers. Results are shown as individual and mean $\pm$ SD virus titers in nasal washes. $^{\circ}P < 0.05$ compared with the unvaccinated control group. Abbreviations: rg-VN - rg-A/VN/1203/04 vaccine; rg-HK - rg-A/HK/213/03 vaccine; rg-JWE - rg-A/JWE/HK/1038/06 vaccine; Multi - multiple-clade vaccine.

Fig. 3.

Serological data suggestive of infection after non-lethal and lethal challenge with H5N1 viruses. Vertical bars represent the number of ferrets (y-axis) in each vaccine group (x-axis) that had a 4-fold antibody rise to the challenge virus and to heterologous viruses. Stars represent data obtained with homologous virus. Serum samples were collected 1 week after boost vaccination and 21 days after challenge with A/VN/1203/04 (A, B), A/JWE/HK/1038/06 (C), or A/WS/MG/244/05 (D) virus. Vaccines used were inactivated whole viruses either without (A) or with (B, C, D) MF59 adjuvant. Single-clade vaccines contained 7.5 μ g HA; multiple-clade vaccine contained 3.75 μ g HA of each of four viruses. Control ferrets challenged with A/VN/1203/04 were euthanized or died before day 21 post-challenge, and as a result the 4-fold increase in antibody titer could not be determined (N/A – not applicable). Abbreviations: rg-VN – rg-A/VN/1203/04 vaccine; rg-HK – rg-A/HK/213/03 vaccine; rg-JWE – rg-A/JWE/HK/1038/06 vaccine; Multi – multiple-clade vaccine.

TABLE 1
Experimental H5N1 influenza vaccine groups for cross-protection studies

H5N1 vaccine ^a	H5N1 challenge virus ^b		
	A/VN/1203/04	A/WS/MG/244/05	A/JWE/HK/1038/06
Adjuvanted vaccine			
rg-A/VN/1203/04	5	5	5
rg-A/HK/213/03	5	5	5
rg-A/JWE/HK/1038/06	5	5	5
Multiple-clade	5	5	5
Control (1 X STE)	5	5	5
Nonadjuvanted vaccine			
rg-A/VN/1203/04	5	- ^c	-
rg-A/HK/213/03	5	-	-
rg-A/JWE/HK/1038/06	5	-	-
Multiple-clade	5	-	-
Control (1 X STE)	5	-	-

Note: Numbers indicate ferrets per experimental group.

^a Single-clade vaccines contained 7.5 µg HA per dose; multiple-clade vaccine contained 3.75 µg HA of each antigen per dose. All vaccines were given in 2 doses (0.5 ml each) injected intramuscularly at a 3-week interval. Multiple-clade vaccine contained rg-A/VN/1203/04 (clade 1), rg-A/HK/213/03 (clade 1), rg-A/JWE/HK/1038/06 (clade 2.2), and rg-A/WS/MG/244/05 (clade 2.3) antigens.

^b One week after the second (boost) dose of vaccine, ferrets were anesthetized with isoflurane and inoculated intranasally with 10⁶ EID₅₀ of a wild-type H5N1 challenge virus in 1 ml sterile PBS.

^c Challenge experiments were not conducted.

TABLE 2
Serum HI antibody titers in ferrets vaccinated with inactivated whole-virus H5N1 influenza vaccines

Vaccine regimen	Mean serum HI antibody titer against ^a					
	rg-A/VN/1203/04		rg-A/HK/213/03		rg-A/JWE/HK/1038/06	
	1 st dose ^c	2 nd dose ^c	1 st dose ^c	2 nd dose ^c	1 st dose ^c	2 nd dose ^c
Nonadjuvanted vaccine						
rg-A/VN/1203/04 ^b	≤ (0/5)	<u>60</u> (4/5)	< (0/5)	64 (5/5)	< (0/5)	< (0/5)
rg-A/HK/213/03 ^b	< (0/5)	13 (3/5)	<u>13</u> (4/5)	<u>416</u> (5/5)	< (0/5)	17 (3/5)
rg-A/JWE/HK/1038/06 ^b	< (0/5)	< (0/5)	< (0/5)	25 (4/5)	≤ (0/5)	< (0/5)
Multiple-clade vaccine ^b	< (0/5)	23 (4/5)	36 (5/5)	384 (5/5)	< (0/5)	17 (3/5)
Adjuvanted vaccine^d						
rg-A/VN/1203/04 ^b	<u>13</u> (7/13)	<u>194</u> (14/14)	13 (6/13)	497 (14/14)	< (0/13)	20 (4/13)
rg-A/HK/213/03 ^b	< (0/15)	42 (12/15)	<u>128</u> (15/15)	<u>1579</u> (15/15)	< (0/15)	20 (3/15)
rg-A/JWE/HK/1038/06 ^b	< (0/15)	10 (3/15)	84 (12/15)	720 (15/15)	<u>14</u> (5/15)	40 (1/15)
Multiple-clade vaccine ^b	< (0/14)	57 (15/15)	116 (14/14)	1173 (15/15)	10 (1/14)	22 (6/14)

Note: Titers for homologous vaccine and virus are underlined.

^aExpressed as the reciprocal of the highest serum dilution that inhibited 4 hemagglutinating units of virus. Results are the arithmetic mean titer of positive sera (HI titer >10). Number of positive sera/total is indicated in parentheses. < indicates that all ferrets had HI titers < 10.

^bSingle-clade vaccines contained 7.5 µg HA per dose; multiple-clade vaccine contained 3.75 µg HA of each antigen per dose. Second (boost) dose was given 3 weeks after the initial dose.

^cSerum samples were collected 3 weeks and 1 week, respectively, after the 1st and 2nd doses of vaccine.

^dVaccination schedule and dosage for adjuvanted vaccines remained unchanged during the study so we averaged the results of the pre-challenge HI titers for ferrets in all challenge studies.

TABLE 3
Neutralizing antibody titers in ferrets vaccinated with inactivated whole-virus H5N1 influenza vaccine

Vaccine regimen	Mean serum neutralizing antibody titer against ^d							
	rg-A/VN/1203/04		rg-A/HK/213/03		rg-A/JWE/HK/1038/06		rg-A/WSN/MG/244/05	
Nonadjuvanted vaccine	1 st dose ^c	2 nd dose ^c	1 st dose ^c	2 nd dose ^c	1 st dose ^c	2 nd dose ^c	1 st dose ^c	2 nd dose ^c
rg-A/VN/1203/04 ^b	<u>43</u> (3/3)	<u>90</u> (3/3)	40 (2/3)	242 (3/3)	40 (3/3)	56 (3/3)	62 (3/3)	95 (3/3)
rg-A/HK/213/03 ^b	40 (1/3)	44 (2/3)	<u>58</u> (3/3)	<u>653</u> (3/3)	40 (1/3)	71 (3/3)	46 (3/3)	170 (3/3)
rg-A/JWE/HK/1038/06 ^b	40 (1/3)	40 (2/3)	40 (3/3)	132 (3/3)	<u>43</u> (3/3)	<u>55</u> (3/3)	69 (3/3)	69 (3/3)
Multiple-clade vaccine ^b	43 (3/3)	62 (3/3)	178 (3/3)	689 (3/3)	60 (2/3)	123 (3/3)	122 (3/3)	413 (3/3)
Adjuvanted vaccine ^d								
rg-A/VN/1203/04 ^b	<u>43</u> (6/8)	<u>1045</u> (9/9)	60 (2/8)	1073 (9/9)	48 (5/8)	214 (9/9)	52 (8/8)	511 (9/9)
rg-A/HK/213/03 ^b	40 (4/9)	100 (9/9)	<u>97</u> (9/9)	<u>3889</u> (9/9)	40 (7/9)	260 (9/9)	53 (7/9)	449 (9/9)
rg-A/JWE/HK/1038/06 ^b	40 (1/9)	70 (9/9)	105 (7/9)	1633 (9/9)	<u>82</u> (8/9)	<u>1648</u> (9/9)	59 (8/9)	255 (9/9)
Multiple-clade vaccine ^b	48 (2/9)	343 (9/9)	125 (9/9)	2547 (9/9)	61 (8/9)	714 (9/9)	74 (9/9)	1707 (9/9)

Note: Titers for homologous vaccine and virus are underlined.

^aExpressed as the reciprocal of the highest serum dilution that neutralized 50% of the HA activity of 100 TCID₅₀ of virus infected cells. Results are the arithmetic mean titers of positive sera (titer ≥ 40). Number of positive sera/total is indicated in parentheses. Neutralizing antibody titers were measured for three ferrets per vaccine group.

^bSingle-clade vaccines contained 7.5 μ g HA per dose; multiple-clade vaccine contained 3.75 μ g HA of each antigen per dose. Second (boost) dose was given 3 weeks after the initial dose.

^cSerum samples were collected 3 weeks and 1 week, respectively, after the 1st and 2nd doses of vaccine.

^dVaccination schedule and dosage for adjuvanted vaccines remained unchanged during the study so we averaged the results of the pre-challenge virus neutralizing antibody titers for ferrets in all challenge studies.