MAJOR ARTICLE

Immunization with Reverse-Genetics–Produced H5N1 Influenza Vaccine Protects Ferrets against Homologous and Heterologous Challenge

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(See the editorial commentary by Hampson, on pages 143–5.)

Background. Multiple cases of transmission of avian H5N1 influenza viruses to humans illustrate the urgent need for an efficacious, cross-protective vaccine.

Methods. Ferrets were immunized with inactivated whole-virus vaccine produced by reverse genetics with the hemagglutinin (HA) and neuraminidase genes of A/HK/213/03 virus. Ferrets received a single dose of vaccine (7 or 15 μg of HA) with aluminum hydroxide adjuvant or 2 doses (7 μg of HA each) without adjuvant and were challenged with 10^6 50% egg infectious doses of A/HK/213/03, A/HK/156/97, or A/Vietnam/1203/04 virus.

Results. One or 2 doses of vaccine induced a protective antibody response to the vaccine strain. All immunization regimens completely protected ferrets from challenge with homologous wild-type A/HK/213/03 virus: no clinical signs of infection were observed, virus replication was significantly reduced ($P < .05$) and was restricted to the upper respiratory tract, and spread of virus to the brain was prevented. Importantly, all vaccinated ferrets were protected against lethal challenge with the highly pathogenic strain A/Vietnam/1203/04. The 2-dose schedule induced higher levels of antibodies that were cross-reactive to antigenically distinct H5N1 viruses.

Conclusions. H5N1 vaccines may stimulate an immune response that is more cross-protective than what might be predicted by in vitro assays and, thus, hold potential for being stockpiled as "initial" pandemic vaccines.

The pandemic potential of the avian H5N1 influenza viruses for humans is well documented [1–3]. These viruses can cause severe disease in humans, with multiple-organ failure and death of the infected individuals [4, 5]. Improvements in biosecurity in poultry markets [6], surveillance, and monitoring of avian-human transmission are important measures for control of the emergence of highly pathogenic viruses. Antiviral drugs can be helpful at the early stage of a pandemic [7], but vaccination of poultry and humans is the main preventive strategy against pandemic influenza.

An effective influenza vaccine is urgently needed as H5N1 viruses continue to spread in Asia, not only causing an increasing number of human infections and high mortality rates [3, 5] but also showing evidence of probable human-to-human transmission [8]. Influenza vaccines based on wild-type H5N1 virus cannot be produced on a large scale, because of high virus virulence and the requirements for working under biosafety level (BSL) 3+ conditions [9]. Vaccines based on less virulent H5 influenza strains that are antigenically similar to circulating strains [10, 11] have so far been poorly immunogenic in human trials [12]. Recombinant hemagglutinin (HA) proteins and DNA vaccines have also been explored [13–15]. Gene-gun–delivered DNA vaccine encoding H5 HA from A/HK/156/97 virus provided immunity against homologous and heterologous H5N1 infection of mice [13]. Baculovirus-expressed H5 HA vaccine was extremely well tolerated in human trials but failed to elicit substantial antibody responses; higher doses of antigen and/or addition of adjuvants have yet to be tested [15].

One of the important requirements for a pandemic vaccine is availability on short notice. Reverse-genetics technology allows the generation of safe vaccine strains

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Table 1. Serum antibody response in ferrets after vaccination with rgHK213/03xPR8 (H5N1) vaccine.

Vaccination regimen, HA content[a]	Serum antibody titer, mean (range)[b]		
	Anti-HA	Anti-NA	Virus neutralizing
Single dose, with adjuvant			
7 μg	107 (40–160)	27 (<10–80)	751 (226–1280)
15 μg	120 (40–160)	50 (20–160)	1145 (320–2153)
Two doses, no adjuvant			
1st dose: 7 μg	115 (40–320)	65 (20–160)	443 (80–1076)
2nd dose: 7 μg	982 (160–1280)	340 (80–640)	5077 (538–10,240)

NOTE. HA, hemagglutinin; NA, neuraminidase.

[a] Four- to 6-month-old ferrets were immunized with inactivated whole-virus rgHK213/03xPR8 vaccine containing 7 or 15 μg of HA that was administered as a single dose with aluminum hydroxide adjuvant or as 2 doses (7 μg each) without adjuvant.

[b] Serum samples (9–11 per vaccine group) were collected 4 weeks after a single dose of vaccine and 3 weeks and 1 week, respectively, after the first and second doses of a 2-dose regimen. The titers were determined against homologous A/HK/213/03 (H5N1) virus and are expressed as the reciprocal value (e.g., as 80 vs. 1:80).

with known properties and offers the important advantage of rapid vaccine preparation, which could be crucial in a pandemic [16]. An inactivated vaccine virus that has the internal genes of A/PR/8/34 (H1N1) virus and the HA and neuraminidase (NA) genes of A/HK/491/97 (H5N1) virus has been found to be immunogenic and to protect mice from challenge with homologous and heterologous H5N1 viruses [17]. The reverse-genetics approach has also been used to generate an effective agricultural H5N3 vaccine that induced anti-HA antibodies and prevented death in chickens [18]. Clinical evaluation of a candidate H5N1 reverse-genetics vaccine based on A/Vietnam/1203/04 virus is planned in the United States and Europe [9].

The use of reverse genetics to produce influenza vaccines is still relatively new, and questions remain about the safety and immunogenicity of these vaccines, as well as what dosage and regimen are needed to protect immunologically naive persons from severe infection. One of the important questions for pandemic preparedness is not only whether the protective immunity provided by a vaccine is effective against viruses that are antigenically closely matched with those in the vaccine but also whether these vaccines can be effective against viruses that have undergone antigenic drift. Cross-protection within an HA subtype would allow the use of a stockpiled vaccine until a strain-specific vaccine is available.

Ferrets are considered to be the most suitable animal model for preclinical evaluation of human influenza vaccines. In the present study, we used this model to assess the immunogenicity, protective efficacy, and cross-reactivity of various regimens of vaccination with inactivated whole-virus A/HK/213/03xA/PR/8/34 (rgHK213/03xPR8) influenza vaccine generated by reverse genetics. Cross-protection was determined by challenge with antigenically diverse H5N1 viruses, including A/HK/156/97 and A/Vietnam/1203/04.

MATERIALS AND METHODS

Viruses and cells. The H5N1 human influenza viruses A/HK/156/97, A/HK/213/03, and A/Vietnam/1203/04 were obtained from the World Health Organization influenza collaborating laboratories. Stock viruses were propagated in the allantoic cavities of 10-day-old embryonated chicken eggs at 35°C for 36 h and stored at −70°C. All experiments with highly pathogenic viruses were conducted in a BSL 3+ containment facility approved for use by the US Department of Agriculture and the US Centers for Disease Control and Prevention. MDCK cells were obtained from the American Type Culture Collection and were grown in MEM supplemented with 5% fetal calf serum in a humidified atmosphere of 5% CO_2.

Antigenic analysis. The antigenic characterization of the H5N1 viruses was performed by the hemagglutination inhibition (HI) test with a panel of polyclonal antisera and monoclonal antibodies (MAbs) against H5 HA. MAbs were prepared using a modification of the method described elsewhere [19].

Generation of the candidate vaccine. Recombinant virus containing the HA and NA genes of A/HK/213/03 (H5N1) influenza virus in the background of the A/PR/8/34 (H1N1) virus was generated and rescued in Vero cells certified for vaccine production at the St. Jude Children's Research Hospital under good manufacturing practice conditions [16]. The vaccine strain was propagated in eggs at 37°C for 48 h, and virus-containing allantoic fluid was inactivated by use of β-propiolactone at a ratio of 1:2000 (vol/vol). Vaccine preparations were concentrated by Amicon ultrafiltration and ultracentrifugation through a 25% and 70% sucrose cushion, pelleted at 76,000 *g* at 4°C for 1 h, and purified as described elsewhere [18]. The HA protein content of vaccine preparations was determined by single radial immunodiffusion assay.

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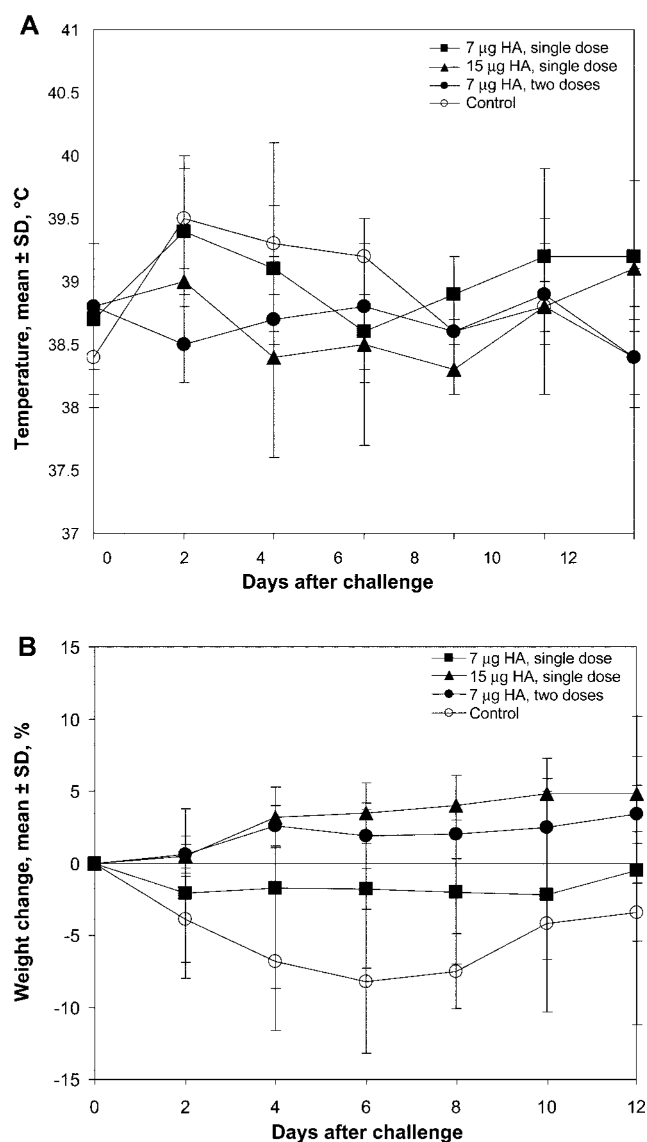

Figure 1. Change in body temperature *(A)* and weight *(B)* of vaccinated and control ferrets after challenge with homologous A/HK/213/03 (H5N1) virus. Groups of 3 or 4 ferrets either were vaccinated with a single dose (7 or 15 μg) or 2 doses (both 7 μg) of hemagglutinin (HA) of HK213/03xPR8 (H5N1) vaccine or not vaccinated and then were inoculated with 10^6 EID_{50} of the wild-type A/HK/213/03 (H5N1) virus. Temperatures were monitored daily by use of subcutaneous implantable temperature transponders. Each data point represents the mean ± SD value. The mean body temperature of an uninfected ferret is 38.8°C. Loss or gain of weight was calculated for each ferret as the percentage change in the initial mean starting weight on day 0. Data are the mean ± SD values from 2–4 ferrets for each group.

Immunization and challenge. Young adult ferrets 4–6 months of age and seronegative for currently circulating influenza A H1N1 and H5N1 and influenza B viruses were obtained from Marshall Farms or the breeding program at St. Jude Children's Research Hospital. More than 90% of ferrets had HI titers (1:20 to 1:160) against A/Panama/2007/99 (H3N2) virus obtained through natural infection. Groups of 3 or 4 ferrets were vaccinated intramuscularly on 2 schedules: (1) with 1 dose of inactivated vaccine containing 7 or 15 μg of HA plus 0.5 mg of aluminum hydroxide adjuvant or (2) with 2 doses of vaccine, each containing 7 μg of HA without adjuvant, administered 3 weeks apart. Control ferrets received PBS. Ferrets that received 1 dose of vaccine were challenged with wild-type H5N1 virus 4 weeks after vaccination. Those that received 2 doses were challenged 1 week after the last dose. Ferrets were anesthetized with isoflurane and inoculated intranasally with 10^6 EID_{50} of either homologous A/HK/213/03 or heterologous A/HK/156/97 and A/Vietnam/1203/04 challenge virus in 1.0 mL of sterile PBS. Clinical signs of infection, relative inactivity index [20], and body temperature were monitored daily for 12 days.

Nasal washes and tissue samples. On days 3, 5, and 7 after virus inoculation, ferrets were anesthetized with ketamine (25 mg/kg) and nasal washes were collected. Lung, brain, olfactory bulb, and intestinal tissues were collected from groups of 2 ferrets vaccinated with 2 doses of vaccine and inoculated with A/HK/213/03 virus, as described elsewhere [21]. Virus was titrated in eggs and expressed as $\log_{10}$ EID_{50} per milliliter or gram, as calculated by the method of Reed and Muench [22]. The limit of virus detection was <0.75 $\log_{10}$ EID_{50}/mL. Virus titers were compared by a 2-tailed *t* test.

Serologic testing. Serum samples were obtained 3 weeks after the first dose and 1 week after the second dose of vaccine or 4 weeks after a single dose. Serum samples were treated with receptor-destroying enzyme, heat inactivated at 56°C for 30 min, treated with packed chicken red blood cells (CRBCs), and tested by HI assay with 0.5% CRBCs. Anti-NA antibodies were assayed by the standard method, with fetuin (50 μg/mL) as a substrate [23]. Virus neutralizing titers were determined by infection of MDCK cells and are expressed as the reciprocal of the highest dilution of serum that gave 50% neutralization of 100 $TCID_{50}$ of virus after incubation at 37°C for 72 h [24].

RESULTS

Immunogenicity of rgHK213/03xPR8 (H5N1) influenza vaccine. We tested the serum antibody response of ferrets to various regimens of vaccination with rgHK213/03xPR8. After a single dose of either 7 or 15 μg of HA with aluminum hydroxide adjuvant, the mean anti-HA titers were 1:107 and 1:120, respectively (table 1). Importantly, HI titers against homologous A/HK/213/03 virus were ⩾1:40 in all vaccinated ferrets. Because HI tests can be relatively insensitive in detecting antibodies induced by avian influenza viruses in mammals [15, 24], we confirmed the HI titers by performing virus-neutralization assays in MDCK cells. There appeared to be a dose response: immunization with 15 μg of HA resulted in mean virus-neutralizing titers ~1.5 times higher than those induced by 7 μg of HA. Both single-dose regimens induced low anti-

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Table 2. Efficacy of rgHK213/03xPR8 (H5N1) vaccine in reducing virus replication in the upper respiratory tracts of ferrets after homologous virus (A/HK/213/03 [H5N1]) challenge.

Vaccination regimen, HA content	3 days after challenge		5 days after challenge		7 days after challenge	
	No. shedding/ total no.	Virus titer[a]	No. shedding/ total no.	Virus titer[a]	No. shedding/ total no.	Virus titer[a]
Single dose, with adjuvant						
7 μg	3/3	3.3 ± 0.7[b]	1/3	2.3	0/3	…
15 μg	2/3	1.0 ± 1.0[c]	0/3	…	0/3	…
Two doses, no adjuvant						
7 μg each	2/4	1.1 ± 1.5[b]	1/4	1.8	0/4	…
Control (PBS)	4/4	5.3 ± 0.5	4/4	5.2 ± 0.3	0/4	…

NOTE. HA, hemagglutinin. Ellipses indicate that the titer was below the limit of detection (<0.75 $\log_{10}$ EID_{50}/mL).

[a] Virus titers were determined in eggs and are expressed as $\log_{10}$ EID_{50}/mL. Data are mean ± SD titers determined in nasal washes.

[b] $P<.05$, vs. virus titers in control group (2-tailed *t* test).

[c] $P<.01$, vs. virus titers in control group.

NA titers, although titers elicited by 15 μg of HA were approximately twice those elicited by 7 μg of HA.

The presence of adjuvant did not affect the level of anti-HA antibodies in postvaccination serum, although it did increase the mean titer of virus-neutralizing antibodies (table 1). A large increase in the mean anti-HA antibody titer was observed after booster immunization. A second dose of vaccine also increased the mean titers of anti-NA antibodies (by a factor of ∼5) and virus-neutralizing antibodies (by a factor of ∼11.5) (table 1). Although 1 dose of rgH5N1 vaccine with adjuvant induced anti-HA titers ranging from 1:40 to 1:160, the 2-dose schedule was more effective in raising serum antibodies.

Protection against challenge with homologous virus. After vaccination, ferrets were challenged with 10^6 EID_{50} of the wild-type A/HK/213/03 (H5N1) virus. Ferrets in the unvaccinated control group ($n = 4$) showed lethargy ($n = 2$), respiratory symptoms ($n = 2$), fever ($n = 4$; range, 38°C–41°C) (figure 1*A*), and weight loss ($n = 4$). On day 6 after inoculation, the ferrets had lost 4%–12% of their initial weight (figure 1*B*). One of 3 ferrets that received a single dose of 7 μg of HA showed increased body temperature and weight loss. None of the remaining vaccinated ferrets showed signs of illness.

To compare the efficacy of the vaccination regimens in inhibiting virus replication in the upper respiratory tract, we collected nasal washes on days 3, 5, and 7 after inoculation with A/HK/213/03 virus (table 2). Unvaccinated control ferrets shed virus at mean titers of 5.3 $\log_{10}$ EID_{50}/mL on day 3 and 5.2 $\log_{10}$ EID_{50}/mL on day 5. Ferrets that received a single dose of 7 μg of HA had significantly lower virus titers than control ferrets on day 3 ($P<.05$), and only 1 of 3 shed virus on day 5 after inoculation. A single dose equivalent to 15 μg of HA reduced the number of ferrets shedding virus on day 3 after inoculation (2/3 ferrets) and completely inhibited virus replication on day 5. Administration of 2 doses of 7 μg of HA significantly reduced virus titers and decreased the number of ferrets that shed virus, compared with control ferrets (table 2).

We tested the efficacy of the 2-dose vaccination in inhibiting the spread of homologous A/HK/213/03 (H5N1) virus to organs. In control ferrets, virus titers on day 3 after inoculation were 5.8 and 6.8 $\log_{10}$ EID_{50}/g in lung (2/2 ferrets), 2.5 $\log_{10}$ EID_{50}/g in brain (1/2 ferrets), and 1.3 and 3.0 $\log_{10}EID_{50}$/g in the olfactory bulb (2/2 ferrets). All vaccinated ferrets were protected against systemic spread of homologous virus, which was not detected in any organ tested.

All vaccine regimens were effective in reducing A/HK/213/03 (H5N1) influenza virus replication in the upper respiratory tract. No statistically significant difference was observed between virus titers after the various regimens. Even a single vaccination with 7 μg of HA appeared to be sufficient to block virus replication in the nasal cavities of ferrets. The 2-dose schedule was superior in reducing clinical signs of illness and completely inhibited virus spread to the internal organs.

Cross-reactivity of antibodies induced by rgHK213/03xPR8 (H5N1) vaccine. To determine whether the schedule of vaccination affected the extent of cross-reactivity, we measured serum antibody responses in ferrets, after vaccination with rgHK213/03xPR8 vaccine, to 2 H5N1 viruses (A/HK/156/97 and A/Vietnam/1203/04). Antigenic analysis with a panel of polyclonal antisera and MAbs revealed that these 2 H5N1 viruses were antigenically distinct from the vaccine strain (table 3). The H5N1 strain that appeared in 2004 did not react with reference antiserum against A/tern/S.Africa/61 and possessed low reactivity with other sera. The reactivity of the viruses to a panel of H5 MAbs also differed: influenza A/HK/213/03 virus was recognized by 7 of 8 MAbs, whereas A/HK/156/97 and A/Vietnam/1203/04 viruses were recognized by 4 and 5 MAbs, respectively (table 3).

The anti-HA and virus-neutralizing antibody responses to vaccine and heterologous viruses induced after immunization

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Table 3. Antigenic analysis of vaccine and challenge H5N1 influenza viruses.

Challenge virus	Polyclonal antiserum against			MAb against							
	A/tern/S.Africa/61	A/gs/HK/437-4/99	A/HK/213/03	HK03-3[a]	VN04-7[b]	VN04-8[b]	VN04-12[b]	VN04-14[b]	VN04-15[b]	CP 24[c]	CP 46[c]
A/HK/156/97	1280	2560	40	<100	<100	<100	3200	3200	<100	3200	12,800
A/HK/213/03	5120	1280	1280	3200	3200	12,800	6400	200	6400	1600	<100
A/Vietnam/1203/04	<20	320	40	<100	<100	1600	1600	1600	800	800	<100

NOTE. Data are hemagglutination inhibition (HI) titers. HI assays were performed in microtiter plates with 0.5% chicken red blood cells. Titers are the reciprocal lowest dilutions of antibodies that inhibited hemagglutination by 4 hemagglutinin (HA) units of virus. Polyclonal antiserum against purified HA from A/tern/S.Africa/61 (H5N3) and A/HK/213/03 (H5N1) viruses was obtained by immunization of goats; antiserum against A/gs/HK/437-4/99 (H5N1) was prepared in chickens. MAb, monoclonal antibody.

[a] Anti-HA MAb against A/HK/213/03 virus.

[b] Anti-HA MAb against A/Vietnam/1203/04 virus.

[c] Anti-HA MAb against A/chicken/Pennsylvania/1370/83 virus.

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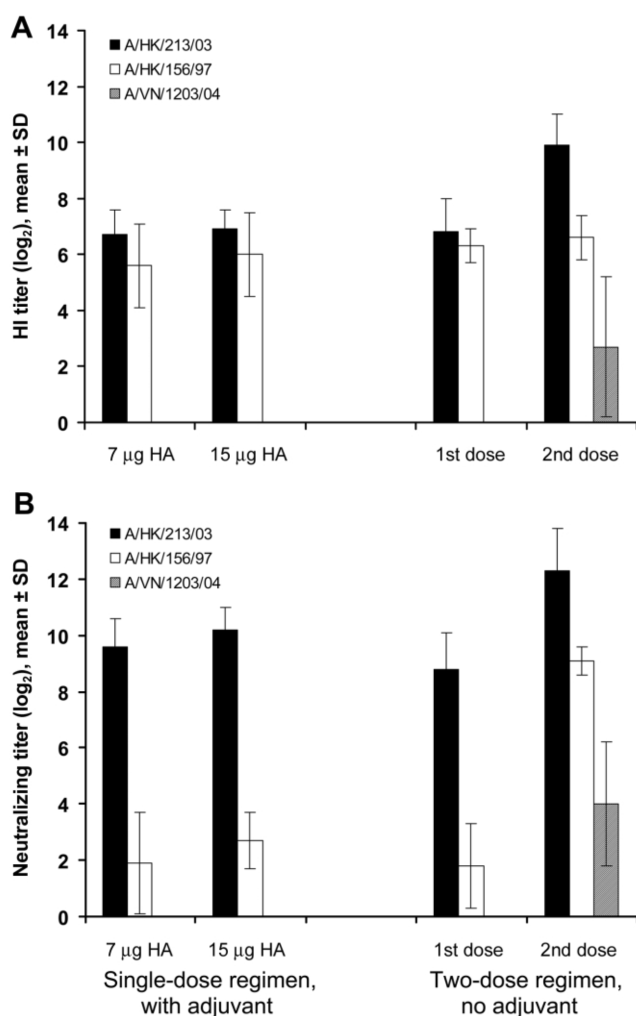

Figure 2. Cross-reactivity of serum antibodies induced in ferrets by 1 or 2 doses of rgHK213/03xPR8 (H5N1) vaccine. Hemagglutination inhibition (HI) *(A)* and neutralizing-antibody *(B)* titers in ferret serum after vaccination are shown. Samples were obtained 4 weeks after a single dose of vaccine and 3 weeks and 1 week, respectively, after the first and second doses of a 2-dose vaccination regimen. HI titers are expressed as the reciprocal of the highest dilution of serum (log_2) that inhibited the hemagglutination of 4 hemagglutinin (HA) units of virus. Neutralizing-antibody titers are expressed as the reciprocal of the highest dilution of serum (log_2) that neutralized 100 $TCID_{50}$ of virus-infected MDCK cells. Data are mean ± SD titers.

of ferrets with rgHK213/03xPR8 vaccine are shown in figure 2. After immunization of ferrets with 1 dose of adjuvanted vaccine (both 7 and 15 μg of HA), the mean HI titers against A/HK/156/97 virus were similar to those against the vaccine strain (figure 2*A*). The second dose of vaccine without adjuvant increased the mean HI titer against the vaccine strain but did not affect titers against the A/HK/156/97 strain. In contrast, the mean titers of virus-neutralizing antibodies against A/HK/156/97 virus were substantially lower than those against the vaccine strain after 1 dose of either adjuvanted or nonadjuvanted vaccine, but the titer was significantly increased by a booster dose of vaccine without adjuvant (figure 2*B*). Tests for serum antibodies against A/Vietnam/1203/04 showed dramatic results. There were no detectable HI or virus-neutralizing antibodies against this virus after 1 dose of vaccine, with or without adjuvant (figure 2). Only a second dose of vaccine resulted in measurable mean HI and virus-neutralizing antibody titers against A/Vietnam/1203/04 virus, although some vaccinated ferrets had no detectable antibodies.

Protection against challenge with antigenically distinct H5N1 influenza viruses. A vaccine that provides cross-protection within an HA subtype could be stockpiled for use in a pandemic until a vaccine antigenically matching the pandemic virus is available. To determine the extent of cross-protection induced by rgHK213/03xPR8 (H5N1) vaccine, we challenged vaccinated ferrets with H5N1 viruses that were antigenically and genetically distinct from the vaccine strain. All 4 unvaccinated ferrets inoculated with 10^6 EID_{50} of A/HK/156/97 virus survived but showed signs of disease. All had fever for 7 days after inoculation; the maximum temperature increase (mean, 1.6°C) (table 4) was observed on day 3 after challenge. Ferrets in the control group lost 5.9% of their initial weight and were shedding virus at titers of 4.0–7.0 log_{10} EID_{50}/mL on days 3 and 5 after inoculation (figure 3*A*). Vaccinated ferrets were protected against virus challenge, although ferrets that received a single dose of 7 μg of HA showed minor clinical signs, and 1 had fever and weight loss (table 4). Greater protection was observed with 2 doses of vaccine: no clinical signs were observed, and virus replication in the upper respiratory tract was reduced significantly, compared with that in control ferrets ($P < .05$) (figure 3*A*).

All 3 unvaccinated ferrets inoculated with influenza A/Vietnam/1203/04 virus died. All had fever, lost ~20.5% of their initial body weight, were extremely lethargic, and showed neurological signs of disease (hindlimb paresis); these ferrets were killed on day 6 or 7 after virus inoculation. Importantly, no deaths were observed in vaccinated ferrets after challenge with A/Vietnam/1203/04 virus (table 4). There was a dose-dependent effect in the protective efficacy of the single-dose vaccine. Fever and weight loss were observed only in ferrets that received the lower single dose for 6 and 14 days, respectively, and virus replication in the upper respiratory tract was observed on days 3 and 5 after inoculation, although no ferrets died. After vaccination with a single dose of 15 μg of HA, ferrets showed decreased activity (table 4), and virus was cleared by day 5 after challenge (figure 3*B*). Although protection against lethal challenge with A/Vietnam/1203/04 (H5N1) virus was achieved with

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Table 4. Cross protection of rgHK/213/03xPR8 (H5N1)–vaccinated ferrets against challenge with antigenically distinct H5N1 influenza viruses.

		Clinical sign					
Challenge virus, vaccination regimen[a]	No. dead/ total no.	Relative inactivity index[b]	Temperature increase, °C[c]	Weight change, %[c]	Respiratory symptoms, no. of ferrets/ total no.	Neurological symptoms, no. of ferrets/ total no.	Diarrhea, no. of ferrets/ total no.
A/HK/156/97							
Single dose (7 μg), with adjuvant	0/3	0.8	0.5	−1.8	1/3	1/3	0/3
Single dose (15 μg), with adjuvant	0/3	0.7	0.1	+3.9	0/3	0/3	0/3
Two doses (7 μg each), no adjuvant	0/4	0.4	0.2	+1.3	0/4	0/4	0/4
Control (PBS)	0/4	0.9	1.6	−5.9	2/4	1/4	2/4
A/Vietnam/1203/04							
Single dose (7 μg), with adjuvant	0/3	1.2	1.0	−9.0	2/3	1/3	1/3
Single dose (15 μg), with adjuvant	0/3	0.8	0.2	+0.1	0/3	0/3	0/3
Two doses (7 μg each), no adjuvant	0/3	0.7	0.4	+0.6	0/3	0/3	0/3
Control (PBS)	3/3	2.4	2.4	−20.5	2/3	3/3	3/3

[a] Ferrets were immunized with either a single dose of aluminum hydroxide–adjuvanted rgHK213/03xPR8 (H5N1) vaccine containing 7 or 15 μg of HA or 2 doses of a 7-μg HA vaccine without adjuvant. Three weeks after the single vaccine dose or 1 week after the 2 doses, ferrets were challenged with 10^6 EID_{50} of either A/Hong Kong/156/97 (H5N1) or A/Vietnam/1203/04 (H5N1) virus.

[b] Determined from observations for 12 days, as described elsewhere [20]. Ferrets in the control group inoculated with A/Vietnam/1203/04 (H5N1) virus were observed for 7 days only, because of mortality.

[c] Maximal change in temperature or weight.

all 3 vaccine regimens tested, 2 doses of nonadjuvanted vaccine and a single dose of 15 μg of HA with adjuvant were the most beneficial.

DISCUSSION

We evaluated the immunogenicity and cross-protective efficacy of an H5N1 influenza vaccine, generated by reverse genetics, that possessed HA and NA genes from the A/HK/213/03 human virus in the genetic background of the high-yield strain A/PR/8/34 [16]. This is the first study in the ferret model, to our knowledge, to characterize the cross-reactive immunity induced by an H5N1 vaccine and to evaluate its protectiveness against both homologous and antigenically distinct viruses.

Although a single dose of vaccine is presently used for immunization of adults, our findings confirmed that a 2-dose regimen is preferable for vaccination of an immunologically naive population against a novel H5N1 strain. The second dose of vaccine significantly increased serum antibody production and conferred complete protection against challenge with a high dose of the homologous H5N1 virus. This observation is consistent with the findings of clinical trials in which 2-dose regimens of candidate H5N3, H2N2, and H9N2 influenza vaccines induced a superior response in unprimed populations [12, 25]. The dose-dependent effect we observed in the induction of neutralizing antibodies and the reduction of clinical signs of illness suggested that an increased dosage of antigen offers better protection. However, in the event of an influenza pandemic, there is likely to be a significant gap between vaccine production capacity and demand. Therefore, the use of adjuvanted vaccines, mucosal adjuvants, or immunostimulating complexes to enhance immune stimulation is considered prudent [26, 27]. Adjuvanted vaccines have only recently been licensed and are not widely available, and there is uncertainty about the safety of some adjuvants in humans, although that excludes vaccines containing aluminum adjuvants [9, 25].

There is still limited information available on the extrapulmonary replication of H5N1 influenza viruses in humans, with respect to high mortality rates observed in documented human infections in 2004 [5]. Virus isolation from cerebrospinal fluid, fecal, throat, and serum specimens [3] underlines that the clinical spectrum of influenza H5N1 is wider than previously thought. The lethality and marked neurotropism of some 1997 and 2004 virus isolates have also been reported in the mouse and ferret models [21, 28, 29]. Because of this lethality, the primary goal of a pandemic influenza vaccine must be to prevent death and not necessarily to prevent infection. This distinction between requirements for annual and pandemic immunization is an important point in influenza vaccine development. In our study, 2 doses of rgHK213/03xPR8 vaccine completely prevented the spread of homologous virus to lung and brain. However, additional studies with challenge viruses of different pathogenicity and, more importantly, different neurotropism are needed.

One desirable feature of a pandemic vaccine is the ability to induce cross-reactive immune responses sufficient to protect against variants that have undergone antigenic drift. Our vac-

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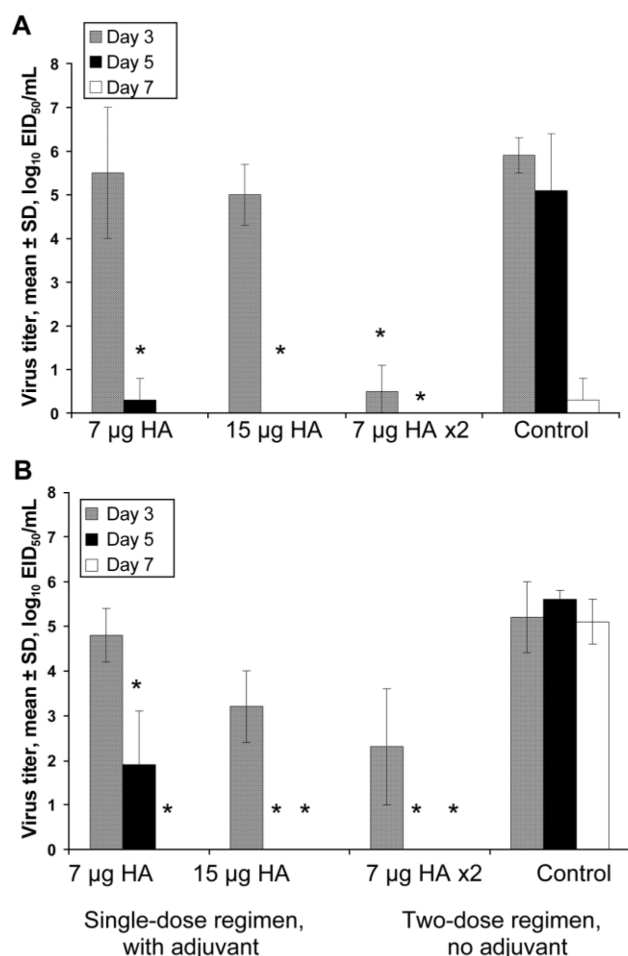

Figure 3. Virus titers in the upper respiratory tracts of vaccinated and control ferrets after challenge with heterologous H5N1 influenza viruses. Groups of 3 or 4 ferrets vaccinated with rgHK213/03xPR8 (H5N1) vaccine and control ferrets were inoculated intranasally with 10^6 EID_{50} of A/HK/156/97 (H5N1) virus *(A)* or A/Vietnam/1203/04 (H5N1) virus *(B)*. Nasal washes were collected on days 3, 5, and 7 after virus inoculation. Data are the mean ± SD virus titers ($\log_{10}$ EID_{50}/mL) on the indicated day. $^*P < .05$, vs. virus titers in the control group (2-tailed *t* test).

cine was tested against H5N1 viruses with substantial antigenic differences. Phylogenetic analysis of the H5 HA genes showed that all 3 H5N1 viruses used in the study belonged to different clades [30]. Sequence analysis revealed that the HA1 regions of A/HK/156/97 and A/Vietnam/1203/04 viruses differ from those of the vaccine strain by 18 and 10 aa, respectively [28, 31]. Most of these amino acids are located on the tip of the HA molecule, the primary target for neutralizing antibodies [32]. Nevertheless, we were able to demonstrate the induction of cross-reactive antibodies after vaccination of immunologically naive ferrets with 2 doses of rgHK213/03xPR8 (H5N1) vaccine. These results suggest that 2 doses of vaccine will be the optimal strategy for a pandemic influenza vaccine.

Surprisingly, ferrets that had almost no detectable antibodies against A/Vietnam/1203/04 virus were protected against lethal challenge with that virus. We consider that compromised sensitivity of the serological assay can lead to underestimation of the levels of antibodies. Indeed, in a recent study [33], we demonstrated that H5N1 viruses isolated in 2004 elicited low levels of detectable HI antibody responses both after intranasal inoculation and after intramuscular vaccination with nonadjuvanted vaccine and that the generation of a recombinant virus with a single amino acid substitution (S223N) in HA resulted in improved detection of anti-HA antibodies. Protection of the ferrets by cellular immune responses could play a role. The ferrets used in the study were seronegative for antibodies against H5N1 virus but possessed anti-HA antibodies against the contemporary human H3N2 influenza virus. This situation mimics that in humans, in which a pandemic vaccine will be used in a population primed with H3N2 viruses. However, it seems more likely that T cell responses to internal proteins of the H3N2 viruses would not influence protection, because these proteins are genetically distinct from those of H5N1 viruses [34]. Cytotoxic T lymphocytes (CTLs) play an important role in the control of influenza infection [35]. Alternatively, H5N1-specific CTLs may have been induced by vaccination. However, the extent and significance of previous cellular immunity in the protection of ferrets in our experiments is unknown, and H3-seropositive control ferrets were highly susceptible to infection and disease with A/Vietnam/1203/04 virus. Although intramuscular vaccination with an inactivated influenza vaccine induces little CTL stimulation in humans [36], little is known about the cellular response in ferrets and the potential contribution of cross-priming of antigen-specific CTLs [37, 38].

At least 6 months will probably be required to produce the first dose of H5N1 vaccine after the virus strain is identified. Vaccine availability could be increased through the stockpiling of pandemic vaccines and the development of vaccine virus libraries. The results of our study show that protection can be gained even when the vaccine strain does not match the challenge virus and that the optimal strategy for vaccination of immunologically naive populations will be the 2-dose regimen, which induced more cross-reactive antibodies within the same HA subtype. Therefore, the strategy of stockpiling pandemic vaccines for administration to groups at high risk offers promise. Reverse-genetics technology allows for the rapid generation of a broad spectrum of vaccine virus libraries. Selection of the most promising vaccine candidate will require special attention, because the candidate must not only carry major antigenic epitopes of H5 HA but also be immunogenic. It should also be remembered that other influenza HA subtypes, such as H2, H9, and H7, have pandemic potential.

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