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Evaluation of Live Avian-Human Reassortant Influenza A H3N2 and H1N1 Virus Vaccines in Seronegative Adult Volunteers

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An avian-human reassortant influenza A virus deriving its genes coding for the hemagglutinin and neuraminidase from the human influenza A/Washington/897/80 (H3N2) virus and its six "internal" genes from the avian influenza A/Mallard/NY/6750/78 (H2N2) virus (i.e., a six-gene reassortant) was previously shown to be safe, infectious, nontransmissible, and immunogenic as a live virus vaccine in adult humans. Two additional six-gene avian-human reassortant influenza viruses derived from the mating of wild-type human influenza A/California/10/78 (H1N1) and A/Korea/1/82 (H3N2) viruses with the avian influenza A/Mallard/NY/78 virus were evaluated in seronegative (hemagglutination inhibition titer, ≤1:8) adult volunteers for safety, infectivity, and immunogenicity to determine whether human influenza A viruses can be reproducibly attenuated by the transfer of the six internal genes of the avian influenza A/Mallard/NY/78 virus. The 50% human infectious dose was $10^{4.9}$ 50% tissue culture infectious doses for the H1N1 reassortant virus and $10^{5.4}$ 50% tissue culture infectious doses for the H3N2 reassortant virus. Both reassortants were satisfactorily attenuated with only 5% (H1N1) and 2% (H3N2) of infected vaccines receiving less than 400 50% human infectious doses developing illness. Consistent with this level of attenuation, the magnitude of viral shedding after inoculation was reduced 100-fold (H1N1) to 10,000-fold (H3N2) compared with that produced by wild-type virus. The duration of virus shedding by vaccinees was one-third that of controls receiving wild-type virus. At 40 to 100 50% human infectious doses, virus-specific immune responses were seen in 77 to 93% of volunteers. When vaccinees who had received $10^{7.5}$ 50% tissue culture infectious doses of the H3N2 vaccine were experimentally challenged with a homologous wild-type human virus, only 2 of 19 (11%) vaccinees became ill compared with 7 of 14 (50%) unvaccinated seronegative controls ($P < 0.025$; protective efficacy, 79%). Thus, three different virulent human influenza A viruses have been satisfactorily attenuated by the acquisition of the six internal genes of the avian influenza A/Mallard/NY/78 virus. The observation that this donor virus can reproducibly attenuate human influenza A viruses indicates that avian-human influenza A reassortants should be further studied as potential live influenza A virus vaccines.

We are currently evaluating six-gene avian-human reassortant influenza A viruses, i.e., reassortant viruses containing the genes coding for the hemagglutinin (HA) and neuraminidase (NA) of human influenza A viruses and the other six RNA segments (internal genes) of avian influenza A viruses, as live influenza A vaccine strains for use in humans. We previously identified several avian influenza A viruses that were restricted in replication in the upper and lower respiratory tract of squirrel monkeys (10). Six-gene reassortant viruses derived from one of these avian influenza viruses, A/Mallard/NY/6750/78 (H2N2), were shown to be reproducibly attenuated for squirrel monkeys (5, 14). The six-gene avian-human reassortant virus derived from the mating of the wild-type human influenza A/Washington/897/80 (H3N2) virus with the avian influenza A/Mallard/NY/78 virus was shown to be attenuated and immunogenic in squirrel monkeys and humans (9). The virus was not enterotropic, nor did it spread systemically in either species, and it was not transmitted to susceptible contacts housed together with the infected monkeys or volunteers.

Studies to date have shown that avian influenza A viruses possess many of the characteristics desirable for a live virus vaccine donor strain (6, 9). The genetic divergence between avian and human influenza A viruses suggests that avian-human reassortant influenza A vaccine viruses should retain their attenuation phenotype after replication in humans (1). However, because the surface glycoproteins (HA and NA) of human influenza A viruses undergo frequent antigenic change and because two subtypes of influenza A virus (H1N1 and H3N2) currently circulate, a virus strain under consideration as a donor of attenuating genes for a human influenza A vaccine virus vaccine production must be able to transfer reproducibly the attenuation phenotype to each new virulent human influenza A virus antigenic variant. The resulting reassortant viruses must be infectious and immunogenic. To evaluate further the suitability of the avian influenza A/Mallard/NY/78 virus as an attenuated donor virus in the production of live attenuated human influenza A virus vaccines, two six-gene reassortant viruses were produced with the wild-type influenza A/California/10/78 (H1N1) or A/Korea/1/82 (H3N2) viruses as human influenza virus parents. These reassortants were evaluated for safety and immunogenicity in seronegative volunteers. In addition, the protective efficacy of immunization with an avian-human reassortant virus was evaluated.

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MATERIALS AND METHODS

Virus. The passage histories of the avian influenza A/Mallard/NY/6750/78 (H2N2) virus parent and the wild-type human influenza A/California/10/78 (H1N1) virus have been described previously (8). The wild-type human influenza A/Korea/1/82 (H3N2) virus was provided by Alan Kendal (Centers for Disease Control, Atlanta, Ga.) and passaged in avian leukosis virus-free embryonated eggs (SPAFAS, Inc., Norwich, Conn.) and primary chick kidney tissue culture (PCKC) at 35 and 39°C by H. F. Maassab (University of Michigan, Ann Arbor) as follows: (i) allantoic cavity of eggs at 35°C, (ii) plaque isolation on PCKC at 35°C, (iii) allantoic cavity of eggs at 35°C, (iv) plaque isolation on PCKC at 39°C, and (v) allantoic cavity of eggs at 35°C.

The avian influenza virus parent was mated with each human influenza virus parent at a multiplicity of infection of approximately 1. Reassortant viruses were selected by passage at elevated temperature in the presence of antiserum against the HA and NA of the avian virus parent and biologically cloned by plaque-to-plaque passage as previously reported (5). The final viral suspensions administered to humans were produced by Louis Potash (Flow Laboratories, Inc., McLean, Va.) and tested for adventitious agents as previously described (7). The infectivity titers in MDCK cells (9) of the viral suspensions used in this study were as follows: A/California/78 wild-type (lot E-163), $10^{8.0}$ 50% tissue culture infectious doses ($TCID_{50}$) per ml; A/Korea/82 wild type (lot E-186), $10^{7.2}$ $TCID_{50}$/ml; A/California/78 × A/Mallard/NY/78 (H1N1) avian-human reassortant (lot E-184), $10^{8.3}$ $TCID_{50}$/ml; and A/Korea/82 × A/Mallard/NY/78 (H3N2) avian-human reassortant (lot E-196), $10^{7.8}$ $TCID_{50}$/ml.

Polyacrylamide gel electrophoresis. The parental origin of the RNA segments of the reassortant viruses was determined by comparison of their migration in polyacrylamide gel electrophoresis with that of the corresponding genes of the parental viruses. Viruses were propagated, and their RNAs were purified, electrophoresed, and visualized as reported earlier (5). Each reassortant virus was found to contain RNA segments coding for the HA and NA surface glycoproteins derived from its respective wild-type human virus parent, whereas the internal genes of each reassortant virus were derived from the avian A/Mallard/NY/78 influenza virus parent.

Clinical studies. Study protocols were approved by the Clinical Research Subpanel of the National Institute of Allergy and Infectious Diseases, the Human Volunteer Research Committee at the University of Maryland School of Medicine, and the Committee on Human Investigation at the University of Rochester. Healthy adults between the ages of 18 and 35 years who were not taking medications, did not have a history of influenza vaccination, and had a serum hemagglutination inhibition (HAI) antibody titer of 1:8 or less against the relevant HA were recruited from among college students and community members in Maryland and Rochester. These volunteers are referred to as seronegative adults because of their low to absent HAI antibody titer, although they have been infected previously with influenza A viruses belonging to the same subtype. Each volunteer gave written informed consent to participation in this study. First, a group of three volunteers was evaluated to determine the safety of immunization with a live H1N1 subtype avian-human reassortant influenza virus vaccine. These volunteers were housed on the isolation ward at the Center for Vaccine Development, University of Maryland Hospital, where they received a 0.5-ml inoculum containing $10^{6.0}$ $TCID_{50}$ of the avian-human influenza A/California/78 (H1N1) reassortant virus intranasally and were then observed for 7 consecutive days after inoculation, as previously described (9). Blood and rectal swab specimens for viral isolation were collected from these volunteers daily and were evaluated for evidence of virus spread beyond the respiratory tract. Data from these three volunteers were later pooled with those of a group receiving $10^{6.5}$ $TCID_{50}$. After study of the first group indicated the safety of vaccination, other volunteers received $10^{4.5}$ to $10^{8.5}$ $TCID_{50}$ of the A/California/78 (H1N1) or $10^{4.5}$ to $10^{7.5}$ $TCID_{50}$ of the A/Korea/82 (H3N2) avian-human influenza reassortant viruses in a 0.5-ml inoculum as outpatients. For 4 consecutive days they recorded their temperatures four times per day and were evaluated daily by two physicians or a physician and a physician's assistant.

Nineteen volunteers who had been vaccinated 4 to 6 weeks earlier with $10^{7.5}$ $TCID_{50}$ of the avian-human influenza A/Korea/82 reassortant virus and 14 unvaccinated controls who agreed to participate in a wild-type challenge study were housed on the isolation ward at the Center for Vaccine Development, University of Maryland Hospital, where they received $10^{6.2}$ $TCID_{50}$ of the wild-type human influenza A/Korea/82 virus intranasally in a 0.5-ml inoculum and were observed daily for 10 consecutive days after inoculation. Fourteen unvaccinated seronegative controls received $10^{4.5}$ $TCID_{50}$ of the wild-type human influenza A/California/78 virus intranasally in a 0.5-ml inoculum and were similarly observed on the isolation wards at the University of Maryland or the University of Rochester.

Volunteers were considered ill if they developed the symptoms or physical findings specified in the footnotes to Table 1 and had evidence of influenza virus infection (either recovery of virus from nasal washes or development of an antibody response or both; see below).

Studies with the A/California/78 viruses were performed between April 1983 and November 1984. Those with the A/Korea/82 viruses were performed between May and November 1984.

Nasal wash specimens for virus isolation were collected daily from each volunteer during the period of clinical observation. The methods of collection and virus isolation from nasal wash specimens have been described previously (9).

Serum and nasal wash specimens were collected before and 4 weeks after virus administration to evaluate systemic and local respiratory tract antibody responses. Nasal wash specimens were pooled and concentrated as previously described (9). Methods for the measurement of serum HAI and NA-inhibiting and enzyme-linked immunosorbent assay (ELISA) antibodies in serum and nasal wash specimens were published earlier (6, 9, 11–13). In studies with viruses of the H3N2 subtype, HAI tests were performed with both the A/Korea/82 wild-type virus and a reassortant virus possessing the A/Philippines/2/82 HA (H3) and equine-1 NA (N7) as antigens. A reassortant virus containing the HA (H7) of influenza equine-1 and the NA (N2) of the influenza A/Bangkok/79 virus was used as antigen in the NA inhibition test. Specimens from volunteers who received H1N1 viruses were tested by HAI assay using both the influenza A/Brazil/11/78 (H1N1) wild-type virus and a reassortant virus possessing the A/USSR/92/77 HA (H1) and equine-1 NA antigens. A reassortant virus containing the HA of influenza equine-1 and the NA of the influenza A/USSR/77 (N1) was used as antigen in NI tests. The antigen used in the ELISA to measure serum and nasal wash antibody response

TABLE 1. Response of seronegative volunteers to A/California/10/78 (H1N1) avian-human reassortant or wild-type human influenza virus[a]

Virus	Dose ($TCID_{50}$)	No. of volunteers	% infected[b]	Virus shedding (nasal wash)			% with indicated illness[c]				Antibody response (%)
				% shedding	Mean duration[d] (days ± SE)	Mean peak Titer[d] (log_{10} $TCID_{50}$/ml ± SE)	Febrile, systemic, or both	Upper respiratory	Lower respiratory	Any illness	
Avian-human reassortant	$10^{4.5}$	12	58	25	0.6 ± 0.5[e]	1.0 ± 0.7[f]	0	0	0	0	58
	$10^{5.5}$	14	64	21	1.0 ± 0.5[e]	0.6 ± 0.1[e]	0	0[g]	0	0[g,h]	64
	$10^{6.5}$	25	92	56	1.2 ± 0.3[e]	1.1 ± 0.2[e]	0	8	0	8[h]	83[i]
	$10^{7.5}$	25	92	72	1.8 ± 0.3[e]	1.7 ± 0.2[j]	12	0[g]	0	12[g,h]	88
	$10^{8.5}$	10	100	80	1.5 ± 0.4[e]	1.8 ± 0.4[h]	20	0	0	20	100
Wild-type	$10^{4.5}$	14	93	79	5.8 ± 0.8	3.3 ± 0.5	36	28	21	43	79

[a] Seronegative (HAI titer, ≤1:8) volunteers received 0.5 ml of virus intranasally. *P* values are relative to values of the control group receiving wild-type virus.
[b] Virus isolation, antibody response, or both signified infection.
[c] Volunteers were considered ill if they developed any of the following syndromes: fever (≥37.8°C); systemic illness (the occurrence of myalgia, chills, and sweats); upper respiratory tract illness (rhinitis, pharyngitis, or both observed on 2 consecutive days); and lower respiratory tract illness (a persistent cough lasting for at least 2 days). Only volunteers with evidence of infection were considered to have influenza-related illness.
[d] Data from infected volunteers were used for calculations. The lowest detectable quantity of virus shedding was $10^{0.75}$ $TCID_{50}$/ml.
[e] $P < 0.001$.
[f] $P < 0.025$.
[g] Two volunteers in each of these groups had evidence of respiratory illness without evidence of infection and are considered not to have influenza-related illness.
[h] $P < 0.05$.
[i] Sera and nasal wash specimens from one volunteer who shed virus were not tested.
[j] $P < 0.01$.

to the H3N2 viruses was a fraction of viral protein containing the HA and NA of the A/Korea/1/82 virus prepared as described elsewhere (4a). The purified HA of the A/California/10/78 virus which was used in the ELISA for the H1N1 vaccinees was prepared by Michael Phalen (Center for Drugs and Biologics, Food and Drug Administration, Bethesda, Md.) as previously described (16).

Data were analyzed for statistical significance by the two-tailed Fisher exact test and two-tailed Student *t* test. Protective efficacy against illness was calculated by the formula: (percentage of controls ill − percentage of vaccinees ill)/(percentage of controls ill).

Efficiency of plaque formation. The efficiency of plaque formation on Madin-Darby canine kidney (MDCK) tissue culture of parental viruses, their reassortant progeny, and isolates of avian-human reassortant virus obtained from infected vaccinees was determined at temperatures permissive for both avian and human influenza viruses (35 to 37°C) and at temperatures restrictive for human but permissive for avian influenza viruses (41 to 42°C) as previously described (5, 9). The avian-human reassortant viruses plaqued efficiently at the higher temperature, as did their avian influenza parent.

RESULTS

Dose response, safety, and immunogenicity. (i) H1N1 studies. The 50% human infectious dose (HID_{50}) of the avian-human A/California/10/78 (H1N1) avian-human reassortant virus was $10^{4.9}$ $TCID_{50}$. At doses of $10^{7.5}$ and $10^{8.5}$ $TCID_{50}$ (400 to 4,000 HID_{50}), the H1N1 reassortant virus produced febrile, systemic illness (maximum temperature 38.9°C) unaccompanied by respiratory tract illness in 5 of 33 (15%) volunteers (Table 1). At doses of $10^{4.5}$ to $10^{6.5}$ $TCID_{50}$ of this reassortant, febrile illness was not observed, but 2 of 39 (5%) infected volunteers developed afebrile upper respiratory illness. Upper respiratory illness was also observed in 4 of 14 (29%) volunteers who received the reassortant virus but did not show any evidence of infection. These uninfected volunteers were not considered to have had an influenza-related illness. Illness was produced by the wild-type influenza A/California/78 virus in 6 of 14 (43%) infected volunteers and was more severe than that seen in vaccinees as indicated by evidence of lower respiratory tract illness in three of the volunteers who received wild-type virus.

Replication of the avian-human A/California/78 reassortant virus in the nasopharynx was significantly restricted compared with that of the wild-type virus. The mean duration of shedding was reduced by at least 65% in each group of vaccinees. The mean peak titer of virus shed by vaccinees was reduced by at least 60-fold. At doses below $10^{7.5}$ $TCID_{50}$ (400 HID_{50}), greater than 100-fold reduction in the mean titer of viral shedding was observed. None of 14 blood or 15 rectal swab specimens from three infected vaccinees yielded virus. Each of 54 viral isolates recovered from the nasal washes of infected volunteers retained the ability to grow efficiently at temperatures restrictive for wild-type virus (41 to 42°C).

Antibody responses were detected in 58 to 100% of vaccinees receiving the avian-human A/California/78 reassortant virus (Table 2). An antibody response was observed in 20 of 24 (83%) vaccinees who received $10^{6.5}$ $TCID_{50}$ (40 HID_{50}) of the avian-human reassortant virus compared with 11 of 14 individuals (79%) who received the wild-type virus. The HAI responses of vaccinees who received a dose of $10^{5.5}$ $TCID_{50}$ (4 HID_{50}) or greater and of controls who received wild-type virus were similar in magnitude and frequency. Differences in magnitude and frequency of nasal wash immunoglobulin A (IgA) ELISA antibody responses between vaccinees in this dose range and wild-type controls were not statistically significant.

(ii) H3N2 studies. The HID_{50} of the avian-human A/Korea/1/82 (H3N2) reassortant virus was $10^{5.4}$ $TCID_{50}$. At doses up to $10^{7.5}$ $TCID_{50}$ (100 HID_{50}), only one of 55 (2%) volunteers infected with the H3N2 reassortant virus developed illness (rhinitis) (Table 3) compared with 7 of 14 (50%) of volunteers who developed illness after receiving the wild-type human influenza A/Korea/82 virus.

The mean peak titer of virus shed by vaccinees who received the A/Korea/82 reassortant virus was reduced

TABLE 2. Immune response of seronegative volunteers to A/California/10/78 (H1N1) avian-human reassortant or wild-type human virus[a]

Virus	No. of volunteers	Dose ($TCID_{50}$)	HAI			NA inhibition			Serum ELISA (IgG)			Nasal wash ELISA (IgA)			% With antibody response
			Pre	Post	% with rise	Pre	Post	% with rise	Pre	Post	% with rise	Pre	Post	% with rise	
Avian-human reassortment	12	$10^{4.5}$	2.9 ± 0.3	4.0 ± 0.6	33	NT	NT		12.1 ± 0.3	12.8 ± 0.4	33	4.6 ± 0.8	6.5 ± 0.5	33	58
	14	$10^{5.5}$	3.6 ± 0.4	5.4 ± 0.3	57	NT	NT		11.9 ± 0.4	13.2 ± 0.4	57	4.3 ± 0.8	6.9 ± 0.6	57	64
	24[b]	$10^{6.5}$	3.2 ± 0.3	5.3 ± 0.3	66	0.4 ± 0.3	1.0 ± 0.4	17	12.8 ± 0.3	13.7 ± 0.3	58	5.2 ± 0.3	7.5 ± 0.4	54	83
	25	$10^{7.5}$	3.0 ± 0.2	5.5 ± 0.3	76	0.5 ± 0.4	1.3 ± 0.4	24	11.8 ± 0.3	13.4 ± 0.4	68	5.2 ± 0.5	7.7 ± 0.4	72	88
	10	$10^{8.5}$	3.0 ± 0.5	5.8 ± 0.6	80	0.2 ± 0.3	1.9 ± 0.6	50	11.4 ± 0.4	13.4 ± 0.4	100	4.8 ± 0.7	8.2 ± 0.9	80	100
Wild-type	14	$10^{4.5}$	2.6 ± 0.4	5.2 ± 0.5	71	0.4 ± 0.5	1.8 ± 0.9	14	12.9 ± 0.3	14.6 ± 0.4	64	4.6 ± 0.9	8.7 ± 0.8	88[c]	79

[a] Antibody titers are expressed as reciprocal mean $\log_2$ titers ± standard errors. All volunteers had HAI antibody titers ≦1:8 on initial screening. The preinfection HAI antibody titers of some volunteers were higher when the paired pre- and postinfection sera subsequently were analyzed due to the biologic variability of the HAI assay. Because the preinfection serum IgG and nasal wash IgA ELISA antibody titers of these volunteers were similar to those of other seronegative volunteers, we did not exclude these volunteers from analysis. The values shown are those of the analysis of paired sera. Pre, Preinfection titer; Post, postinfection titer; NT, not tested.

[b] Serologic specimens from one volunteer who shed virus were not tested.

[c] Specimens from 9 of 14 volunteers in this group were tested for nasal wash IgA ELISA antibody.

10,000-fold compared with that of volunteers who received wild-type virus. The duration of shedding was reduced 69% in vaccinees compared with wild-type controls. All 19 viral isolates tested retained the ability to grow efficiently at temperatures restrictive for wild-type virus (41 to 42°C).

Antibody responses to infection with the avian-human A/Korea/82 reassortant virus were similar to those seen with the avian-human A/California/78 reassortant virus (Table 4). At doses of $10^{6.5}$ and $10^{7.5}$ $TCID_{50}$ (10 and 100 HID_{50}), 93 and 87% of vaccinees developed an antibody response, respectively. The HAI response of vaccinees who received doses in this range was similar in magnitude to that of recipients of wild-type virus, and differences in frequency were not statistically significant. The percentage of vaccinees receiving these doses of virus who developed a significant rise in nasal wash IgA ELISA antibody titer was similar to that of controls receiving wild-type virus. Although the magnitude of the rise was lower in the vaccinees than in controls, the difference was not statistically significant.

Response of vaccinees to challenge with wild-type virus. Nineteen vaccinees, 17 of whom had been previously infected with the avian-human A/Korea/82 reassortant virus, were challenged with $10^{6.2}$ $TCID_{50}$ of the homologous wild-type virus. Ten of the 19 (53%) were infected compared with 14 of 14 (100%) seronegative controls ($P < 0.0025$) (Table 5). The titer of virus shed by vaccinees infected with wild-type virus was significantly reduced compared with unvaccinated controls ($P < 0.025$). Illness was observed in two vaccinees (10%) compared with 50% of controls ($P < 0.025$). The two volunteers who were resistant to infection with the vaccine virus also were not infected with the wild-type virus. The protective efficacy of the vaccine against illness was 79%.

DISCUSSION

Previous studies with a six-gene avian-human reassortant virus derived from the mating of the avian influenza A/Mallard/NY/6750/78 (H2N2) virus with the wild-type human influenza A/Washington/897/80 (H3N2) virus showed that a set of properties desirable for a live influenza A virus vaccine were transferred to the wild-type virus together with the six internal genes of the avian influenza virus parent. These properties include (i) satisfactory attenuation, (ii) reduced replication, (iii) phenotypic stability, (iv) lack of transmissibility, and (v) lack of spread of virus infection beyond the respiratory tract. These findings led us to investigate the avian influenza A/Mallard/NY/78 virus further as a candidate donor strain for use in the production of live influenza A virus vaccines.

The present study shows that the avian influenza A/Mallard/NY/78 virus can reproducibly transfer this set of properties to wild-type human influenza A viruses of the H1N1

TABLE 3. Response of seronegative volunteers to A/Korea/1/82 (H3N2) avian-human reassortant or wild-type human influenza virus[a]

Virus	Dose ($TCID_{50}$)	No. of volunteers	% infected[b]	Virus shedding (nasal wash)			% with indicated illness[c]			% with antibody response
				% shedding	Mean duration[d] (days ± SE)	Mean peak titer[d] ($\log_{10}$ $TCID_{50}$/ml ± SE)	Febrile, systemic or both	Upper or lower respiratory (or both)	Any illness	
Avian-human reassortant	$10^{4.5}$	10	10	0	0 ± 0.0[e]	≦0.5 ± 0.0[e]	0	0	0[f]	10
	$10^{5.5}$	15	67	27	1.1 ± 0.5[g]	1.0 ± 0.3[g]	0	0	0[f]	67
	$10^{6.5}$	14	100	57	1.7 ± 0.5[g]	1.0 ± 0.2[g]	0	0	0[f]	93
	$10^{7.5}$	31	87	32	0.7 ± 0.2[g]	0.8 ± 0.1[g]	0	3	3[g]	87
Wild-type	$10^{6.2}$	14	100	100	5.5 ± 0.5	4.9 ± 0.4	29	50	50	100

[a] Seronegative (HAI titer, ≦1.8) volunteers received 0.5 ml of virus intranasally. *P* values are relative to the control group receiving wild-type virus. Studies were not done concurrently.

[b] Virus isolation, antibody response, or both signified infection.

[c] See footnote *c* in Table 1.

[d] Data from infected volunteers were used for calculations. The lowest detectable quantity of virus shed was $10^{0.75}$ $TCID_{50}$/ml.

[e] $P < 0.025$.

[f] $P < 0.01$.

[g] $P < 0.001$.

TABLE 4. Immune response of seronegative volunteers to A/Korea/82 (H3N2) avian-human reassortant or wild-type human virus[a]

Virus	Dose ($TCID_{50}$)	No. of volunteers	HAI			NI			Serum ELISA (IgG)			Nasal wash ELISA (IgA)			% with antibody response
			Pre	Post	% with rise	Pre	Post	% with rise	Pre	Post	% with rise	Pre	Post	% with rise	
Avian-human reassortant	$10^{4.5}$	10	2.3 ± 0.2	2.3 ± 0.3	10	2.0 ± 0.5	2.2 ± 0.5	0	12.5 ± 0.3	12.5 ± 0.3	10	5.2 ± 0.4	5.5 ± 0.5	10	10
	$10^{5.5}$	15	1.9 ± 0.2	2.7 ± 0.4	33	2.4 ± 0.5	3.5 ± 0.4	27	11.2 ± 0.4	12.0 ± 0.3	40	4.5 ± 0.4	6.0 ± 0.4	40	67
	$10^{6.5}$	14	1.6 ± 0.2	3.5 ± 0.5	57	2.7 ± 0.5	3.9 ± 0.5	43	11.5 ± 0.3	13.3 ± 0.5	64	4.5 ± 0.4	6.7 ± 0.5	64	93
	$10^{7.5}$	31	2.2 ± 0.2	4.3 ± 0.3	68	2.6 ± 0.3	3.9 ± 0.3	42	11.5 ± 0.3	13.8 ± 0.3	77	5.5 ± 0.3	7.7 ± 0.3	55	87
Wild-type	$10^{6.2}$	14	1.9 ± 0.2	3.6 ± 0.2	71	1.9 ± 0.4	4.4 ± 0.5	71	11.3 ± 0.5	14.2 ± 0.3	93	5.9 ± 0.4	9.1 ± 0.4	57	100

[a] Antibody titers are expressed as reciprocal mean $\log_2$ titers ± standard errors. All volunteers had HAI antibody titers ≤1:8 on initial screening. The preinfection HAI antibody titers of some volunteers were higher when the paired pre- and postinfection sera subsequently were analyzed due to the biologic variability of the HAI assay. Because the preinfection serum IgG and nasal wash IgA ELISA antibody titers of these volunteers were similar to those of other seronegative volunteers, we did not exclude these volunteers from analysis. The values shown are those of the analysis of paired sera. Abbreviations are as in Table 1.

and H3N2 serotypes. Transmissibility was not studied. Febrile illnesses were observed in vaccinees infected with doses of 400 to 4,000 HID_{50} of the avian-human influenza A/California/78 reassortant virus. A similar response to viral inocula of this magnitude has been seen with other attenuated viruses (11, 15). In the range of 10 to 100 HID_{50}, both of the avian-human reassortant viruses caused mild illness with low frequency, comparable to that seen with cold-adapted reassortant viruses derived from the influenza A/Ann Arbor/6/60 cold-adapted virus (2, 4). The percentage of vaccinees developing an immune response after inoculation with 10 to 100 HID_{50} of either of the avian-human reassortant viruses was similar to that seen in the control groups inoculated with wild-type virus.

The HID_{50} of the avian-human reassortant viruses were $10^{4.9}$ $TCID_{50}$ for the H1N1 reassortant virus and $10^{5.4}$ $TCID_{50}$ for the H3N2 reassortant virus. These are similar to the HID_{50} for the previously reported A/Mallard/NY/78 × A/Washington/80 (H3N2) avian-human reassortant virus ($10^{5.9}$ $TCID_{50}$) (9) and those of reassortant viruses derived from the A/Ann Arbor/60 cold-adapted virus ($10^{5.5}$ to $10^{6.2}$ $TCID_{50}$) (4, 8).

Vaccination with the avian-human A/Korea/82 reassortant virus provided protection against illness induced by experimental intranasal challenge with wild-type influenza A/Korea/82 virus similar to that provided by inactivated influenza A virus vaccine (3). Protection against infection and viral shedding after challenge was lower than in previous studies with cold-adapted vaccine viruses (3). The relative efficacy of various live influenza virus vaccines will be addressed by simultaneous studies with cold-adapted and avian-human reassortant viruses whose surface antigens are derived from the same wild-type donor virus.

Because avian influenza viruses infect the gastrointestinal tract of birds in nature (17), it is possible that avian-human influenza viruses could spread beyond the human respiratory tract. In the current study we were unable to detect evidence of spread of viral infection beyond the respiratory tract of the three volunteers studied. Thus far, a total of 44 susceptible adult volunteers who have received avian-human reassortant viruses have been studied intensively for evidence of extra-respiratory tract spread of infection; evidence of viral replication outside the respiratory tract has not been found (9).

Studies of reassortant viruses derived from the mating of the influenza A/Udorn/72 × A/Mallard/NY/78 viruses have shown that more than one RNA segment of the influenza A/Mallard/NY78 virus is capable of transferring attenuation to wild-type human virus (18). Reassortant viruses which possessed only one or two RNA segments derived from the avian influenza virus parent and all other RNA segments derived from the human virus parent have been evaluated for their level of replication in squirrel monkeys. The RNA segments of avian influenza virus coding for the matrix proteins and the nucleoprotein can each specify the phenotype of restricted replication in squirrel monkeys. Additionally, a combination of RNA segments one (coding for a polymerase protein) and eight (coding for the nonstructural proteins) are capable of causing restriction of replication, although these RNA segments do not produce this effect singly. Furthermore, comparison of the nucleotide sequences of the genes encoding matrix proteins and

TABLE 5. Response to wild-type influenza A/Korea/1/82 (H3N2) challenge of volunteers previousy immunized with avian-human reassortant virus vaccine and seronegative controls[a]

Group	No. of volunteers	% infected[b]	Virus shedding (nasal wash)			% with indicated illness[c]		
			% shedding	Mean duration[d] (days ± SE)	Mean peak titer[d] ($\log_{10}$ $TCID_{50}$/ml ± SE)	Febrile, systemic or both	Upper or lower respiratory (or both)	Any illness
Vaccinees	19	53[e]	53	4.3 ± 0.5	3.0 ± 0.6[f]	5	10[f]	10[f]
Controls	14	100	100	5.5 ± 0.5	4.8 ± 0.4	29	50	50

[a] Volunteers received 0.5 ml of wild-type virus ($10^{6.2}$ $TCID_{50}$) intranasally. Studies were not done concurrently. *P* values are relative to the unvaccinated control group.

[b] Virus isolation, antibody response, or both signified infection.

[c] See footnote *c* to Table 1.

[d] Data from infected volunteers were used for calculations. The lowest detectable quantity of virus shed was $10^{0.75}$ $TCID_{50}$/ml.

[e] $P < 0.0025$.

[f] $P < 0.025$.

nucleoprotein of the avian A/Mallard/NY/78 virus with those of the corresponding genes of human influenza A viruses revealed significant sequence divergence at both the nucleotide and amino acid levels (1). This suggests that true reversion or suppression of all mutations responsible for attenuation of avian-human influenza virus reassortants for humans is unlikely to occur during restricted replication of virus in vaccines.

Avian-human reassortant viruses are promising candidates as live virus influenza A vaccines. Their growth to high titer in the allantoic cavity of embryonated eggs combined with the relatively small quantity of virus needed to infect vaccinees would provide the economic advantage of multiple vaccine doses per egg. It is known that avian influenza viruses exhibit a spectrum of virulence for primates, and hence it should be possible to select an avian influenza donor virus whose internal genes code for a greater or a lesser degree of attenuation than the A/Mallard/NY/78 donor strain. Additional studies are planned to assess the safety and immunogenicity of reassortant viruses derived from the avian influenza A/Mallard/NY/78 virus in young children who have not experienced previous influenza A virus infection, to compare directly the immunogenicity and protective efficacy of avian-human and cold-adapted reassortant viruses which have the same wild-type human influenza virus parent, and to investigate other avian influenza viruses as donors of attenuating genes.

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