

Comparison of the Virologic and Immunologic Responses of Volunteers to Live Avian-Human Influenza A H3N2 Reassortant Virus Vaccines Derived from Two Different Avian Influenza Virus Donors

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We compared the abilities of the six internal RNA segments of two avian influenza viruses, A/Mallard/Alberta/88/76 (H3N8) and A/Mallard/NY/6750/78 (H2N2), to confer attenuation on wild-type human influenza A/Bethesda/1/85 (H3N2) virus in seronegative adult volunteers. Live avian-human influenza A reassortant virus vaccines derived from either avian virus parent were comparable in the following properties: safety, infectivity, immunogenicity, and genetic stability. Since the avian influenza A/Mallard/Alberta/76 virus offered no clear advantage as a donor virus, we will conduct our future evaluations on live influenza A virus reassortants derived from the more extensively characterized avian influenza A/Mallard/NY/78 virus.

Avian influenza A viruses differ in their virulence and their ability to replicate in the lower respiratory tracts of squirrel monkeys (3, 7). Previously, we evaluated the ability of three different avian influenza A viruses to serve as donors of attenuating genes to the wild-type human influenza A/Korea/1/82 (H3N2) virus (10). We found a disassociation between the degree of virulence and the restriction of replication of the three avian influenza A viruses in squirrel monkeys and of their avian-human influenza A reassortant viruses in susceptible adult volunteers (10). Avian-human reassortant viruses bearing the six internal RNA segments from the avian influenza A/Mallard/NY/6750/78 (H2N2), A/Pintail/Alberta/119/79 (H4N6), and A/Mallard/Alberta/88/76 (H3N8) viruses (which replicated to low, intermediate, or high levels, respectively, in the lower respiratory tracts of squirrel monkeys [7]) and the H3N2 surface glycoproteins of the virulent human influenza A/Korea/82 (H3N2) virus were similarly attenuated in adult volunteers. Compared with the other two avian-human influenza A reassortants, however, the avian-human influenza reassortant A/Pintail/Alberta/119/79 × A/Korea/82 appeared to be somewhat overattenuated for adult volunteers, as indicated by decreased immunogenicity at the single dose tested ($10^{7.0}$ to $10^{7.5}$ 50% tissue culture infective dose [TCID₅₀]).

In the present study, we sought to further compare the avian influenza A/Mallard/Alberta/76 and A/Mallard/NY/78 viruses to determine whether either virus has an identifiable advantage as a donor virus. Since the infectivity of the reassortants derived from these two donor viruses had not been compared previously, we evaluated both avian-human reassortant viruses containing the H3N2 surface glycoproteins of the wild-type human influenza A/Bethesda/85 virus and the six internal genes of the avian influenza A/Mallard/Alberta/76 or A/Mallard/NY/78 virus for safety and immu-

nogenicity at doses of $10^{4.5}$ to $10^{7.5}$ TCID₅₀ in adult volunteers.

The wild-type human influenza A/Bethesda/1/85 (H3N2) virus and the avian-human influenza reassortant A/Mallard/NY/78 × A/Bethesda/85 virus were previously evaluated in seronegative volunteers (S. D. Sears, M. L. Clements, R. F. Betts, H. F. Maassab, B. R. Murphy, and M. H. Snyder, J. Infect. Dis., in press); these data are included herein for the purpose of comparison. The isolation and biological cloning of the avian influenza A/Mallard/Alberta/88/76 (H3N8) virus have been described previously (3, 7). The production and characterization of the avian-human influenza A/Bethesda/1/85 × A/Mallard/Alberta/88/76 reassortant virus were similar to those of the avian-human influenza A/Bethesda/1/85 (H3N2) × A/Mallard/NY/6750/78 (H2N2) reassortant virus which were reported elsewhere (Sears et al., in press). The parental origin of the genes in the avian-human influenza A/Bethesda/1/85 × A/Mallard/Alberta/88/76 reassortant virus was determined by comparing the migration of the eight virion RNA segments of the reassortant with that of the two parental viruses by polyacrylamide gel electrophoresis as described previously (3). Electrophoresis was carried out for 16 h at 90 V and 0.5°C on 16-cm gels containing 2.6% polyacrylamide and 7 M urea. The results revealed that the reassortant virus contained the HA and NA genes of the human influenza A virus parent and the internal genes of the avian influenza A parent virus. The avian-human influenza A reassortant virus, like its avian influenza A/Mallard/Alberta/76 parent virus, produced plaques efficiently at 42°C, a temperature restrictive for the wild-type human influenza A/Bethesda/85 virus. The avian-human influenza A/Bethesda/1/85 × A/Mallard/Alberta/88/76 reassortant virus suspension (Clone 1-1, Lot E248) administered to humans was grown in the allantoic cavity of specific-pathogen-free eggs (SPAFAS, Inc., Norwich, Conn.) by Louis Potash (Flow Laboratories, Inc., McLean, Va.) and had a titer of $10^{7.6}$ TCID₅₀ per ml.

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TABLE 1. Antibody response of seronegative volunteers to influenza A/Bethesda/1/85 (H3N2) avian-human reassortants or wild-type human influenza A virus^a

Influenza virus	Virus dose (TCID ₅₀)	No. of volunteers (% with antibody rise)	Serum HAI reciprocal antibody titer ^b (mean log ₂ [SE])		% of volunteers with rise	Serum KELISA IgG rate (geometric mean [RSE] ^c)		% of volunteers with rise	Nasal-wash KELISA IgA rate (geometric mean [RSE])		% of volunteers with rise	% of volunteers with any illness ^d
			Preinoculation	Postinoculation		Preinoculation	Postinoculation		Preinoculation	Postinoculation		
A/Bethesda/85 × A/Mallard/Alberta/76 reassortant	10 ^{7.5}	15 (87)	2.0 (0.3)	4.2 (0.3)	80	56.6 (1.2)	102.9 (1.1)	53	18.2 (1.3)	32.8 (1.4)	40	7
	10 ^{6.5}	14 (57)	1.5 (0.2)	3.6 (0.4)	50	49.8 (1.2)	81.3 (1.1)	36	18.6 (1.2)	43.3 (1.3)	50	0
	10 ^{5.5}	12 (33)	2.1 (0.2)	2.8 (0.3)	17	53.6 (1.1)	67.5 (0.5)	25	23.2 (1.3)	25.4 (1.4)	8	0
	10 ^{4.5}	12 (33)	2.0 (0.2)	2.4 (0.3)	8	46.8 (1.2)	57.1 (1.3)	8	15.6 (1.3)	23.1 (1.3)	25	0
A/Bethesda/85 × A/Mallard/NY/78 reassortant	10 ^{7.5}	22 (73)	2.4 (0.2)	3.9 (0.3)	45	48.0 (1.2)	93.2 (1.1)	50	37.3 (1.2)	85.9 (1.2)	29 ^e	0
	10 ^{6.5}	11 (55)	1.6 (0.3)	3.2 (0.4)	45	32.6 (1.2)	70.5 (1.2)	45	27.4 (1.2)	90.7 (1.6)	45	0
	10 ^{5.5}	13 (8)	1.8 (0.2)	2.3 (0.2)	8	56.1 (1.2)	64.4 (1.2)	0	55.5 (1.3)	55.0 (1.2)	0	0
	10 ^{4.5}	12 (25)	1.8 (0.3)	1.9 (0.3)	0	42.0 (1.2)	46.9 (1.2)	17	62.2 (1.2)	72.6 (1.3)	18 ^e	0
A/Bethesda/85 wild-type	10 ^{7.0}	10 (100)	1.7 (0.3)	3.9 (0.5)	60	29.2 (1.4)	100.0 (1.4)	80	75.0 (1.3)	178.7 (1.4)	50	30

^a Antibody values shown are those for paired serum or nasal-wash specimens.

^b All volunteers had HAI antibody titers less than or equal to 1:8 in serum on initial screening.

^c RSE, Relative standard error.

^d Only ill volunteers with evidence of infection were included. Among the unvaccinated recipients of wild-type virus, 30% had fever, systemic illness, or both, and 10% had upper or lower respiratory tract illness.

^e Nasal-wash specimens from one volunteer were not available.

Virus suspensions were tested for the presence of adventitious agents by Louis Potash; none were found.

Study protocols were approved by the Clinical Research Subpanel of the National Institute of Allergy and Infectious Diseases and the Joint Committee on Clinical Investigation of The Johns Hopkins Medical Institutions. Healthy adults, 18 to 40 years of age, who had a hemagglutination inhibition (HAI) antibody titer in serum of $\leq 1:8$ for influenza A/Bethesda/85 hemagglutinin (HA) were recruited from Towson State University and from the Baltimore community. These volunteers are referred to as seronegative because of their low or absent HAI antibody titer, although they most likely had been infected previously with influenza A virus of the H3N2 subtype. Persons who had a history of influenza vaccination or who were taking medication were not eligible. Those who participated in these studies gave written, informed consent.

The clinical procedures have been detailed previously (1, 6, 9; Sears et al., in press). Seronegative volunteers were randomly assigned to receive 10^{4.5}, 10^{5.5}, 10^{6.5}, or 10^{7.5} TCID₅₀ of one of the reassortant viruses intranasally as outpatients to evaluate their immune responses to the reassortant. All volunteers were examined each day separately by two physicians for up to 9 days after inoculation, and their oral temperatures and pulses were recorded four times a day. Volunteers were considered ill if they developed any of the following signs or symptoms within 5 days after inoculation: fever (>37.8°C), systemic illness (the occurrence of myalgia or chills and sweats), upper respiratory tract illness (rhinitis, pharyngitis, or both) observed on 2 consecutive days, and lower respiratory tract illness (persistent cough lasting for at least 2 days). An illness was attributed to influenza A virus when confirmed by laboratory evidence of influenza A infection, i.e., virus shedding, development of a significant rise in specific antibody, or both.

Nasal-wash specimens were collected for up to 10 days after virus inoculation from each volunteer who received the avian-human influenza A/Mallard/Alberta/76 reassortant virus, from 18 of 22 volunteers inoculated with 10^{7.5} TCID₅₀ of

the avian-human influenza A/Mallard/NY/78 reassortant virus, and from each volunteer inoculated with wild-type virus. These specimens were used for isolation and quantitation of virus. Serum and nasal-wash specimens were collected before virus inoculation and 3 to 4 weeks after administration of the virus; these specimens were used to evaluate systemic and local respiratory tract antibody responses. The methods for virus isolation and the HAI antibody assay have been described previously (2, 5, 7). Influenza A/Mississippi/1/85 (H3N2) virus, which is serologically closely related to the influenza A/Bethesda/85 virus, was used as antigen in the HAI test because of its greater sensitivity in detecting serum antibody than the influenza A/Bethesda/85 virus.

In addition, HA-specific immunoglobulin antibody in serum and HA-specific immunoglobulin A (IgA) antibody in nasal-wash specimens were measured concurrently by a kinetic-based enzyme-linked immunosorbent assay (KELISA) described elsewhere (8). Purified HA from the influenza A/Mississippi/85 virus (provided by Michael Phelan, Center for Drugs and Biologics, Food and Drug Administration, Bethesda, Md.) was used as the antigen in the KELISA. Briefly, the reagents consisted of HA, which was absorbed to the plate in carbonate buffer followed by single dilutions of serum (1:4,000 and 1:16,000) or nasal-wash specimen (1:64), immunoglobulin class-specific rabbit anti-human IgG (for serum) or IgA (for nasal wash) antibodies, goat anti-rabbit IgG antibody conjugated with alkaline phosphatase, and substrate. After the addition of substrate, each well of the 96-well plate was read by a Vmax Kinetic microplate reader (Molecular Devices, Palo Alto, Calif.). The rate of color development (milli-optical density units per minute) was calculated as the slope of the regression line defined by the optical density readings for that well. A 1.8-fold change in KELISA rates between preinoculation and postinoculation serum specimens and a 2.9-fold change in KELISA rates between preinoculation and postinoculation nasal-wash specimens were considered significant antibody responses, as described elsewhere (8).

Dose-response studies revealed that the 50% human infec-

TABLE 2. Recovery of virus from seronegative adult volunteers administered avian-human influenza A/Bethesda/85 (H3N2) reassortant or wild-type human virus^a

Influenza virus	Dose (TCID ₅₀)	No. of volunteers (% infected) ^b	Virus shedding (nasal wash)		
			% Shedding	Mean duration ^c (days [SE])	Mean peak titer ^c (log ₁₀ TCID ₅₀ /ml [SE])
A/Bethesda/85 × A/Mallard/Alberta/76 reassortant	10 ^{7.5}	15 (93 ^d)	20	0.3 (0.2)	0.7 (0.1)
	10 ^{6.5}	14 (57)	21	0.6 (0.4)	0.8 (0.2)
	10 ^{5.5}	12 (33)	0	0	≤0.5
	10 ^{4.5}	12 (33)	0	0	≤0.5
A/Bethesda/85 × A/Mallard/NY/78 reassortant	10 ^{7.5}	18 (78 ^e)	17	0.7 (0.4)	0.6 (0.04)
A/Bethesda/85 wild-type	10 ^{7.0}	10 (100)	100	5.9 (0.4)	4.2 (0.5)

^a Volunteers received 0.5 ml of virus intranasally. Studies were not done concurrently.

^b Virus isolation, antibody response, or both signified infection.

^c Data from infected volunteers were used for calculations. The lowest detectable quantity of virus shed was 10^{0.75} TCID₅₀. Culture-negative samples were assigned a value of 0.50 TCID₅₀ for calculations.

^d Of the 14 infected volunteers, 1 shed virus but did not mount an antibody response.

^e Nasal washes were collected for virus isolation from 18 of 22 volunteers; 14 of the 18 volunteers were infected with the reassortant virus.

tious doses of the avian-human influenza A/Bethesda/85 × A/Mallard/Alberta/76 reassortant virus and the avian-human influenza A/Bethesda/85 × A/Mallard/NY/78 reassortant virus were 10^{5.8} and 10^{6.3} TCID₅₀, respectively (Table 1). These differences were not statistically significant ($P = 0.22$, Mantel Haenszel chi-square test). Overall, 14 (93%) of the 15 recipients of the avian-human influenza A/Mallard/Alberta/76 reassortant virus were infected after receiving 80 times the 50% human infectious dose, compared with 16 (73%) of the 22 vaccinees who were infected after receiving 30 times the 50% human infectious dose of avian-human influenza A/Mallard/NY/78 reassortant virus (Table 2).

After challenge with the wild-type human influenza A virus, 3 (30%) of the 10 unvaccinated control volunteers developed influenzalike illness. In contrast, both of the avian-human influenza A reassortant viruses were relatively nonreactogenic; illness occurred in only one vaccinee. This person, who received 10^{7.5} TCID₅₀ of the avian-human influenza A/Mallard/Alberta/76 reassortant virus, had fever, myalgia, chills, nausea, and abdominal cramps that began 7 h after vaccination and lasted only 1 day. The ill vaccinee did not shed virus but did mount serum and nasal-wash HA-specific antibody responses after vaccination. Volunteers infected with the reassortant viruses shed significantly less virus over a shorter interval than did individuals infected with wild-type human influenza A virus (Table 2). Importantly, the amounts of virus shed by each group of vaccinees were comparable. Each of three nasal-wash isolates of the influenza A/Mallard/Alberta/76 reassortant virus tested retained the ability to replicate efficiently at a temperature (42°C) restrictive for wild-type human influenza A viruses.

The avian-human influenza A/Bethesda/85 × A/Mallard/Alberta/76 and A/Bethesda/85 × A/Mallard/NY/78 reassortant viruses were comparable in terms of their infectivity, level of attenuation, level of virus replication, and immunogenicity. The levels of attenuation and replication of the two avian-human influenza A/Bethesda/85 reassortant viruses in adult volunteers were similar to those of the avian-human influenza A/Korea/82 (H3N2) reassortant viruses derived from the same avian influenza A viruses (10).

A definite gradient of attenuation of vaccine candidates derived from the same wild-type virus and different donors of attenuating genes can be demonstrated in studies with a small number of volunteers, as has been shown previously in studies with the temperature-sensitive *ts-1* (A) and *ts-1* (E)

reassortant viruses (4). Our finding that the levels of virus replication, infectivity, and immunogenicity of the two avian-human influenza A/Bethesda/85 reassortant viruses and of previously studied avian-human influenza A/Korea/82 reassortant viruses (10) were not statistically different suggests that the internal genes of these two avian viruses reproducibly confer a similar restriction of replication on wild-type human viruses for adults. In another study, we demonstrated protective efficacy of avian-human influenza reassortant viruses (derived from A/Mallard/NY/78 and A/Bethesda/85 or A/Texas/85) for adults against experimental challenge with wild-type human influenza A/Bethesda/85 (H3N2) and A/Texas/85 (H1N1) viruses that was comparable with that of live, attenuated cold-adapted influenza A reassortant viruses derived from the same parental wild-type human viruses or inactivated influenza vaccine (Sears et al., in press).

In summary, the present study has demonstrated that each avian donor virus confers a similar set of properties on wild-type human influenza A virus. Considered in the context of previous studies (9; Sears et al., in press), our results suggest that the avian influenza A/Mallard/Alberta/76 virus offers no clear advantage over the avian influenza A/Mallard/NY/78 virus as a donor of attenuating genes for construction of live influenza virus vaccines for use in humans. For this reason, we have decided to restrict future evaluation of live attenuated influenza A vaccine candidates in children and high-risk populations to reassortants derived from the well-characterized avian influenza A/Mallard/NY/78 virus.

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