Evaluation of Avian-Human Reassortant Influenza A/Washington/897/80 × A/Pintail/119/79 Virus in Monkeys and Adult Volunteers

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A reassortant influenza A virus was produced by mating an avian influenza A/Pintail/Alberta/119/79 (H4N6) virus with wild-type human influenza A/Washington/897/80 (H3N2) virus. The avian-human influenza A reassortant virus contained the genes coding for the hemagglutinin and neuraminidase surface antigens of the human influenza wild-type virus and the six other RNA segments (internal genes) of the avian influenza A virus donor. In the lower respiratory tract of squirrel monkeys, this avian-human influenza reassortant virus, like its avian influenza A parent virus, was restricted approximately 100-fold in replication compared with the wild-type human influenza A virus. Despite this restriction of replication, infection of monkeys with the avian-human influenza A reassortant virus induced resistance to wild-type human influenza A virus challenge. In comparison with the wild-type human influenza A virus, the avian-human influenza A reassortant was also fully attenuated when 106.8 to 107.4 50% tissue culture infective doses were administered to susceptible adult volunteers. Attenuation was indicated by a more than 300-fold reduction in virus shedding and lack of reactogenicity. The reassortant virus did not spread to susceptible contacts and could not be isolated from the blood or stools of infected adults. The 50% human infectious dose was 109.0 to 109.5 50% tissue culture infective dose, indicating that this reassortant virus is only slightly less infectious for adults than a similarly derived avian-human influenza A/Washington/80 × A/Mallard/78 reassortant virus. These findings suggest that the avian influenza A/Pintail/79 virus may be a satisfactory donor of attenuating genes for production of live, attenuated avian-human influenza A reassortant virus vaccines.

A novel approach for the development of live influenza A virus vaccines involves the use of an avian influenza A virus as the donor of genes for attenuation of wild-type human influenza A viruses (10). The avian-human influenza A reassortant virus produced by this method contains the hemagglutinin (HA) and neuraminidase (NA) genes of a human influenza A virus and the other six RNA segments (i.e., internal genes) of an avian influenza A virus. One advantage of this approach is that avian influenza A viruses have evolved over a long period in birds and, thus, contain nucleotide sequences that are significantly different from those of the corresponding genes of human influenza A viruses (2, 13). Because of this genetic divergence, avian influenza A viruses would be expected to replicate poorly in respiratory epithelial cells of humans and to retain their attenuation phenoyte following such a restricted infection. Results of previous studies have demonstrated that the avian influenza A/Mallard/New York/6750/78 (H2N2) virus can be used as a donor of its six internal genes to attenuate virulent influenza A viruses for monkeys and humans (4, 6, 10, 14). The avian-human influenza A/Mallard/78 × A/Korea/1/82 (H3N2) reassortant virus induced protective immunity in adult volunteers against challenge with the wild-type human influenza A/Korea/1/82 (H3N2) virus (14).

Several other avian influenza A viruses that exhibit restriction of replication in monkeys were identified in our previous studies (7). One of these strains, the influenza A/Pintail/Alberta/119/79 (H4N6) virus, replicated slightly more efficiently in the lower respiratory tract of squirrel monkeys than did the A/Mallard/78 strain (7). This finding suggests that avian-human influenza A virus reassortants derived from the influenza A/Pintail/79 donor virus might be more infectious for humans than reassortants derived from the A/Mallard/78 strain. To answer this question, we produced and characterized a reassortant virus containing the HA and NA genes of the wild-type human A/Washington/897/80 (H3N2) virus and the six internal genes from the A/Pintail/Alberta/119/79 virus. This reassortant was evaluated for its safety, infectivity, transmissibility, and immunogenicity in monkeys and seronegative adult volunteers. The capacity of this reassortant virus to induce resistance against experimental challenge with wild-type human influenza A virus in monkeys was also assessed.

MATERIALS AND METHODS

Viruses. The isolation and cloning of the avian influenza A/Pintail/Alberta/119/79 (H4N6) virus and wild-type human influenza A/Washington/897/80 (H3N2) virus have been described previously (4, 7). The isolation and characterization of the avian-human influenza A/Washington/897/80 × A/Mallard/6750/78 reassortant virus used in the current studies on monkeys were also described previously (4). The wild-type human influenza A/Washington/897/80 virus was previously evaluated in seronegative volunteers (3); the data are in-

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cluded herein for the purpose of comparison. The production and characterization of the avian-human influenza A/Washington/897/80 × A/Pintail/Alberta/115/79 reassortant virus were similar to those of the avian-human influenza A/Washington/897/80 × A/Mallard/New York/6750/78 reassortant virus which were reported elsewhere (4). The avian-human influenza A/Washington/897/80 × A/Pintail/Alberta/115/79 reassortant virus suspension (Lot E 201) and reassortant virus were grown in the allantoic cavity of specific-pathogen-free eggs (SPAFAST, Inc., Norwich, Conn.) by Louis Potash (Flow Laboratories, Inc., McLean, Va.) and had a titer of 10^6.5 50% tissue culture infectious doses (TCID_{50}) per ml. Virus suspensions were tested for the presence of adventitious agents by Louis Potash; none were found.

**Polyacrylamide gel electrophoresis.** The parental origin of the genes in the avian-human influenza A/Washington/80 × A/Pintail/79 reassortant virus was determined by comparing the migration of the eight virion RNA segments of the reassortant with those of the two parental viruses by polyacrylamide gel electrophoresis, as described previously for the avian-human influenza A/Washington/80 × A/Mallard/78 reassortant virus (4). Electrophoresis was carried out for 16 to 20 h at 200 V and 5°C on 32-cm gels containing 2.6% polyacrylamide and either 6.0 or 4.5 M urea.

**Replication of virus in monkeys.** The studies in monkeys were performed as described previously (7, 9). Briefly, a 10^7.0 TCID_{50} of virus in a 0.5-ml inoculum was administered intratracheally to squirrel monkeys. Nasopharyngeal (NP) and rectal swab specimens were collected daily for 10 days. Tracheal lavages were performed on days 2, 4, and 6 after inoculation. The quantity of virus present in each NP swab and tracheal lavage specimen was determined by titration in Madin Darby canine kidney cells, and mean log_{10} titer were calculated. Serum was collected before and 28 days after virus administration and assayed for hemagglutination-inhibiting (HAI) antibody, using homologous virus as antigen.

Pairs of infected and uninoculated control monkeys were housed together in cages to determine if transmission of reassortant virus occurred. Evidence of infection was sought by sampling contact monkeys for evidence of virus replication and the development of HAI antibody in serum.

To determine if the avian-human influenza A reassortant virus initiated a systemic infection, six monkeys (three males and three females) were sacrificed, two each on days 2, 4, and 8. The following tissues were removed and examined grossly and histologically for pathological changes and for the presence of infectious virus in a 10% (wt/vol) suspension: nasal mucosa, trachea, lung, esophagus, small intestine, colon, kidney, bladder, spleen, liver, thymus, inguinal muscle, myocardium, pancreas, testis, ovary, cerebellum, brain stem, ventercle with ependyma, spinal cord, meninges, blood, bone marrow, skin, and uterus.

**Clinical studies in volunteers.** Study protocols were approved by the Clinical Research Subpanel of the National Institute of Allergy and Infectious Diseases and the Human Volunteer Research Committee at the University of Maryland. Healthy adults (18 to 35 years of age) who had a HAI antibody titer in serum of ≤ 1:8 for A/Washington/80 H3 HA were recruited from the University of Maryland and from the Baltimore community. 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 (6). Briefly, initial clinical studies of the avian-human influenza A reassortant virus 10^6.5 TCID_{50} (four volunteers) and 10^7.5 TCID_{50} (19 volunteers) were conducted on the isolation ward of the Center for Vaccine Development. Either virus (10^7.5 TCID_{50}) or placebo (allantoic fluid) was administered intranasally (0.25 ml per nostril) to 19 volunteers in a double-blind manner to evaluate the safety and transmissibility of the avian-human influenza A reassortant virus; four placebo recipients served as control contact controls. Volunteers were isolated for 3 days before and 7 to 9 days after virus inoculation. After studies performed on the isolation ward indicated that the avian-human influenza A reassortant virus was attenuated and nontransmissible, 29 additional seronegative volunteers were randomly assigned to receive 10^6.5, 10^6.5, or 10^7.5 TCID_{50} of virus intranasally as outpatients to more fully evaluate the immune response of susceptible adults to the reassortant. All volunteers were examined each day separately by two physicians for 4 to 9 days after inoculation, and their oral temperature and pulse were recorded four times a day.

Volunteers were considered ill if they developed any of the following 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; lower respiratory tract illness as a 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, or both of a significant rise in antibody titer.

**Laboratory studies.** Nasal wash specimens were collected daily for virus isolation and quantitation. In addition, blood and rectal swab specimens were obtained daily for 5 days after virus administration from each of the volunteers housed on the isolation ward to determine if viremia or virus replication in the intestinal tract occurred. Serum and nasal wash specimens were collected before and 3 to 4 weeks after administration of the virus to evaluate systemic and local respiratory tract antibody responses. The methods for collection of nasal-wash specimens, culture and quantitation of virus, determination of the efficiency of plaque formation at permissive and restrictive temperatures, and measurement of HAI in serum, NA-inhibiting, and HA immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) antibodies in serum, and HA IgA ELISA antibodies in nasal wash have been described previously (1, 3, 5, 8, 11, 12). A reassortant virus possessing the influenza A/Texas/77 HA (H3) and equine-1 NA (N7) was used as antigen in the HAI test because this antigen was more efficient for detection of antibody responses in serum than a similar reassortant containing the influenza A/Washington/80-like HA (H3) of the influenza A/Bangkok/79 virus. A reassortant containing the HA (H7) of the influenza equine-1 and the NA (N2) of the influenza A/Bangkok/79 virus was used as antigen in the NA-inhibiting test. Purified HA (H3) of the X-73 influenza (A/Bangkok/7/79) virus was used in the ELISA (8).

### RESULTS

Characterization of the avian-human influenza A/Washington/80 × A/Pintail/79 reassortant virus. Polyacrylamide gel electrophoresis of virion RNAs of the human and avian influenza A virus parents and the reassortant revealed that the reassortant contained the HA and NA genes of the human influenza A parent virus and the internal genes of the avian influenza A parent virus. The avian influenza A/Pin-
A/Pintail/79 virus produced plaques efficiently at 42°C, a temperature restrictive for the wild-type human influenza A/Washington/80 virus. The avian-human influenza A reassortant virus, like its avian influenza A virus parent, produced plaques efficiently at 42°C. This suggests that one or more of the internal genes of this avian virus strain is responsible for growth at 42°C.

Evaluation of the avian-human influenza A reassortant virus in squirrel monkeys. The level of replication of the avian-human influenza A/Washington/80 × A/Pintail/79 reassortant virus in the nasopharynx and trachea was compared with that of its parental viruses (Table 1). In addition, the level of replication of the avian-human influenza A/Washington/80 × A/Pintail/79 reassortant virus was compared with that of the avian-human influenza A/Washington/80 × A/Mallard/78 reassortant virus to assess the degree of growth restriction specified by the two sets of internal avian influenza virus genes. The acquisition of either set of internal genes by the wild-type human influenza A/Washington/80 virus resulted in a similar level of restriction of replication in squirrel monkeys. In this experiment, the level of replication of the two avian influenza A viruses was similar, as was that of their reassortant progeny. As previously observed (4), the reassortant viruses were even more restricted in replication in the upper respiratory tract than were their avian influenza A parental viruses; the reason for this finding is not clear. Despite their restricted replication, the avian-human influenza A reassortant viruses induced HAI antibodies in serum.

**Lack of transmissibility and systemic spread of reassortant virus.** Virus did not spread from four monkeys infected with avian-human influenza A/Washington/80 × A/Pintail/79 reassortant virus to four seronegative monkeys sharing the same cages. Evidence for the lack of transmission was based on the finding that control monkeys did not shed virus or develop an HAI antibody response.

Because avian influenza A viruses can cause enteric and systemic infections in their natural hosts (7), a study was conducted to determine whether extrarespiratory spread of the reassortant virus occurred in infected primates. Six monkeys were sacrificed, and different tissues were harvested to determine if reassortant virus could be recovered. Virus was recovered only from the respiratory tract of the infected monkeys, and pathological changes were not observed.

**Resistance induced by the avian-human influenza A reassortant virus in squirrel monkeys.** Resistance conferred by infection with the avian-human influenza A/Washington/80 × A/Pintail/79 reassortant virus was assessed by challenge

### Table 1. Evaluation of the replication of avian-human influenza A reassortant viruses in squirrel monkeys

<table>
<thead>
<tr>
<th>Virus administered</th>
<th>No. of monkeys</th>
<th>Nasopharynx</th>
<th>Trachea</th>
<th>Serum HAI antibody response (reciprocal mean log2 ± SE) on day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Pintail/79</td>
<td>4</td>
<td>3.5 ± 1.2</td>
<td>2.4 ± 0.2</td>
<td>3.0 ± 1.3</td>
</tr>
<tr>
<td>A/Washington/80 × A/Pintail/79</td>
<td>4</td>
<td>1.0 ± 0.6</td>
<td>0.9 ± 0.4</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>A/Mallard/78</td>
<td>4</td>
<td>2.5 ± 0.3</td>
<td>2.0 ± 0.5</td>
<td>3.0 ± 0.6</td>
</tr>
<tr>
<td>A/Washington/80 × A/Mallard/78</td>
<td>4</td>
<td>1.2 ± 0.5</td>
<td>0.8 ± 0.2</td>
<td>2.5 ± 1.0</td>
</tr>
<tr>
<td>A/Washington/80</td>
<td>4</td>
<td>6.5 ± 0.3</td>
<td>4.4 ± 0.3</td>
<td>5.5 ± 0.5</td>
</tr>
</tbody>
</table>

* Each monkey received 10⁴ TCID₅₀ of virus intratracheally in a 0.5-ml inoculum, and infection was indicated by a fourfold or greater rise in antibody. The studies were done concurrently.
* Each monkey was tested daily for 10 days, as described in the text.
* The amount of virus in the NP swab specimens or tracheal lavage from each monkey was determined by titration in Madin Darby canine kidney cell tissue culture, and the average maximum amount of virus shed by each monkey was determined.
* Each monkey was tested on days 2, 4, and 6.
* The preincubation vires were ±1.0 for each monkey. Each group tested against homologous virus by HAI assay.

### Table 2. Resistance of monkeys previously infected with the A/Washington/80 × A/Pintail/119/79 reassortant virus to challenge with A/Washington/80 (H3N2) wild-type virus

<table>
<thead>
<tr>
<th>Previously administered</th>
<th>No. of monkeys</th>
<th>Nasopharynx</th>
<th>Trachea</th>
<th>H3 HAI antibody titer (reciprocal mean log2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Washington/80 × A/Pintail/79</td>
<td>6 (50)</td>
<td>0.0 ± 0.5</td>
<td>0.0 ± 0.5</td>
<td>4.7 ± 0.0</td>
</tr>
<tr>
<td>A/Washington/80</td>
<td>2 (50)</td>
<td>0.0 ± 0.5</td>
<td>0.0 ± 0.5</td>
<td>4.0 ± 0.0</td>
</tr>
<tr>
<td>A/Pintail/79</td>
<td>2 (100)</td>
<td>6.5 ± 0.5</td>
<td>4.0 ± 1.3</td>
<td>5.0 ± 1.0</td>
</tr>
<tr>
<td>Placebo</td>
<td>6 (100)</td>
<td>7.5 ± 0.7</td>
<td>4.3 ± 0.2</td>
<td>5.7 ± 0.3</td>
</tr>
</tbody>
</table>

* Each monkey received 10⁴ TCID₅₀ of virus intratracheally in a 0.5-ml inoculum.
* Infection was indicated by a fourfold or greater increase in antibody titer, evidence of virus shedding, or both.
* Each monkey was tested daily for 10 days, as described in the text.
* The amount of virus in the NP swab specimens or tracheal lavage from each monkey was determined by titration in Madin Darby canine kidney cell tissue culture, and the average maximum amount of virus shed by each monkey was determined.
* Each monkey was tested on days 2, 4, and 6.
with $10^{7.0}$ TCID$_{50}$s of wild-type human influenza A/Washington/80 virus intratracheally 1 month later (Table 2). Significant reduction in virus replication in the upper and lower respiratory tracts was observed in monkeys previously infected with the avian-human influenza A reassortant or wild-type human influenza A/Washington/80 influenza A parental virus but not in monkeys infected with the avian influenza A parental virus. These results indicate that resistance induced by infection with the reassortant is specific for the HA and NA surface antigens of the wild-type human influenza A parental virus.

**Evaluation of the avian-human influenza A reassortant virus in humans.** Dose-response studies revealed that the 50% human infectious dose (HID$_{50}$) of the avian-human influenza A/Washington/80 x A/Pintail/79 reassortant virus was $10^{6.2}$ TCID$_{50}$ (Table 3). Eighty-four percent of the vaccinees were infected at 20 HID$_{50}$ The avian-human influenza A reassortant virus was safe in adult volunteers; illness was not observed after intranasal vaccination; and virus was not recovered from the blood or stools of 19 vaccinees, 9 of whom shed virus from the respiratory tract. In contrast, 46% of the unvaccinated control volunteers developed influenza-like illness after challenge with the wild-type human influenza A/Washington/80 virus. Volunteers infected with the reassortant virus shed significantly less virus over a shorter interval than did individuals infected with wild-type human influenza A virus. These results, together with the lack of reactogenicity, indicate that the avian-human influenza A/Washington/80 x A/Pintail/79 reassortant virus is satisfactorily attenuated at the doses given ($10^{6.5}$ to $10^{7.5}$ TCID$_{50}$).

Infection was not transmitted from 13 infected vaccinees ($10^{5.6}$ TCID$_{50}$) to four susceptible, unvaccinated volunteers (controls) who were housed together for a period of 9 days following inoculation of the avian-human influenza A reassortant virus.

The reassortant virus induced antibody responses in serum and nasal wash. At 20 HID$_{50}$, 80% of the vaccinees developed an antibody response in serum, and 40% had an antibody response in nasal wash.

**DISCUSSION**

Results of this study demonstrate that the avian influenza A/Pintail/Alberta/119/79 virus can be used as a donor of six internal genes to attenuate a virulent human influenza A virus for squirrel monkeys and humans. In comparison with the wild-type human virus parent, the avian-human influenza A/Washington/80 x A/Pintail/79 reassortant virus was attenuated, as indicated by restriction of virus replication in the upper and lower respiratory tracts of experimentally infected monkeys. Likewise, in comparison with the wild-type human influenza A virus, the avian-human influenza A reassortant was fully attenuated for seronegative, susceptible adult volunteers, as evidenced by a markedly reduced amount of virus shedding in nasal washes and lack of reactogenicity following intranasal administration of the reassortant. This low level of viral replication of the influenza A/Washington/80 x A/Pintail/79 reassortant virus in adult volunteers was similar to that of other attenuated influenza A reassortant viruses containing the HA and NA genes of the wild-type human influenza A/Washington/80 (H3N2) virus and the six internal genes of the avian influenza A/Mallard/6750/78 donor virus or the cold-adapted (ca) influenza A/Ann Arbor/6/60 donor virus (3, 4, 6, 10, 14). The avian-human influenza A/Washington/80 x A/Pintail/79 reassortant virus was similarly well tolerated by adult volunteers in this study.
sorant virus, like these other reassortants, was not transmitted to four susceptible unvaccinated monkey cagemates or human contacts and did not spread beyond the respiratory tract (4, 10).

Results of previous studies have indicated that the avian influenza A/Pintail/Alberta/119/79 virus replicates slightly better in squirrel monkeys than the avian influenza A/Mallard/6750/78 virus (4). Studies in which squirrel monkeys received reassortants containing one RNA segment from the avian influenza A/Pintail/79 virus and seven segments from the wild-type human influenza A/Washington/80 virus demonstrated that the NP gene of the influenza A/Pintail/79 virus specified restriction of replication; a similar finding had been reported for the avian influenza A/Mallard/78 donor virus (B. R. Murphy, M. H. Snyder, A. J. Buckler-White, M. L. Clements, R. F. Betts, W. T. London, and R. M. Chanock, Virus Res., in press). However, unlike the latter virus, the M gene of the influenza A/Pintail/79 virus did not appear to restrict replication. The effect of the internal genes of these two avian viruses on virus replication in squirrel monkeys was assessed in this study by comparing the restriction of replication of reassortants of the two avian donor viruses prepared from the same wild-type human influenza A virus. The previous difference in replication of the two donor viruses in monkeys was not confirmed when their reassortants were compared in the same study. The level of replication of the avian-human influenza A/Washington/80 × A/Pintail/79 and A/Washington/80 × A/Mallard/78 reassortant viruses was comparable in squirrel monkeys, indicating that the internal genes of these two avian viruses imposed a similar restriction on replication of the wild-type human influenza A/Washington/80 virus. However, the avian-human influenza A/Washington/80 × A/Pintail/79 reassortant virus was slightly less infectious for susceptible adults in this study than was the avian-human reassortant influenza A/Washington/80 × A/Mallard/78 reassortant virus in an earlier study (6). In seronegative adults, the HID50 for the influenza A/Washington/80 × A/Pintail/79 reassortant was 10^−2 TCID50, whereas the HID50 for the influenza A/Washington/80 × A/Mallard/78 reassortant was 10^5.9 TCID50. Each of these reassortants stimulated an immunologic response in at least 80% of seronegative adults to whom were administered 20 to 100 HID50 of virus intranasally (3, 6).

Protective efficacy of the avian-human influenza A/Washington/80 × A/Pintail/79 reassortant virus was demonstrated by experimental challenge of squirrel monkeys with wild-type human influenza A/Washington/80 virus. Significant resistance to replication of wild-type virus was observed in the nasopharynx and trachea of monkeys previously infected with this reassortant. There was also a 50% reduction in frequency of infection in the vaccinated monkeys compared with that in unvaccinated placebo controls. These results are encouraging and suggest that reassortants derived from the avian influenza A/Pintail/79 donor virus should be evaluated further for their suitability for use in live attenuated influenza A virus vaccines.

**LITERATURE CITED**