Ortho- and Paramyxoviruses from Migrating Feral Ducks: Characterization of a New Group of Influenza A Viruses

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SUMMARY

Ortho- and parainfluenza viruses isolated from the cloacas of migrating feral ducks shot on the Mississippi flyway included three strains of influenza A virus (Hav6 Nav1, Hav6 Nl, Hav7 Neq2) as well as Newcastle disease virus. One influenza virus, A/duck/Memphis/546/74, possessed Hav3 haemagglutinin, but the neuraminidase was not inhibited by any of the known influenza reference antisera. The neuraminidase on this virus was related to the neuraminidases on A/duck/GDR/72 (H2 N?), A/turkey/Ontario/7732/66 (Hav 5 N?), A/duck/Ukraine/1/60 (Hav3 N?) and A/turkey/Wisconsin/68. We therefore propose that the neuraminidase on this group of influenza viruses be designated Nav6.

The A/duck/Memphis/546/74 influenza virus caused an ocular discharge in 1 of 5 ducks and was shed in faeces for 10 days; it was stable in faecal samples for up to 3 days at 20 °C. These results suggest that ecological studies on influenza in avian species should include attempts to isolate virus from faeces. Faecal–oral transmission is an attractive explanation for the spread of influenza virus from feral birds to other animals.

INTRODUCTION

Influenza A viruses cause pandemics of disease in man at irregular intervals, but the origin of these pandemic strains remains elusive. Increasing evidence suggests that the new pandemic strains of human influenza virus originate from the influenza viruses present in lower animals and birds—either by direct transmission or after genetic recombination between animal or avian strains and the influenza A virus prevalent in man at that time (Webster & Laver, 1975; Laver & Webster, 1972). Experimental studies have demonstrated that new influenza A viruses can be selected in vivo and that these viruses can cause epidemics of disease in laboratory animals (Webster & Campbell, 1974). Despite the appeal of this argument, the evidence for an animal or avian origin of pandemic strains of influenza is still circumstantial; to date no pandemic strains of influenza A viruses have been isolated from lower animals before they were isolated from man.

The long-term aims of our study are (i) to determine if it is possible to isolate a future pandemic strain of virus from lower mammals and birds before it occurs in man and (ii) to

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determine if there is a finite number of influenza A viruses in nature. This report describes the isolation of a wide range of both ortho- and paramyxoviruses from the cloacas of migrating ducks. One of the isolated influenza viruses possessed an antigen that had not been characterized before.

METHODS

Collection of materials. Tracheal and cloacal swabs were collected from ducks (mallard [Anas platyrhynchos], gadwall [Anas strepera] and wood ducks [Aix sponsa]) shot by hunters on the Mississippi flyway at West Memphis, Arkansas, during the hunting season, from November 1974 to January 1975 (20 Nov to 7 Dec and 18 Dec to 18 Jan). Samples were collected within 4 to 6 hours after the birds were shot. The swabs were collected and placed in bacterial broth (Brain Heart Infusion) containing penicillin, streptomycin and polymyxin B, at 0 °C and stored at -70 °C until assayed. Recent unpublished studies in this laboratory have shown that a more satisfactory support medium is phosphate-buffered saline (pH 7.2) containing 50 % glycerol and the above antibiotics.

Virus isolation. The samples were thawed and 0.1 ml volumes were inoculated into the allantoic cavity of 11-day-old chick embryos. The eggs were incubated at 36 °C for 48 h and tested individually for haemagglutinin activity.

Antisera. Antisera specific for the isolated haemagglutinins and for most of the neuraminidase subunits of the reference strains of influenza A viruses (WHO report, 1971) were prepared in goats (Webster, Isachenko & Carter, 1974). Antisera to the other neuraminidase antigens were prepared in rabbits with antigen hybrid viruses possessing an irrelevant haemagglutinin subunit. Antisera to the following parainfluenza and related viruses were prepared in guinea pigs: type 1 HA-2 (C-39), type 1 Sendai, type 2 SV5, type 2 (Greer), type 3 SF, type 3 HA-1, type 4A (M25), type 4B (19503), mumps (Enders), canine distemper, Newcastle disease virus (Roakin), measles (Edmonston) and respiratory syncytial virus. The above reference sera were generously provided by the Resource Service Branch of the National Institutes of Health.

Serological tests. Haemagglutinin (HA) titrations and haemagglutination inhibition (HI) tests were performed in plastic trays containing receptor-destroying enzyme-treated sera (Fazekas de St. Groth & Webster, 1966). Neuraminidase (NA) titrations and neuraminidase inhibition (NI) tests were done as previously described (WHO report, 1973).

Immunodiffusion tests were done in 1.5 % agarose (A37) dissolved in phosphate-buffered saline (pH 7.2) containing 0.1 % Sarkosyl NL97 and 0.1 % sodium azide. Purified virus (HA 6.0 log10 units/ml) was disrupted with 0.1 % Sarkosyl NL97; the same concentration of Sarkosyl was added to the antisera before it was applied to the plates in order to prevent formation of non-specific precipitation bands. The precipitation lines were photographed without staining.

Ribonucleoprotein (RNP) antigen. Chick embryos (11-day-old) were infected with high multiplicities of the haemagglutinating agents (0.05 ml of undiluted infectious allantoic fluid) and incubated at 36 °C for 20 h. After the eggs had been de-embryonated and washed to remove red blood cells, the chorioallantoic membranes were homogenized, frozen and thawed for use as a source of RNP antigen for gel diffusion tests (Beard, 1970).

Actinomycin sensitivity. The haemagglutinating agents were inoculated on to primary chick embryo fibroblast cultures (Darlington, Portner & Kingsbury, 1970) in the presence and absence of 5 μg/ml of actinomycin D. The cultures were incubated at 37 °C and assayed for haemagglutinin activity 24 and 48 h later.

Inoculation of ducks. Four-week-old ducks (Pekin white) were inoculated intratracheally.
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with approx. $1 \times 10^{7.5}$ EID$_{50}$ of the first cloned passage of the duck/546 influenza A virus. Swabs from tracheas, cloacas and broth extracts of the droppings were tested daily in 11-day-old chick embryos for infectious virus. The ducks were observed for signs of disease.

RESULTS

Isolation of haemagglutinating agents

A total of 13 haemagglutinating agents were isolated from 829 feral ducks (Table I), comprising 669 mallards, 51 gadwalls, 31 teals, 15 wood ducks and 63 ducks of other species. Samples were collected from ducks twice weekly during the hunting season. The number of agents isolated was not correlated with either the species of duck tested or the time of year when swabs were taken. This suggests that these viruses may be endemic in the duck population and that an epidemic of infection was not in progress at the time of our study.

Separation of ortho- and paramyxoviruses

Actinomycin D, when added to cultures at the time of infection will inhibit the replication of orthomyxoviruses (Granoff & Kingsbury, 1964), but not paramyxoviruses. The different viruses were inoculated on to primary chick embryo fibroblast cultures in the presence and absence of actinomycin D and virus growth was detected by haemagglutination tests (Table I). Gel diffusion tests with specific antisera to the nucleoprotein of influenza A viruses were used to characterize the RNP from the isolated haemagglutinating agents (Table I, Fig. 1). Each isolated haemagglutinating agent was examined by electron microscopy and characterized as either ortho- or parayxovirus by its morphology, particularly the appearance of the ribonucleoprotein (RNP; Fig. 2). Six of the 13 virus isolates reacted with antisera to influenza A virus RNP and, by all other criteria, 5 of the 6 were influenza A viruses. Seven isolates were classified as paramyxoviruses and the remaining agent behaved in an ambiguous fashion, reacting with antiserum to influenza A ribonucleoprotein but also growing in the presence of actinomycin D. When examined by electron microscopy, this isolate appeared to be a mixture of orthomyxo- and paramyxoviruses.

Antigenic analysis of virus isolates

The viral isolates were also subjected to HI and NI tests against antisera to all the known reference strains of influenza A viruses and to 13 different parainfluenza viruses. A total of 6 influenza A viruses were identified: three possessed the antigenic configuration Hav6 Nav1, one possessed Hav6NI, and one possessed Hav7 Neq2. Only one of the paramyxoviruses was identified – NDV from a wood duck (no 604) – despite the use of antisera to a wide range of known paramyxoviruses. This virus did not kill chick embryos in 2 days and would be considered a lentogenic (avirulent) strain of NDV.

Studies on the influenza virus present in the mixedly infected duck

Since the above studies show that the viruses present in duck/546 were a mixture of influenza and paramyxovirus it was necessary to separate them. Antisera prepared in rabbits to isolate number no. 406 also reacted with the paramyxovirus present in the mixed-virus preparation isolated from duck no. 546. The virus mixture from duck/546 was inoculated into 11-day-old chick embryos in the presence of antiserum to the paramyxoviruses (no. 406). The virus that grew was cloned by two further limit-dilution passages in chick embryos.
Table 1. *Myxoviruses isolated from feral ducks on the Mississippi flyway at West Memphis, Arkansas*

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Type of duck</th>
<th>Source of samples</th>
<th>Influenza A RNP</th>
<th>Growth in presence of actinomycin</th>
<th>Antigenic configuration</th>
<th>Morphological identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>346</td>
<td>Mallard</td>
<td>C</td>
<td>+</td>
<td>ND†</td>
<td>Hay6</td>
<td>Influenza</td>
</tr>
<tr>
<td>546</td>
<td>Mallard</td>
<td>C</td>
<td>+</td>
<td>ND†</td>
<td>Hay6</td>
<td>Influenza</td>
</tr>
<tr>
<td>928</td>
<td>Mallard</td>
<td>C</td>
<td>+</td>
<td>ND†</td>
<td>Hay6</td>
<td>Influenza</td>
</tr>
<tr>
<td>994</td>
<td>Mallard</td>
<td>C</td>
<td>+</td>
<td>ND†</td>
<td>Hay6</td>
<td>Influenza</td>
</tr>
<tr>
<td>1070</td>
<td>Mallard</td>
<td>C</td>
<td>+</td>
<td>ND†</td>
<td>Hay6</td>
<td>Influenza</td>
</tr>
<tr>
<td>1072</td>
<td>Mallard</td>
<td>C</td>
<td>+</td>
<td>ND†</td>
<td>Hay6</td>
<td>Influenza</td>
</tr>
<tr>
<td>116</td>
<td>Mallard</td>
<td>C</td>
<td>-</td>
<td>ND†</td>
<td>Hay6</td>
<td>Influenza</td>
</tr>
<tr>
<td>319</td>
<td>Gadwall</td>
<td>T</td>
<td>-</td>
<td>+</td>
<td>Hay6</td>
<td>Influenza</td>
</tr>
<tr>
<td>330</td>
<td>Gadwall</td>
<td>C</td>
<td>-</td>
<td>+</td>
<td>Hay6</td>
<td>Influenza</td>
</tr>
<tr>
<td>406</td>
<td>Mallard</td>
<td>C</td>
<td>-</td>
<td>+</td>
<td>NDV</td>
<td>Paramyxovirus</td>
</tr>
<tr>
<td>604</td>
<td>Wood Duck</td>
<td>C</td>
<td>-</td>
<td>+</td>
<td>NDV</td>
<td>Paramyxovirus</td>
</tr>
<tr>
<td>1330</td>
<td>Mallard</td>
<td>C</td>
<td>-</td>
<td>ND†</td>
<td>ND</td>
<td>Paramyxovirus</td>
</tr>
</tbody>
</table>

* C = cloacal, T = tracheal.
† ND = not determined.

Fig. 1. Double-immunodiffusion tests showing that the haemagglutinating agent 546 from ducks is an influenza A virus. The influenza viruses were disrupted with Sarkosyl as given in Methods; Port Chalmers = A/Port Chalmers/1/73, a human influenza A virus.
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Fig. 2. Electron micrographs of ortho- and paramyxoviruses isolated from the cloaca of duck 546. (a) Paramyxovirus with RNP. (b) A/duck/Memphis/546/74 influenza virus isolated from the original mixture. (Electron micrographs by courtesy of Dr N. Wrigley.)
Table 2. Identification of the haemagglutinin antigen on A/duck/Memphis/546/74

<table>
<thead>
<tr>
<th>Antisera to:</th>
<th>Duck/England/56</th>
<th>Duck/546 HS*</th>
<th>Duck/Ukraine</th>
<th>A/duck/Germany/N/49</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duck/England/56 (Hav3) to isolated haemagglutinin</td>
<td>2700</td>
<td>260</td>
<td>56</td>
<td>&lt;↑</td>
</tr>
<tr>
<td>Duck/Memphis/546/74 to whole virus</td>
<td>10000</td>
<td>10000</td>
<td>7000</td>
<td>&lt;</td>
</tr>
</tbody>
</table>

* Values represent reciprocal of serum dilution causing inhibition of three out of four haemagglutinating doses of virus.
↑ < = less than 20.

Morphology of the A/duck/546 influenza virus

The duck/546 virus was concentrated from allantoic fluid by adsorption-elution on chicken red blood cells and further purified on a sucrose gradient. In the electron microscope, this preparation appeared to contain a heterogeneous population of particles varying from circles to long bulbous filaments (Fig. 2). The surface of the particles was covered with short spikes of typical influenza viruses; a number of spikeless particles were also present. Because of the high concentration of ‘spikes’ in the background, and the presence of one partly denuded particle, it is possible that the bald particles lost their spikes during preparation.

Preparation of antigenic hybrids of duck/546 influenza virus

Unambiguous identification of the surface antigens on influenza viruses requires separation of the haemagglutinin and neuraminidase subunits on antigenic hybrid viruses. The technique of genetic reassortment (Webster, 1970) permits preparation of antigenic hybrids that possess irrelevant haemagglutinin and neuraminidase antigens which are labile in sodium dodecyl sulphate (SDS).

Both the haemagglutinin and neuraminidase subunits of duck/546 influenza virus were stable in SDS, and the two surface subunits could not be separated electrophoretically after disruption of the virion. Antigenic hybrids possessing NWS(57)-duck/546(57) and duck/546(57)-Bel(20) were prepared and used in the following section for unambiguous identification of the surface antigens on duck/546 influenza virus.

Identification of the antigens on duck/546 virus

Purified duck/546 virus reacted in gel diffusion tests with specific antisera to both the matrix and RNP of influenza A viruses (Fig. 1). The haemagglutinin subunits on this virus reacted with specific antisera to Hav3 (Table 2). Although the antigen was not identical to the haemagglutinin on duck/England/56, it showed an asymmetrical cross-reaction with this virus. Antiserum to Nav 3 and to duck/546 virus also reacted with A/duck/Ukraine/1/60 influenza virus. The neuraminidase on duck/546 virus did not react with antiserum to any of the known subtypes of influenza A, but was related to the neuraminidase on A/duck/GDR/72 (H2 N?), A/turkey/Ontario/7732/66 (Hay5 N?), A/duck/Ukraine/1/60 (Hav3 N?), and A/turkey/Wisconsin/68 influenza viruses (Table 3).

Infection of ducks with A/duck/546 influenza virus

Pekin white ducks inoculated with the duck/546 virus produced signs of disease in two out of five birds (Table 4). One duck had an ocular discharge and ate less than normal from
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Table 3. Identification of the neuraminidase antigen on A/duck/Memphis/546/74

<table>
<thead>
<tr>
<th>Antisera to the following whole viruses</th>
<th>NI titres with the following influenza viruses:*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duck/546</td>
<td>800 320 200 100 200 1,000</td>
</tr>
<tr>
<td>Duck/GDR/72</td>
<td>320 100 56 32 100 630</td>
</tr>
<tr>
<td>Turkey/Ontario/7732/66</td>
<td>200 32 56 20 20 200</td>
</tr>
</tbody>
</table>

* Values represent the reciprocal of the serum dilution causing 50% inhibition of virus giving an approximate extinction reading of 0.5.

Table 4. Virus isolation and signs of disease in ducks after inoculation with A/duck/Memphis/546/74 influenza virus*

<table>
<thead>
<tr>
<th>Day</th>
<th>Trachea</th>
<th>Cloaca</th>
<th>Faeces†</th>
<th>Signs of disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0/5‡</td>
<td>0/5</td>
<td>NT§</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>2/5</td>
<td>2/5</td>
<td>NT</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>2/5</td>
<td>2/5</td>
<td>NT</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>1/5</td>
<td>2/5</td>
<td>NT</td>
<td>None</td>
</tr>
<tr>
<td>6</td>
<td>1/5</td>
<td>1/5</td>
<td>1/5</td>
<td>One duck with ocular discharge and poor appetite and a second duck with diarrhoea</td>
</tr>
<tr>
<td>7</td>
<td>2/5</td>
<td>0/5</td>
<td>3/5</td>
<td>1/5 with ocular discharge and poor appetite</td>
</tr>
<tr>
<td>8–10</td>
<td>0/5</td>
<td>0/5</td>
<td>1/5</td>
<td>1/5 with ocular discharge and poor appetite</td>
</tr>
</tbody>
</table>

* Ducks were inoculated by the intratracheal route with approximately $1 \times 10^7$ EID$_{50}$ of virus as given in Methods.
† Faecal samples were collected on plastic sheets placed in the bottom of the individual cages.
‡ Number of ducks with virus v. number inoculated.
§ NT, not tested.

the 6th to the 10th day after infection; a 2nd duck had diarrhoea on the 6th day. Virus was recovered on the 2nd day after infection from the trachea and cloaca of the two ducks that showed signs of infection. The virus was isolated for 10 days, and was recovered from the cloaca and droppings as well as from the trachea. Serum samples collected 10 days after infection from all ducks contained HI antibodies to duck/546 virus, indicating that infection had been initiated in all birds despite the absence of disease signs. Faecal samples were collected and kept at room temperature (20 °C) for varying lengths of time before assays for virus. A/duck/546 influenza virus was isolated consistently for a period of 3 days from the faecal samples.

DISCUSSION

Several different ortho- and paramyxoviruses were isolated from apparently healthy migrating feral ducks shot on the Mississippi flyway during the 1974 migration season. Of the 14 viruses isolated, 6 were influenza A viruses: three had the antigenic configuration Hav6 Navi, one had Hav6 N1, and one had Hav7 Neq2. One virus, A/duck/Memphis/546/74, possessed a haemagglutinin subunit related to Hav3 influenza viruses. The neuraminidase activity of this virus was not inhibited by antisera to any of the designated reference strains (WHO report, 1971), but is related to the neuraminidases on A/duck/Ukraine/1/60
(Hav3 N?), A/turkey/Ontario/7332/66 (Hav5 N?), A/turkey/Wisconsin/68 and A/duck/GDR/72 (H2 N?). We therefore propose that the neuraminidase subunits on this group of viruses constitute a new group of avian neuraminidases, i.e. Nav6. The majority of viruses (13/14) were isolated from cloacal samples collected from feral ducks; the only virus obtained from a tracheal sample was also isolated from the cloaca of the same duck. The relatively high frequency of isolation of ortho- and paramyxoviruses from the cloacas instead of from the respiratory tract of avian species (Rosenberger, Krauss & Slemons, 1974; Slemons & Easterday, 1975) is borne out in these studies. In the present study, samples were collected twice weekly during the approx. 2-month-long hunting season. There was no correlation between the number of agents isolated and the time of isolation or species of ducks tested, suggesting that these viruses may be endemic in the duck population.

The isolation of duck/546 influenza virus from the faeces of experimentally infected ducks for 10 days after inoculation raises the question of how the virus gets into the faeces. Whether these viruses reach the cloaca by the urinary, lymphatic or digestive tract is not known, but since influenza viruses are labile at low pH the digestive tract seems an unlikely route. The stability of duck/546 influenza virus in faeces (up to 3 days at 20 °C) suggests that these viruses may be transmitted by the faecal–oral route, at least in avian species. These experiments also suggest that ecological studies on influenza viruses in avian species should include virus isolation studies on ‘fresh’ faecal material. Samples of faecal material from sea birds have already been used in some studies (G. C. Schild, personal communication).

One of the paramyxoviruses was identified as a lentogenic (avirulent) strain of Newcastle disease virus, but the other isolates failed to react with a wide range of antisera to the known paramyxoviruses. The unidentified agents have tentatively been called paramyxoviruses on the basis of their morphology and resistance to actinomycin D, but further studies are required for complete characterization.

Serological evidence for influenza A viruses in migratory waterfowl (Easterday et al. 1968; WHO report, 1972; Zakstelskaya, 1973) and the subsequent isolation of many influenza A and paramyxoviruses from migrating waterfowl (Rosenberger et al. 1974; Slemons et al. 1974; Slemons & Easterday, 1975), as well as from domestic and feral birds (for review see Easterday, 1975) has made it apparent that there is a large number of influenza A viruses present in these species. Some of these avian influenza viruses possess antigens that are closely related to influenza A viruses isolated from man and to the influenza viruses from other animal and avian species (WHO report, 1971). The above observations suggest that migratory birds might be involved in the dissemination of influenza viruses over vast areas of the world. Indeed, the influenza virus isolated from shearwaters in Australia (Downie & Laver, 1973) has also been isolated from turkeys in California (V. S. Hinshaw & R. A. Bankowski, personal communication).

In addition to spreading influenza viruses, the lower animal and avian species may serve as a reservoir from which new pandemic strains of human influenza viruses arise. Transmission of influenza viruses by the faecal–oral route would provide an ideal method of transmitting the influenza viruses in untreated water from migrating ducks to domestic ducks and pigs, or even to man. All the known human influenza viruses that have been isolated since 1934 possess haemagglutinin and neuraminidase subunits that are related immunologically to influenza viruses in lower mammals and birds (Webster, Laver & Tumova, 1975). Whether the viruses in the lower orders serve as the source of new genetic information for the pandemic strains of human influenza viruses has not been resolved. By studying the ecology of influenza viruses in lower animals and birds it might be possible to isolate a future pandemic human strain before the virus appears in man.
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