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Intestinal Influenza: Replication and Characterization of Influenza Viruses in Ducks

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Influenza A viruses isolated from the cloaca of naturally infected feral ducks replicate in the lungs and in the cells lining the intestinal tract of feral and domestic ducks. Despite the low pH of the gizzard, the duck influenza viruses reach the intestines via the digestive tract and are found in high concentration in the feces. The viruses retain infectivity in fecal material for at least 30 days at 4° and for 7 days at 20°. The morphology of one strain of intestinal duck influenza virus (Hav7 Neq2) that had never been passed in embryonated eggs and was isolated from the feces was roughly spherical and fairly uniform in size and shape. However, another strain of duck influenza virus studied (Hav3 Nav6) was predominantly filamentous, suggesting that the morphology of influenza viruses in their natural hosts varies from strain to strain. After passage in the chick embryo each strain retained the morphological characteristics found in the feces. In contrast to duck influenza viruses, representative human influenza viruses of the HON1, H3N2, and Hsw1 N1 subtypes replicate only in the upper respiratory tract of ducks. The duck influenza viruses are more stable to low pH than human strains and retain infectivity for over 30 days in nonchlorinated river water at 0° and for 4 days at 22°. The susceptibility of ducks to infection with human and avian strains of influenza virus and the possibility of transmission to animal species through the water supply suggests that ducks may be important in the ecology of influenza viruses. The possibility of "intestinal influenza" virus vaccines is considered.

INTRODUCTION

Avian influenza viruses have been postulated to play a role in the origin of new human pandemic strains (Laver and Webster, 1972; Webster and Campbell, 1974). Biochemical and biological studies suggest that at least one of the known human pandemic strains [(A/Hong Kong/68 (H3N2)] arose by genetic reassortment between a human and an animal strain of influenza virus. The most likely source of the genetic information coding for the hemagglutinin of A/Hong Kong/68 (H3N2) influenza virus was from a virus related to A/duck/Ukraine/63 or to A/equine/Miami/2/63.

Earlier studies have shown that human influenza viruses are naturally transmitted from man to lower animals and birds; this was particularly evident with the A/Hong Kong/68 strains that transmitted to such diverse species as pigs, domestic chickens, and feral birds (Easterday, 1975). Until recently, there has been no evidence for the spread of animal influenza viruses to man; however, the transmission of swine influenza viruses from pigs to man (1976-1977) has now established that this can occur.

The opportunity for interaction between influenza viruses of feral avian species and mammalian species would not appear to be high if influenza viruses were transmitted only by droplet spread to the respiratory tract. However, recent studies on the ecology of influenza viruses in avian species has shown that many more influenza viruses have been isolated from cloa-

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cal samples than from the respiratory tract (Webster *et al.*, 1976; Shortridge *et al.*, 1977). The question arises as to the site of replication of these influenza viruses, and whether the biological and biochemical properties of the viruses would permit physical contact between feral avian species and mammalian species.

Since many influenza A viruses have been isolated from the cloaca of ducks, the site of replication of these viruses was studied. The present report shows that A/duck/Memphis/546/74 influenza virus replicates in both the respiratory tract and in the lower intestinal tract of ducks and is shed in high concentration in the feces. In contrast, human influenza A viruses replicated in the respiratory tract of ducks but not in the intestinal tract. The temperature and pH stability of the duck and human strains of influenza viruses were compared in an attempt to elucidate the properties of the duck strain that would permit replication in the lower intestinal tract of ducks.

MATERIALS AND METHODS

Viruses. The following influenza A viruses were used in this study: Duck/Memphis/546/74 (Hav3 Nav6); WSN/34 (HON1); Japan/305/57 (H2N2); Aichi/2/68 (H3N2); Memphis/110/76 (H3N2); Texas/1/77 (H3N2); and swine/Tn/1/75 (Hsw1 N1). In addition, representative influenza A viruses isolated from the cloaca of feral mallard ducks were also used. These included duck/Alberta/49/76 (Hav1 Nav2); duck/Alberta/24/76 (Hav4 Nav1); duck/Alberta/57/76 (Hav5 N2); and duck/Alberta/19/76) (H? N?); the ? indicates that the hemagglutinin and neuraminidase on these viruses are probably new subtypes.

The viruses were grown in the allantoic sac of 11-day-old chick embryos and the virus particles were purified by adsorption–elution on erythrocytes followed by differential centrifugation and sedimentation through a sucrose gradient (10 to 40% sucrose in 0.15 *M* NaCl) as previously described (Laver, 1969).

Animals. Mallard ducks (*Anas platyrhynchos*) or muscovey ducks (*Cairina moschata*) at 6–8 months of age were used in these experiments.

Antisera and assay systems. Preparation of antisera to the hemagglutinin and neuraminidase antigens of influenza viruses and the serological methods of assays of these antigens were as previously described (Webster *et al.*, 1976).

Infection of ducks with influenza virus and collection of samples. Groups of four mallard ducks were inoculated both orally and intratracheally with 0.5 ml of allantoic fluid containing A/duck/Memphis/546/74 influenza virus. Each duck received 10^7 EID_{50} of virus. At hourly intervals for 12 hr after infection and twice during the first day after infection, blood samples were drawn by syringe from the wing vein. Tracheal and cloacal samples were collected into 1.0 ml of buffered glycerol saline (Hinshaw *et al.*, 1977) at the above time intervals. The samples were collected with dacron swabs, if cloacal swabs were not stained with fecal material the swabs were inserted into the rectum. On the second day after infection, and at daily intervals thereafter, one duck was exsanguinated and samples of the organs were collected aseptically for virus titrations. The pH of the gizzard cavity was measured by inserting an electrode directly into the organ immediately after exsanguination. The organs were ground in a mortar with powdered glass to give a 10% suspension with antibiotics and saline (Hinshaw *et al.*, 1977). The samples were tested undilute for viral infectivity in 11-day-old embryonated eggs, and were stored at −70°. Samples that contained virus were titrated to determine the EID_{50} and the agents recovered were identified with specific antiserum to duck/Memphis to verify that the virus isolated was the one inoculated. For inoculation directly into the crop or rectum of ducks, the virus was put into gelatin capsules (0.2 ml per capsule 10^7 EID_{50}) and inserted by plastic tube into the crop or 15 cm into the rectum.

Assay of the intestinal tract of ducks for duck/Memphis influenza virus. Mallard ducks were inoculated intratracheally with $10^{4.0}$ EID_{50} of A/duck/Memphis/546/74 influenza virus. Four days later the ducks were exsanguinated and lungs and

15-cm sections of the intestinal tract were collected. Samples of the small intestine immediately posterior to the ascending loop of the duodenum, of the small intestine immediately anterior to the caecal junction, and of the large intestine (from the caecal junction to rectum) were studied. The intestinal contents were expelled and the segment of intestine was washed extensively with six to eight washes of phosphate-buffered saline (PBS) from a large syringe. The intestinal segment was cut open and was gently scraped with a blade to remove the surface cells; the cells were suspended to a 10% suspension in PBS and disrupted by freezing, thawing, and sonication. The virus content of the samples was assayed as described above.

Collection and processing of fecal material for virus isolation. Fecal material was collected from ducks on plastic sheets placed under the cages on the second to the fifth day after infection. The fecal material was stored at 0° in ice, then diluted to make a 10% suspension with PBS. The material was centifuged to remove the large particulate material and the supernatant was centrifuged at 35,000 *g* for 60 min and the pellet obtained was resuspended in a small volume of PBS. The resuspended pellet was layered onto a 25–70% sucrose gradient and centrifuged at 52,000 *g* for 2 hr. Samples were collected from the bottom of the tube and the peak activity was determined by hemagglutination tests on the samples. The samples containing HA activity were diluted in PBS and centrifuged at 52,000 *g* for 2 hr to pellet the virus.

An alternative approach to purifying influenza virus from the fecal material was to adsorb and elute the initially clarified fecal material on chicken erythrocytes. Vibrio cholera neuraminidase was added to the extensively washed chicken erythrocytes to ensure virus elution. The virus was sedimented as given above.

Assay of stability of duck/Memphis influenza virus. Fecal material collected from muscovey ducks 3 days after infection was stored either at 4° or at 22° in screw-capped glass vials without dilution. At 2- to 5-day intervals aliquots were diluted in antibiotic saline and assayed for infectivity in embryonated eggs. In a second assay the infected fecal material was diluted in untreated Mississippi River water (pH 6.8), stored at either 4 or 22°, and assayed for infectivity at 3- to 7-day intervals.

In order to determine the pH stability of duck/Memphis influenza virus, infectious allantoic fluid was diluted in 0.1 *M* citrate–phosphate-buffered saline, pH 3.0, 4.0, 5.0, and 7.0 at 0°. The infectivity titers of the preparations were determined in embryonated eggs after 10 min exposure to the above pH.

Inoculation of ducks with mammalian influenza A viruses. Groups of two muscovey ducks each were inoculated orally and intratracheally with approximately 10^7 EID_{50} of representative human and swine influenza viruses. Tracheal and cloacal samples were collected daily into 1.5 ml of 50% glycerol–antibiotic saline and were assayed for virus infectivity in embryonated eggs. Samples of virus isolated from each bird were identified with specific antisera in HI tests. Blood samples were collected prior to infection and 14 days later and the sera were assayed for specific antibodies in HI tests.

RESULTS

Site of Replication of Duck/Memphis Influenza Virus in Ducks

Since duck/Memphis influenza virus was isolated from the cloaca of mallard ducks (Webster *et al.*, 1976), the question of how the virus reached the cloaca was studied. The possible ways whereby the virus could reach the cloaca were via the blood stream, via the urinary tract, via the digestive tract, or by external contamination.

Mallard ducks were infected with approximately 10^7 EID_{50} of A/duck/Memphis/546/74 in 0.5 ml by both oral and intratracheal inoculation. Samples collected from ducks at hourly intervals after infection showed that virus was first detected in cloacal samples 1 hr after infection (Table 1), but was never isolated from the blood, kidneys, spleen, or liver. On the second day after infection, virus was detected in

TABLE 1

SITE OF REPLICATION OF A/DUCK/MEMPHIS/546/74 INFLUENZA VIRUS IN MALLARD DUCKS[a]

Days after inoculation	Number of samples with virus/number of ducks inoculated			Log of virus titer (EID_{50}/g) in the following organs					Signs of disease
	Tracheal swab	Cloacal swab	Blood	Lung	Kidney, liver, spleen	Gizzard (pH)[b]	Caecum	Rectum	
0[c]	4/4	2/4	0/4						
1	3/4	4/4	0/4						
2	2/4	4/4	0/4	3.5[d]	<1.0	<1.0 (2.9)	ND[e]	6.5	Nil
3	1/3	3/3	0/3	<1.0	<1.0	<1.0 (4.4)	6.0	4.5	Nil
4	1/2	2/2	0/2	<1.0	<1.0	<1.0 (4.0)	4.0	4.0	Nil
5	0/1	1/1	0/1	<1.0	<1.0	<1.0 (3.5)	ND	3.5	Nil

[a] Mallard ducks were infected with approximately 10^7 EID_{50} of A/duck/Memphis/546/74 in 0.5 ml by both oral and intratracheal inoculation; swab samples and tissues were prepared and assayed as given in Materials and Methods.

[b] Figures in brackets give the pH of the gizzard.

[c] Virus detected at 1, 5, 7, and 9 hr.

[d] Samples of virus isolated from each organ were identified in HI tests with specific antiserum to A/duck/Mem/546/74.

[e] Not determined.

the lungs and to high titers in the rectum; on subsequent days the virus was detected in the caecum and rectum, but not in the lungs or other organs. No overt disease was manifest. This experiment suggests that the duck/Memphis influenza virus can pass through the digestive tract, (despite the low pH in the gizzard) and replicate in the lower intestinal tract, without producing any signs of disease.

Is Replication in the Lungs Necessary to Establish Infection in the Intestines?

The above studies did not determine if virus replication in the lungs was necessary to establish infection in the lower intestinal tract. To answer this question, ducks were inoculated directly into the crop and rectum. In order to avoid inoculation of the upper respiratory tract, duck/Memphis influenza virus was put into gelatin capsules and inserted directly into the crop or into the rectum of the ducks.

These studies gave results that were very similar to those described above (Table 1) and are not presented; influenza virus was not isolated from the trachea or lungs, indicating that encapsulation of the virus had bypassed infection of the respiratory tract. The virus was not isolated from blood, kidney, liver, spleen, or from the duodenum, but was isolated from the lower intestinal tract. The virus was isolated from cloacal samples each day after infection and on the third and fourth days after inoculation the virus was isolated from both the caecum and the rectum ($EID_{50} \geq 5.8$/g).

These studies indicate that duck/Memphis influenza virus can transit the digestive tract of the duck and replicate in the lower intestinal tract of ducks without first replicating in the lungs.

Replication of Duck/Memphis Influenza Virus in the Intestinal Tract of Ducks

The above studies showed that duck/Memphis influenza virus could not be detected in the stomach (proventriculus plus gizzard) or duodenum of ducks after infection but was present in the lower intestinal tract. To determine where the virus was replicating, mallard ducks were infected both orally and intratracheally with lower doses of duck/Memphis influenza virus (10^4 EID_{50}/duck) and different segments of the intestinal tract were assayed for influenza virus.

Duck/Memphis influenza virus was detected in all segments of the intestines posterior to the duodenum (Table 2). Higher titers of influenza virus were de-

TABLE 2

REPLICATION OF A/DUCK/MEMPHIS/546/74 IN THE INTESTINAL TRACT OF DUCKS

Sample	Infectivity titers (log/ml)[a] in the following organs			
	Small intestine (duodenal end)	Small intestine (caecal end)	Caecum	Rectum
Intestinal contents	3.8	7.5	7.8	7.5
Intestinal mucosal scrapings	4.8	7.8	4.5	7.8

[a] Figures represent the EID_{50} in log terms. The intestinal tract was extensively washed before the surface cells were removed and disrupted (see Materials and Methods for details).

tected in the cells than in the intestinal contents at the duodenal end of the small intestine. High titers of virus (10^{7-8} EID_{50}/ml) were detected both in the cells and in the contents of the small intestine before the caecal junction and in the large intestine. The high titers of virus obtained from the mucosal cells of the intestines suggests that the virus was replicating in these cells. Since mallard ducks were not readily available on a year-round basis, domestic ducks (muscovey) were used to determine if duck/Memphis influenza virus would replicate in this species. Tracheal and cloacal samples, collected at daily intervals after infection, showed that duck/Memphis influenza virus would replicate in both the respiratory and intestinal tract of muscovey ducks and was shed in high titers in the feces for 6 days (results not shown). On the seventh day half of the ducks shed virus and on the eighth day postinfection one out of four birds shed virus.

Stability of Duck/Memphis Influenza Virus in Fecal Material

Since duck/Memphis influenza virus was shed in high concentration in the feces, the most likely method of spread of the virus in ducks in their natural habitat would be through the water. In order to determine the stability of this virus in fecal material and in nonchlorinated water, samples from infected ducks were stored at 0° and at 22°.

The fecal material had a pH of 7.68 and the infectivity titer of the virus in the feces showed no detectable decrease over a period of 2 weeks at 0°, but over the following 2-week period there was an appreciable loss of infectivity (3.0 log EID_{50}) (Table 3). After 32 days of storage at 4° there was significant residual infectivity. An identical sample stored at 22° showed a more rapid drop in infectivity; infectious virus was present for at least 8 days but was not detectable after 13 days. Fecal material diluted in nonchlorinated water showed no drop in infectivity over a 7-day period at 4° and gradually decreased thereafter, but residual infectivity was still detectable after 32 days. At 22° the infectivity of virus in untreated water decreased more rapidly but significant levels of virus were detected after 4 days. The marked variation in the infectivity titrations (Table 3) was probably due to uneven distribution of virus in the fecal samples, for such variation was not found in samples of fecal material diluted in water and subsequently titrated for infectivity.

Replication of other Avian influenza A Viruses in Ducks

Since the above studies were done with a single avian influenza A strain [(duck/Memphis (Hav3 Nav6)], other strains were

TABLE 3

STABILITY OF A/DUCK/MEMPHIS/546/74 IN FECAL MATERIAL

Time in days	Infectivity titers (log/ml)[a] after storage in			
	Fecal material		River water	
	4°	22°	4°	22°
0	6.8	6.8	8.1	8.1
4	8.8	7.5	8.1	3.6
5	8.3	4.8		
7	7.8	2.3	8.1	<1.0
8	8.6	3.3		
13	7.6	<1.0	5.1	<1.0
15	7.1	<1.0		
22	6.4	<1.0		
32	3.3	<1.0	4.3	<1.0

[a] Figures give the EID_{50} in log per ml.

tested to determine if they also replicated in the intestinal tract. Influenza A viruses isolated from the cloaca of feral mallard ducks and possessing different surface antigens were inoculated orally and intratracheally into mallard ducks. Each of the viruses tested (Hav1 Nav2, Hav4 Nav1, Hav5 N2, and H? N?) replicated in the upper respiratory tract of the duck, were shed in high concentrations in fecal material (Table 4) for 6 to 7 days, and caused no signs of disease. These data show that a number of avian influenza A viruses possessing different hemagglutinin and neuraminidase antigens can replicate in the intestinal tract of ducks.

Morphology of Influenza Viruses Shed in Fecal Material

The morphology of influenza viruses in their natural hosts has not been described due to the relatively low concentration of virus particles in respiratory secretions. To date, the morphology of influenza viruses has been described after passage of host material in chick embryos; after one egg passage, influenza viruses from mammals or birds are usually filamentous with some spherical particles (Chu *et al.*, 1949; Choppin *et al.*, 1960).

Since relatively high concentration of influenza viruses were shed in the feces of ducks, it was possible to obtain sufficient material to examine the structure of influenza virus from its natural host by electron microscopy. The fecal material obtained from ducks 3 days after infection with duck/Memphis influenza virus was purified by differential centrifugation followed by equilibrium centrifugation. An electron microscopic examination of a negatively stained preparation of this material revealed a small number of typical influenza viruses and a large number of another group of morphologically different viruses. The latter were heterogeneous in size and shape, had surface projections similar to those of corona viruses, and contained proteins of electrophoretic mobilities distinct from typical influenza viruses as revealed by polyacrylamide-gel analysis. Subsequent studies have shown that these viruses did not multiply in chick embryos and that they were also present in the feces of ducks that were not infected with influenza virus.

Since the viruses mentioned above masked influenza virus, it was necessary to purify the influenza virus. To achieve this, fecal material from a swab sample of a feral mallard duck known to contain Hav7 Neq2 influenza virus was inoculated into mallard ducks and fecal material was collected from the second to the fifth day postinoculation. The influenza virus in the fecal material was separated by adsorption to and elution from chicken erythrocytes. The virus concentrated in this fashion was inhibited by specific antiserum to the hemagglutinin subunits of the influenza virus used to infect the ducks. Negatively

TABLE 4

MULTIPLICATION OF REPRESENTATIVE AVIAN INFLUENZA A VIRUSES IN MALLARD DUCKS[a]

Virus strain	Sample	EID_{50}/swab on the following days after inoculation							Disease signs
		1	2	3	4	5	6	7	
Hav1 Nav2	Trachea	2.3	3.3	3.5	4.8	3.3	1.5	<1.0	None
	Cloaca	3.3	5.8	5.8	5.3	4.5	2.6	2.3	
Hav4 Nav1	Trachea	2.5	5.8	4.6	2.8	1.5	<1	<1	None
	Cloaca	3.5	4.8	3.5	2.3	2.0	1.5	<1	
Hav5 N2	Trachea	1.8	3.8	5.9	5.8	4.3	1.4	<1	None
	Cloaca	1.5	2.3	3.4	1.6	1.4	<1	<1	
H? N?[b]	Trachea	3.5	4.8	5.3	3.3	2.5	2.4	<1	None
	Cloaca	4.0	5.8	5.8	5.3	5.8	4.5	3.5	

[a] Mallard ducks were infected both by the oral and intratracheal routes as described in Materials and Methods.

[b] Influenza A virus possessing HA and NA antigens that are probably new subtypes that have not been fully characterized.

stained preparations from this material showed particles that were roughly spherical and measured 80 to 120 nm in diameter (Fig. 1A); the surface of the virions was covered with closely spaced spikes of 10 to 12 nm in length. There was a surprising lack of heterogeneity and pleomorphism in the size and morphology of the viruses obtained from the fecal material. Passage of the influenza virus from the feces in embryonated eggs caused no change in the morphology of the virus.

On the other hand, another influenza virus from ducks (Hav3 Nav6) was extremely heterogenous in size and shape after isolation from fecal material and after one passage in the allantoic cavity of the chick embryo. The particles were large with bizarre shapes (Figs. 1B and C). Thus, some strains of duck influenza virus are fairly uniformly spherical after isolation from their natural host and others are filamentous and heterogenous. After passage in chick embryos, each influenza strain retained the morphology described above.

Replication of Mammalian Influenza Viruses in the Respiratory Tract of Ducks

Since influenza viruses antigenically related to each of the major human subtypes have been isolated from avian species (Webster, 1976), studies were done to determine if mammalian influenza viruses could replicate in ducks. A limited number of mammalian viruses representative of each of the major subtypes were inoculated intratracheally into ducks. The WSN/33 (HON1), Memphis/110/76 (H3N2) (identical with A/Victoria/3/75), Texas/1/77 (H3N2), and swine/Tn/1/75 (Hsw1 N1) (identical with A/New Jersey/8/76) strains each replicated in the upper respiratory tract of ducks, but these viruses were not detected in cloacal samples (Table 5). The A/Japan/305/57 strain did not multiply in ducks. The relatively high titers of virus obtained from tracheal swab samples (in excess of 1000 EID_{50}/swab and as high as 50,000 EID_{50}/swab with A/WSN) together with the relatively high HI antibody titers 14 days after infection, leaves no doubt that the above-mentioned mammalian influenza A viruses can replicate in ducks. The Memphis/110/76 (H3N2) strain was also inoculated rectally into ducks but no virus was detected in cloacal swabs collected 24 hr later, indicating that the human influenza viruses do not replicate in the cells lining the intestinal tract of ducks.

Stability of Duck/Memphis and Human Influenza A Viruses at Low pH

The above studies suggest that duck/Memphis influenza virus can pass through the low pH of the digestive tract of the duck and can subsequently replicate in the intestines, whereas the mammalian strains tested did not replicate in or pass through the intestinal tract. To determine if duck/Memphis influenza virus was more stable at lower pH than human influenza strains, the infectivity of two human strains [WSN/33 (HON1) and Aichi/2/68 (H3N2)] were tested over a pH range from 3.0 to 7.0. These studies showed that duck/Memphis influenza virus was more stable at pH 4.0 than the human strains tested; however, no detectable infectivity remained after exposure to pH 3.0 in the duck/Memphis or human strains (Table 6).

DISCUSSION

The above studies show that a number of influenza A viruses isolated from the cloacae of feral ducks replicate in the upper respiratory tract and also in the intestinal tract of feral and domestic ducks. Representative human influenza viruses of the HON1, H3N2, and Hsw1 N1 subtypes replicate in the upper respiratory tract of ducks but not in the intestinal tract. The duck influenza viruses studied reached the intestines via the digestive tract and replicated in the intestinal tract when inoculation of the respiratory tract was circumvented by inoculation into the rectum. The virus replicates in the cells lining the intestine and is shed in high concentration in the feces. In fecal material, the viruses retain infectivity for over 30 days at 4°, and for over 7 days at 20°. When diluted in untreated water, the virus retains infectivity for over 30 days at

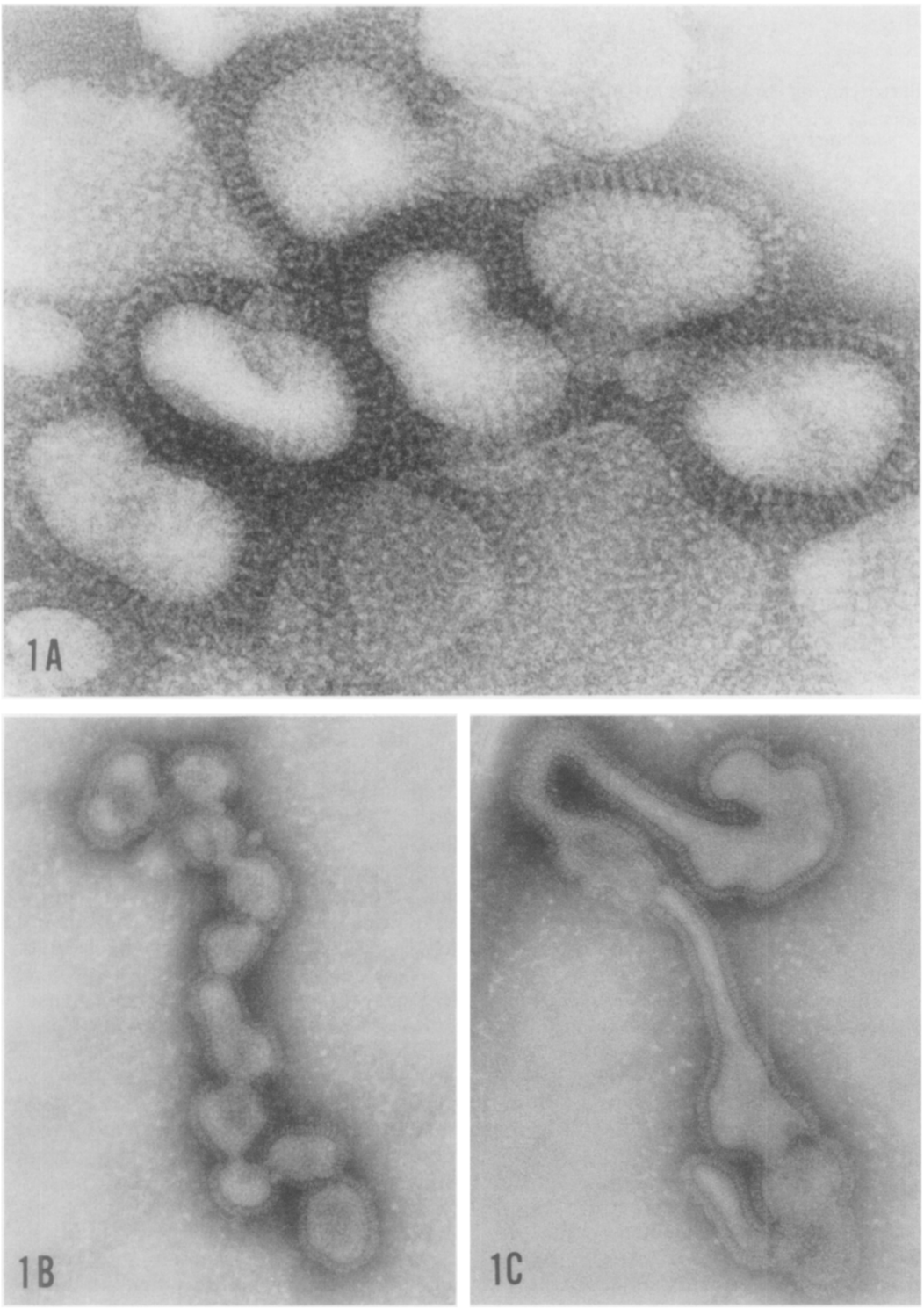

FIG. 1. Electron micrographs of influenza viruses negatively stained with 2% phosphotungstic acid Figure 1A shows Hav7 Neq2 influenza virus in the fecal material of infected ducks. The virus wa adsorbed to chicken erythrocytes and eluted with vibrio cholera neuraminidase. ×391,300. Figures 1B an C illustrate duck/Memphis influenza virus that was purified from fecal material and subjected to on passage in the allantoic cavity of the duck embryo. ×140,000.

TABLE 5

REPLICATION OF MAMMALIAN INFLUENZA A VIRUSES IN THE RESPIRATORY TRACT OF MUSCOVEY DUCKS

Influenza virus[a] inoculated	Infectivity titers (log/ml) of tracheal samples[b] on the following days after infection							HI antibody titers to the following viruses 14 days after infection			
	1	2	3	4	5	6	7	WSN/34	Mem/76	Tex/77	Sw/75
WSN/34 (H0N1)	2.8	4.5[c]	4.3[c]	2.9	1.9	1.5	<0.7	480	<10	<10	<10
Japan/305/57 (H2N2)	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<0.7	<10	<10	<10	<10
Memphis/110/76 (H3N2)	0.7	2.3[c]	3.5[c]	1.7	<0.7	<0.7	<0.7	<10	900	120	<10
Texas/1/77 (H3N2)	0.7	<0.7	1.5	3.3	0.7	<0.7	<0.7	<10	120	420	<10
Swine/TN/1/75 (Hsw1 N1)	2.8	3.6[c]	3.6[c]	2.4	<0.7	<0.7	<0.7	<10	<10	<10	450

[a] Muscovey ducks were infected with the above influenza viruses by the intratracheal route (see Materials and Methods for details).

[b] Cloacal samples were also collected daily but no influenza viruses were isolated.

[c] Virus isolates were identified with specific antisera in HI tests and were identical with the inoculated viruses.

TABLE 6

pH STABILITY OF INFLUENZA A VIRUSES

Virus strain	Infectivity titers after exposure to the following pHs for 10 min			
	3.0	4.0	5.0	7.0
WSN/33 (H0N1)	<1	<1	4.5[a]	8.8
Aichi/2/68 (H3N2)	<1	<1	8.7	8.7
Duck/Memphis/546/76 (Hav3Nav6)	<1	6.4	7.9	8.5

[a] Infectivity titer, EID_{50} log/ml after dilution in media at the above pH. (Details are given in Materials and Methods.)

low temperature (4°), and for 4 days at 22°. Replication of duck influenza viruses in the intestinal tract has also been demonstrated by Slemons and Easterday (personal communication); these investigators have used fluorescent antibody techniques to show that duck influenza viruses multiply in the cells lining the large and small intestines.

The duck influenza virus (Hav7 Neq2) concentrated from the feces was roughly spherical in morphology and was uniform in size and shape. On the other hand, duck/Memphis influenza virus contained a heterogeneous array of virus particles. These results suggest that the morphology of influenza viruses from the intestines of ducks depends on the virus strain studied. Some strains are uniform in size and shape while other strains are very heterogeneous. The two virus strains examined retained their predominantly spherical or filamentous forms after subsequent passages in chicken embryos; after four passages the Hav3 Nav6 virus was still predominantly filamentous with some spherical forms. Studies by Ada *et al.*, (1958) showed that the morphological characteristics of influenza virus are a genetic trait and this is borne out in these studies.

To date there is no evidence that human or mammalian influenza viruses will replicate in the intestinal tract of their natural hosts. The human strains tested in this study replicated in the respiratory tract of ducks but did not replicate in the intestinal tract. Studies on vaccination of humans with influenza virus by ingestion of virus in enteric coated capsules gave no evidence that human influenza viruses replicated in the intestinal tract (Channock, personal communication).

It is not known whether avian influenza viruses will replicate in the intestinal tract of mammalian hosts. Providing that these viruses can replicate in mammalian hosts the possibility of producing recombinant influenza viruses containing the surface antigens of human strains and the growth potential of an avian virus could be considered as a potential means of forming a vaccine strain.

Live influenza vaccines (see review by Murphy *et al.*, 1976) that replicate in the upper respiratory tract and contain known genetic lesions and that can be reassorted

by recombination after an antigenic change are the most likely contenders for future human vaccines. The possibility of an influenza vaccine strain that replicates in the intestinal tract of man is, as yet, only conjectural.

Influenza A viruses from humans lose their infectivity below pH 5 (Chagnon *et al.*, 1965), whereas equine strains show a fall in infectivity between pH 4 and 5. The present studies show that duck/Memphis/546/74 influenza virus was relatively stable at pH 4.0, whereas the human strains tested were destroyed at this pH. The relative stability of this virus probably permits these strains to pass through the digestive tract of the duck. Even though the pH of the gizzard of ducks can be as low as 2.9 (a pH that will inactivate duck influenza viruses), the rapid passage of virus mixed with food through the digestive tract must permit at least a proportion of the virus from being exposed to the low pH and bile salts. Thus in the above studies, when high doses of duck/Memphis/74 influenza virus or of human influenza viruses were fed to ducks, residual infectivity was detected 1 hr later in fecal samples from birds infected with the duck influenza virus but the human strains failed to transit the digestive tract.

The high concentration of duck influenza viruses in fecal material and the relatively long stability of the virus in unchlorinated river water offer a logical mechanism for transmission of avian influenza viruses from feral ducks to domestic avian and mammalian species. In these experiments muscovey ducks shed 6.4 g of fecal material per hr with an infectivity titer of 7.8 log EID_{50}/g. In a 24-hr period one duck would shed approximately 10^{10} EID_{50} with a half-life of at least 24 hr; if this was diluted into the Mississippi River (average flow rate 1.4×10^6 liters/sec) the virus content would be insignificant but in a small lake with hundreds of ducks roosting in one area the virus concentration in that locality could be high.

None of the duck influenza viruses tested caused signs of disease in the feral or domestic ducks tested, but it is possible they could cause disease in other species. Thus, A/turkey/Oregon/71 (Hav1 Nav2) that caused mild respiratory infections in turkeys with low mortality Beard and Easterday, 1972) was a new virus that had not previously been reported. The recent isolation of an antigenically identical avirulent virus from feral ducks (Hav1 Nav2) (Hinshaw, personal communication) suggests that the A/turkey/Oregon/71 strain was introduced in turkeys by feral ducks, or alternatively, that the feral ducks contracted the virus from turkeys. Feral ducks may play a role in the transmission of economically important diseases of animals.

The demonstration in this study that most of the human influenza viruses tested could replicate in the upper respiratory tract of ducks offers the possibility of genetic interaction between human and avian strains in this species. Swine influenza viruses can also replicate in ducks offering the possibility of transmission of these viruses over long distances by migrating birds.

The susceptibility of ducks to infection with human influenza viruses and to a wide range of different avian influenza viruses, suggests that feral and domestic ducks may play an important role in the ecology of influenza A viruses. Thus viruses antigenically related to the H2 human subtype have been isolated from ducks (H2 Nav2 and H2 Nav6) (Webster *et al.*, 1975), and the H3 human subtype is related antigenically to A/duck/Ukraine/1/63 (Hav7 Neq2) (Coleman *et al.*, 1968; Laver and Webster, 1973). The isolation of many different influenza A viruses from feral ducks (Slemons and Easterday, 1975) and from domestic ducks in Hong Kong (Shortridge *et al.*, 1977), together with the wide range of viruses that will replicate in ducks, suggests that the duck may be the natural host of many influenza A viruses.

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REFERENCES

ADA, G. L., PERRY, B. T., and ABBOT, A. (1958). Biological and physical properties of the Ryan strain of filamentous influenza virus. *J. Gen. Microbiol.* **19**, 23–39.

BEARD, C. W., and EASTERDAY, B. C. (1973). A/Turkey/Oregon/71: An avirulent influenza isolate with the hemagglutinin of Fowl Plague virus. *Avian Dis.* **17**, 173–181.

CHAGNON, A., GILKER, J. C., and BOUDREAULT, A. (1965). Infectivity and hemagglutinating properties of influenza virus at varying pH. *Can. J. Microbiol.* **11**, 845–851.

CHOPPIN, P. W., MURPHY, J. S., and TAMM, I. (1960). Studies of two kinds of virus particles which comprise influenza A2 virus strains. III. Morphological characteristics: Independence to morphological and functional traits. *J. Exp. Med.* **112**, 945–952.

CHU, C. M., DAWSON, I. M., and ELFORD, W. J. (1949). Filamentous forms associated with newly isolated influenza virus. *Lancet* **1**, 602.

COLEMAN, M. T., DOWDLE, W. R., PEREIRA, H. G., SCHILD, G. C., and CHANG, W. K. (1968). The Hong Kong/68 influenza A2 variant. *Lancet* **2**, 1384–1386.

EASTERDAY, B. C. (1975). Animal influenza. *In* "The Influenza Viruses and Influenza" (E. D. Kilbourne, ed.), pp. 449–481. Academic Press, New York.

HINSHAW, V. S., BEAN, W. J., JR. WEBSTER, R. G., and EASTERDAY, B. C. (1978). The prevalence of influenza viruses in swine and the antigenic and genetic relatedness of influenza viruses from man and swine. *Virology*. In press.

LAVER, W. G. (1969). Purification of influenza virus. *In* "Fundamental Techniques in Virology" (K. Habel and N. P. Salzman, eds.), pp. 82–86. Academic Press, New York.

LAVER, W. G., and WEBSTER, R. G. (1972). Studies on the origin of pandemic influenza. II. Peptide maps of the light and heavy polypeptide chains from the hemagglutinin subunits of A2 influenza viruses isolated before and after the appearance of Hong Kong influenza. *Virology* **48**, 445–455.

LAVER, W. G., and WEBSTER, R. G. (1973). Studies on the origin of pandemic influenza. III. Evidence implicating duck and equine influenza viruses as possible progenitors of the Hong Kong strain of human influenza. *Virology* **51**, 383–391.

MURPHY, B. R., SPRING, S. B., and CHANNOCK, R. M. (1976). Live vaccine: Production and use. *In* "Influenza: Virus, Vaccines and Strategy" (P. Selby, eds), pp. 179–197. Sandoz Institute for Health and Socio-Economic Studies and Academic Press, New York.

SHORTRIDGE, K. F., BUTTERFIELD, W. K., WEBSTER, R. G., and CAMPBELL, C. H. (1977). Isolation and characterization of influenza A viruses from avian species in Hong Kong. *Bull. W. H. O.* **55**, 15–20.

SLEMONS, R. D., and EASTERDAY, B. C. (1975). The natural history of type-A influenza viruses and wild waterfowl. *In* "Proceedings of the Third International Wildlife Disease Conference, Munich, August, 1975" (L. A. Page, eds.), pp. 215–224. Plenum Publishing Co., New York.

WEBSTER, R. G. (1976). Strain surveillance in animals and birds. *In* "Influenza: Virus, Vaccines and Strategy" (P. Selby, eds.), pp. 33–43. Sandoz Institute for Health and Socio-Economic Studies and Academic Press, New York.

WEBSTER, R. G., and CAMPBELL, C. H. (1974). Studies on the origin of pandemic influenza. IV. Selection and transmission of "new" influenza viruses *in vivo*. *Virology* **62**, 404–413.

WEBSTER, R. G., MORITA, M., PRIDGEN, C., and TUMOVA, B. (1976). Ortho- and paramyxoviruses from migrating feral ducks: Characterization of a new group of influenza A viruses. *J. Gen. Virol.* **32**, 217–225.

WEBSTER, R. G., LAVER, W. G., and TUMOVA, B. (1975). Studies on the origin of pandemic influenza viruses. V. Persistence of Asian influenza virus hemagglutinin (H2) antigen in nature. *Virology* **67**, 534–543.