Bull. World Health Organ. } 1974, **51**, 325-332
Bull. Organ. mond. Santé }

A new avian influenza virus from feral birds in the USSR: Recombination in nature?

ROBERT G. WEBSTER,[1] VALENTINA A. ISACHENKO,[2] & MARTHA CARTER[3]

Six avian influenza A viruses isolated in the USSR were characterized antigenically by using specific antisera to the isolated surface subunits of the known reference strains. Three of the viruses, all isolated from the same region, were characterized as A/duck/Ukraine/63 (Hav7 Neq2), and a virus isolated from a crow was of the Hong Kong/68 (H3 N2) type. The remaining two viruses were novel in that they possessed Hav7 Nav2 antigens, a combination that has not previously been reported. It is suggested that these new influenza viruses might have arisen by recombination in nature between the A/duck/Ukraine/63 (Hav7 Neq2) and A/tern/So. Africa/61 (Hav5 Nav2) strains of avian influenza viruses.

INTRODUCTION

The origin of new strains of influenza A virus that appear in man at irregular intervals of 10–15 years remains unknown. There is increasing evidence that these new viruses do not arise by mutation from existing human strains (10), but rather arise by recombination between the influenza viruses of man, lower mammals, and birds. Studies in animals and birds using antigenically distinct influenza A viruses (16, 18, 19) have demonstrated that recombination and selection of new strains of influenza viruses can occur *in vivo*, under simulated conditions of natural transmission. Such studies provide only circumstantial evidence that antigenic shift in human influenza viruses occurs by recombination. More definitive evidence might be obtained by detecting recombination between different influenza A viruses in nature. The present study provides some evidence that is consistent with recombination between two different avian influenza A viruses in nature.

Several influenza A viruses isolated from avian species in the USSR (8, 12, 21) have been characterized antigenically by using " monospecific " antisera to the isolated haemagglutinin antigens of all the reference strains of influenza A virus. An influenza virus possessing the haemagglutinin antigen from duck/Ukraine/1/63 (Hav7 Neq2) and the neuraminidase antigen from tern/So.Africa/61 (Hav5 Nav2), which was isolated from ducks and terns in the USSR, suggests that recombination could have occurred in nature.

MATERIALS AND METHODS

All of the reference strains of influenza A virus (4) except A/turkey/England/63 (Hav1 Nav3) were used in this study. In addition, the following influenza A virus isolates from the USSR (8, 12, 21) were studied; for simplicity they are referred to in this article by the abbreviations given below:

duck/Chabarovsk/1610/72 = duck/1610
duck/Chabarovsk/698/73 = duck/698
heron/Chabarovsk/700/73 = heron/700
tern/Turkmenistan/18/73 = tern/18
duck/Chabarovsk/1574/72 = duck/1574
crow/Kazan/20/72 = crow/20

The viruses were grown in the allantoic cavity of 11-day-old chick embryos. They were concentrated and partially purified by adsorption and elution from chicken erythrocytes and finally sedimented through a sucrose gradient (10–60% sucrose in phosphate-buffered saline, pH 7.2).

[1] Member, Laboratories of Virology and Immunology, St Jude Children's Research Hospital, P.O. Box 318, Memphis, TN, USA.

[2] Research Scientist, Ivanovskij Institute of Virology Moscow, USSR.

[3] Research Associate, Laboratories of Virology and Immunology, St Jude Children's Research Hospital, P.O. Box 318, Memphis, TN, USA.

Preparation of antigenic hybrid viruses

Antigenic hybrid influenza viruses were prepared as previously described (15).

Preparation of isolated haemagglutinin and neuraminidase subunits

Haemagglutinin subunits were isolated from wild-type virus or from antigenic hybrids possessing A/Bel/42 (H0 N1) haemagglutinin. Neuraminidase subunits were isolated from influenza viruses possessing A/NWS/33 (H0 N1) haemagglutinin. These subunits migrate as cations during electrophoresis of virus particles disrupted with sodium dodecyl sulfate (SDS), and permit isolation of biologically active pure subunits (9, 10). Since the haemagglutinin subunits of the H0 N1 (PR8) and H1 N1 (FM1) influenza viruses are denatured by SDS, it was necessary to isolate these subunits by electrophoretic separation of viruses treated with Tween 20 and sodium deoxycholate (3). The fractions containing biologically active subunits were eluted from cellulose acetate, precipitated with cold ethanol (10), and taken up in physiological saline.

Antisera

Hyperimmune antisera to the isolated haemagglutinin and neuraminidase subunits were made with antigen emulsified in Freund's complete adjuvant. The antigen mixture was injected into goats—into the tail as well as intramuscularly. The animals received a second dose of antigen in adjuvant plus an intravenous injection in saline 30 days later. Blood samples were collected 7 days after the second injection and the serum stored at −20°C. Antisera to antigenic hybrid viruses were prepared in rabbits as previously described (17).

Serological tests

Haemagglutination titrations (H) and haemagglutination inhibition (HI) tests were performed in plastic trays with sera treated with receptor-destroying enzyme (RDE), as previously described (7). Neuraminidase titrations (N) were done by the method of Warren (14), except that the colour was extracted into butanol containing 50 ml of concentrated hydrochloric acid per litre (1). Neuraminidase-inhibition (NI) tests were performed as previously described (2), the virus antibody and substrate (fetuin) being incubated at 37°C for 20 hours prior to assay for free sialic acid. Intact influenza viruses were used in all tests.

Immunodiffusion tests were performed in agarose (A 37) (15 mg/litre) dissolved in phosphate-buffered saline (PBS) (pH 7.2) containing 1 mg of Sarkosyl NL97 and 1 mg of sodium azide per litre. Purified virus (HA $\geqslant$ 6.0 $\log_{10}$ units/ml) was disrupted with Sarkosyl NL97 (1 mg/litre) and the same concentration of Sarkosyl was added to the antisera before addition to the plates to prevent nonspecific precipitation bands. The precipitation lines were either photographed without staining or, if the lines were faint, were first stained with Coomassie Brilliant Blue (1 mg/litre) in a mixture of methanol, water, and acetic acid (5 : 5 : 1, by vol.) and decolorized in the same solution.

Chick embryo fibroblast cultures

Cultures of chick embryo fibroblasts were prepared as described by Darlington et al. (6). The cultures were inoculated 2 days after preparation and incubated at 38°C.

Inoculation of chickens

Day-old chickens (mixed breeds) were inoculated intratracheally with approximately 5.0 $\log_{10}$ EID_{50} of each of the USSR avian influenza viruses. Tracheal swabs were collected daily and assayed for infectious virus in 11-day-old chick embryos. The chickens were observed for overt signs of disease.

RESULTS

Characterization of the haemagglutinin antigens

Initial characterization of 6 avian influenza viruses with antisera to the isolated haemagglutinin of each of the reference strains of influenza A virus (Table 1) showed that these viruses reacted to high titres with antisera to Hav7, to slightly lower titres with H3, and to considerably lower titres with Heq2. The low levels of cross-reactions obtained between the crow/20 and duck/1610 strains and many of the antisera suggest that these reactions may be due to the high sensitivity of these strains to inhibitors found in the sera.

Details of cross-reactions with antisera to the various H3 variants (Table 2) indicated that the influenza virus isolated from a crow (crow/20) gave strong cross-reactions with antisera to the Hong Kong/68 strain of human influenza and gave lower levels of cross-reactions with antisera to England/42/72 and Port Chalmers/1/73. This virus (crow/20) also reacted with antiserum to duck/Ukraine/63 and to equine 2 influenza virus.

Table 1. Identification of recent influenza A virus isolates in haemagglutination tests using antisera to the isolated haemagglutinin subunits of all reference strains

Specific antiserum to:	Haemagglutination-inhibition titres with the following viruses:					
	Duck/1610	Duck/698	Heron/700	Tern/18	Duck/1574	Crow/20
H0 (PR8/34)	150	< 20	< 20	< 20	< 20	100
H1 (FM1/47)	80	< 20	< 20	< 20	< 20	74
H2 (Sing/57)	28	< 20	< 20	< 20	< 20	37
H3 (HK/68)	3 400	480	720	900	1 100	> 10 000
HSW1 (SW/30)	< 20	< 20	< 20	< 20	< 20	56
Heq1 (Eq1/56)	28	< 20	< 20	< 20	< 20	110
Heq2 (Eq2/63)	2 000	120	90	170	150	2 400
Hav1 (FPV/27)	< 20	< 20	< 20	< 20	< 20	110
Hav2 (Chick/N/49)	< 20	< 20	< 20	< 20	< 20	110
Hav3 (Duck/Eng/56)	< 20	< 20	< 20	< 20	< 20	< 20
Hav4 (Duck/Cz/56)	< 20	< 20	< 20	< 20	< 20	< 20
Hav5 (Tern/SA/61)	< 20	< 20	< 20	< 20	< 20	220
Hav6 (Turkey/Mass/65)	< 20	< 20	< 20	< 20	< 20	80
Hav7 (Duck/Ukr/63)	12 000	2 900	2 300	2 000	2 500	> 10 000
Hav8 (Turkey/Ont/6118/68)	< 20	< 20	< 20	< 20	< 20	< 20

[a] Values represent reciprocal of serum dilution causing 50 % inhibition of 4 haemagglutinating doses of virus.

Table 2. Identification of influenza A viruses from avian sources

Antiserum to:	Haemagglutinin-inhibition titres with the following viruses: [a]										
	HK/68	Eng/42$_{(H)}$-Bel$_{(N)}$	Port Chalmers/73	Equine 2	Duck/Ukr$_{(H)}$-Bel$_{(N)}$	Duck/1610	Duck/698	Heron/700	Tern/18	Duck/1574	Crow/20
HK68 (H3) [b]	75 000	35 000	5 100	< 100	1 600	3 400	200	280	430	190	41 000
Eng/42/72 (Eng$_{(H)}$-Eq1$_{(N)}$) [c]	1 700	5 900	2 200	< 100	70	140	< 100	50	50	< 50	13 000
Port Chalmers/73 (Parental) [d]	650	2 200	4 500	< 100	230	650	140	140	100	130	560
Equine 2 (Heq2)	5 100	4 500	3 400	4 800	1 700	650	70	70	70	50	2 400
Duck/Ukr (Hav7)	35 500	19 000	8 900	300	20 400	12 000	1 700	2 200	1 100	850	18 000
Duck/1610 (Parental)	2 200	1 100	400	200	1 100	1 500	120	140	90	< 100	2 200

[a] Values represent reciprocals of serum dilution causing 50 % inhibition of 4 haemagglutinating doses of virus.
[b] Antiserum to isolated haemagglutinin subunits.
[c] Antiserum to recombinant influenza virus possessing an irrelevant neuraminidase.
[d] Antiserum to parental virus.

Table 3. Identification of the neuraminidase antigen on influenza viruses from avian sources

Antiserum to:	Neuraminidase inhibition titres with the following influenza viruses: [a]						
	Equine 2	Duck/1610	Duck/698	Heron/700	Tern/18	Duck/1574	Tern/SA/61
Equine $1_{(H)}$-Equine $2_{(N)}$ (Heq1 Neq2)	1 200	1 000	1 000	1 000	< 10	< 10	< 10
Tern/SA/61 (Hav5 Nav2)	< 10	< 10	< 10	< 10	800	1 000	1 000

[a] Values represent the reciprocal of the dilution of serum causing 50 % inhibition of virus giving an approximate absorbance reading of 0.5.

The other avian influenza viruses (duck/1610, duck/698, duck/1574, heron/700, tern/18) gave strong reactions with antiserum to duck/Ukraine/63 (Table 2) but also reacted with antiserum to Hong Kong and to a lesser degree with antiserum to England/42/72, Port Chalmers/1/73, and equine 2.

From the results reported in Tables 1 and 2 it was difficult to know with certainty whether the avian strains are more closely related to duck/Ukraine/63 or to Hong Kong/68 influenza viruses. Immunodiffusion studies with antiserum to the isolated haemagglutinin of duck/Ukraine/63 influenza virus (Fig. 1A) indicated that duck/1610, duck/1574, tern/18, and duck/Ukraine/63 influenza viruses gave two lines of precipitation and were therefore identical with duck/Ukraine/63 influenza virus. Hong Kong/68 influenza virus in this test gave only one line of precipitation and was therefore different antigenically from the avian viruses. Antiserum to the isolated haemagglutinin of Hong Kong/68 influenza virus (Fig. 1B) showed that duck/1610, duck/1574, tern/18, and heron/700 were identical with duck/Ukraine/63 but gave a definite spur or line of partial identity with Hong Kong/68 influenza virus.

The influenza virus from a crow (crow/20) gave a line of identity with Hong Kong/68 influenza virus (Fig. 1C) and a weak spur with duck/1610 influenza virus. (The concentration of crow/20 virus was low because this virus grows to low levels in embryonated eggs, hence the line of precipitation was weak.)

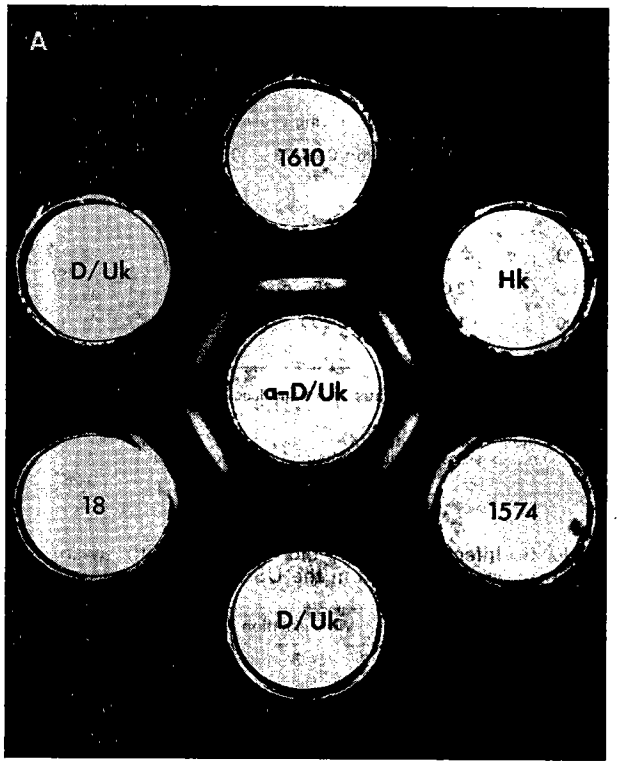
The above studies indicate that the haemagglutinin subunits on duck/698, duck/1610, duck/1574, tern/18, and heron/700 influenza viruses are similar to duck/Ukraine/63 influenza virus and belong to the Hav7 subgroup. The haemagglutinin of the virus from a crow appears to be of the H3 subtype and was most closely related to Hong Kong/68 influenza virus.

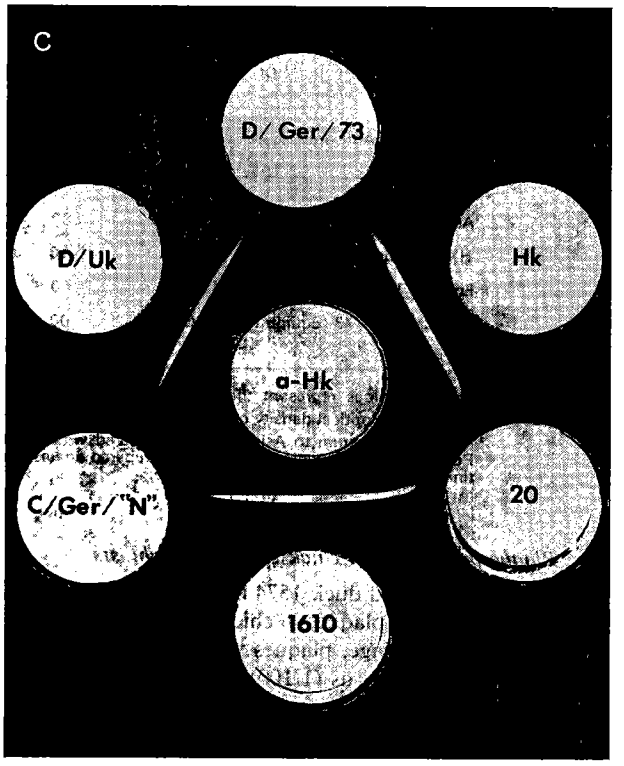
Characterization of the neuraminidase antigens

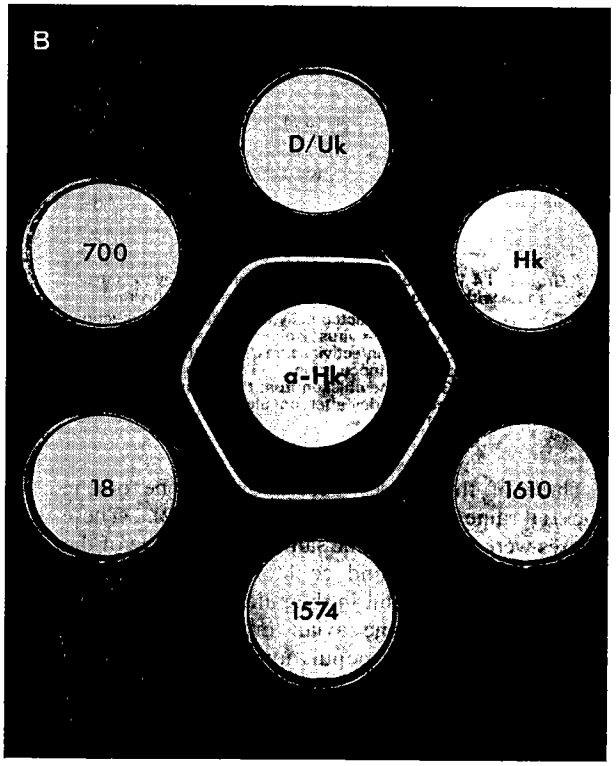
The neuraminidase antigens were characterized in neuraminidase inhibition tests using antisera that possessed no antibodies to the homologous haemagglutinin. The results (Table 3) showed that the neuraminidase antigens on duck/698, duck/1610, and heron/700 influenza viruses were closely related to equine 2 influenza virus and were therefore of the Neq2 subtype. The neuraminidase antigens on duck/1574 and tern/18 influenza viruses were related to the neuraminidase on tern/So. Africa/61 influenza virus (Table 3; Fig. 1D) and were of the Hav2 subtype.

The neuraminidase on the crow/20 influenza virus was inhibited by antibodies to the N2 subtype (Table 4) and appeared to be most closely related to Hong Kong/68 neuraminidase.

Fig. 1. Surface antigens on avian influenza viruses as characterized by double immunodiffusion. The preparation of viruses, antisera, and the immunodiffusion plates is described in *Materials and Methods*. The precipitin patterns were photographed without staining except for Fig. 1D, for which the gel was stained with Coomassie brilliant blue. The centre wells contained antiserum to the isolated haemagglutinin of the virus specified, with the exception of Fig. 1D, which contained antiserum to the whole virus. Abbreviations: 18, tern/18; 20, crow/20; 700, heron/700; 1574, duck/1574; 1610, duck/1610; C/Ger/« N », chicken/Germany « N »/49 (Hav2 Neq2); D/Ger/73, duck/Germany/1215/73 (H2 Nav2); D/Uk, duck/Ukraine/1/63 (Hav7 Neq2); HK, Hong Kong/68 (H3 N2); tern/S.A., tern/South Africa/61 (Hav5 Nav2). In Fig. 1D, Ha is haemagglutinin, NA neuraminidase; M and NP are probably precipitin lines to matrix and nucleoprotein antigens.


A
1610
D/Uk
Hk
α-D/Uk
18
1574
D/Uk


C
D/Ger/73
D/Uk
Hk
α-Hk
C/Ger/"N"
20
1610


B
D/Uk
700
Hk
α-Hk
18
1610
1574

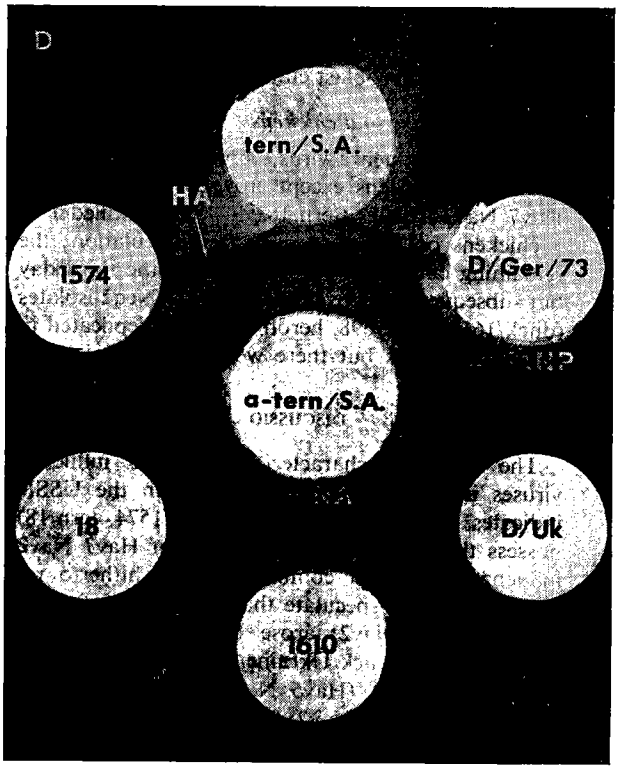

D
tern/S.A.
1574
D/Ger/73
α-tern/S.A.
18
D/Uk
1610

Table 4. Identification of the neuraminidase antigen on an influenza virus isolated from a dove

Antiserum to: [b]	Neuraminidase inhibition titres with the following viruses: [a]				
	Asian/57	Hong Kong/68	Eng/42/72	Port Chal/73	Crow/20
Asian/57 (isolated N2)	1 000	100	150	30	300
HK/68 (Equine $1_{(H)}$-$HK_{(N)}$)	10	200	300	100	500
Eng/42/72 (Equine $1_{(H)}$-$Eng/42_{(N)}$)	10	100	2 000	150	400
Port Chalmers/73 (Equine $1_{(H)}$-$P/C_{(N)}$)	100	100	1 500	2 000	100

[a] Values represent the reciprocal of the dilution causing 50 % inhibition of virus neuraminidase giving an approximate optical density of 0.5.

[b] Antiserum to Asian/57 was prepared with isolated neuraminidase; antisera to HK/68, England/42/72 and Port Chalmers/73 neuraminidase were prepared against antigenic hybrids possessing equine/Miami/1/63 haemagglutinin and the appropriate neuraminidase.

Plaque production in chick embryo fibroblasts

The tern/18 and duck/1574 influenza virus isolates produced turbid plaques in chick embryo fibroblasts. On the first passage, plaques were obtained only at low dilutions of virus (1/100), but after the plaques had been picked out and the viruses passaged in chick embryos there was good correlation between plaque production and infectivity in chick embryos. The other strains of avian influenza viruses from the USSR used in this study failed to produce plaques in chick embryo fibroblast cultures.

Infection of 1-day-old chickens

All of the influenza viruses under study replicated in day-old chickens except crow/20 (Table 5). The Hav7 Nav2 isolates (tern/18, duck/1574) killed 1 of 4 chickens on the third day after inoculation; the remaining chickens were lethargic on the third day but subsequently recovered. The Hav7 Neq2 isolates (duck/1610, duck/698, heron/700) each replicated in day-old chickens but there were no overt signs of disease.

Table 5. Infection of day-old chickens with avian influenza viruses isolated in the USSR

Viruses inoculated: [a]	Virus isolation (days) [b]					
	1	2	3	4	5	6
Tern 18	+	0	+ (3.5)	+	+	+
Duck 1574	0	+	+ (4.0)	+	+	+
Duck 1610	+	+	+ (2.5)	+	+	+
Duck 698	+	+	+ (3.5)	+	+	+
Heron 700	+	+	+ (3.0)	+	+	+
Crow 20	0	0	0	0	0	0

[a] Groups of 4 1-day-old chickens were inoculated by the intratracheal route with approximately 5.0 $\log_{10}$ EID_{50} of each virus.

[b] Tracheal swabs were collected daily and assayed for infectious virus in chick embryos (+ = virus isolation; 0 = no virus). The figures in brackets give the infectivity titres/ml of the samples collected on the third day after inoculation. One chicken from the group infected with tern 18 and one chicken from the group infected with duck 1574 died on the third day after inoculation.

DISCUSSION

The antigenic characterization of 6 influenza viruses isolated from avian sources in the USSR indicates that 2 of the viruses (duck/1574, tern/18) possess the antigenic characteristics of Hav7 Nav2 influenza viruses—a combination not hitherto reported. One can speculate that these new influenza viruses (Hav7 Nav2) arose by recombination in nature between duck/Ukraine/63 (Hav7 Neq2) and tern/So. Africa/61 (Hav5 Nav2). The isolation of these viruses (Hav7 Nav2) from a duck and a tern is consistent with this idea.

Three of the viruses were shown to be of the duck/Ukraine/63 subtype (Hav7 Neq2). These viruses were isolated in the same region as one of the Hav7 Nav2 viruses and could have provided the haemagglutinin subunit in the putative recombinant. Thus, more than one avian influenza virus can circulate in the avian population at the same time, creating ideal conditions for mixed infection leading to the appearance of a new recombinant influenza virus.

The remaining influenza virus, crow/20, was most closely related antigenically to Hong Kong/68 and offers another example of the ubiquity of the Hong Kong strains of influenza in human, animal, and avian species. The possibility that this virus came from a laboratory source cannot be completely ruled out, although its poor growth characteristics in chick embryos and its isolation in a laboratory in the USSR that did not maintain Hong Kong influenza virus do not favour this notion.

Since the haemagglutinin subunits of duck/Ukraine/63, equine 2 (Heq2), and Hong Kong/68 (H3) influenza viruses are fairly closely related immunologically (5, 11, 13, 20), it is difficult to identify new virus isolates that fall into these subgroups. However, with specific antisera to the isolated haemagglutinin subunits and by using haemagglutination inhibition in conjunction with gel diffusion, it was possible to assign the avian virus isolates to their appropriate subgroups.

The isolation of different influenza viruses from inapparent infection of avian species, together with the antigenic similarity between Hav7, Heq2, and H3, suggests that a large number of viruses that exist in nature could be possible progenitors of future human pandemic viruses. The ecological approach to the study of influenza viruses may establish the number of different subtypes that exist in nature and may offer definitive evidence of the origin of new strains.

ACKNOWLEDGEMENTS

These studies were supported by research grant AI 08831 and contract AI 32516 from the National Institute of Allergy and Infectious Diseases, by Childhood Cancer Clinical Center grant CA 08480 from the National Cancer Institute, by the World Health Organization, and by ALSAC. The authors thank the US–USSR Scientific Exchange Program for permitting these cooperative studies to be done.

RÉSUMÉ

UN NOUVEAU VIRUS GRIPPAL AVIAIRE ISOLÉ À PARTIR D'OISEAUX SAUVAGES EN URSS: RECOMBINAISON DANS LA NATURE?

De nouvelles souches de virus grippaux humains peuvent apparaître à la suite d'une recombinaison entre virus grippaux de l'homme, de mammifères inférieurs ou d'oiseaux. D'où l'intérêt d'isoler et de caractériser les virus grippaux que l'on rencontre dans des populations animales partout dans le monde.

On a isolé en URSS six virus aviaires de la grippe A qui ont été caractérisés antigéniquement à l'aide d'antisérums spécifiques des antigènes de surface de souches de référence connues. Trois de ces virus, isolés dans la même région, ont été identifiés comme A/duck/Ukraine/63 (Hav7 Neq2); un autre, isolé chez un corbeau, était du type Hong Kong/68 (H3 N2); les deux virus restants possédaient les antigènes Hav7 Nav2, une combinaison qui n'a pas été signalée jusqu'à présent. On peut supposer que ces deux nouveaux virus grippaux (Hav7 Nav2) sont issus d'une recombinaison dans la nature entre les souches aviaires A/duck/Ukraine/63 (Hav7 Neq2) et A/tern/So. Africa/61 (Hav5 Nav2).

Les nouveaux virus (Hav7 Nav2) ont été isolés dans la même région que les virus Hav7 Neq2. Il semble donc que des virus grippaux aviaires différents circulent en même temps dans les populations d'oiseaux, créant des conditions idéales pour des infections mixtes aboutissant à l'apparition de nouveaux recombinants.

REFERENCES

1. AMINOFF, D. *Biochem. J.*, **81**: 384 (1961).
2. AYMARD-HENRY, M. ET AL. *Bull. World Health Organ.*, **48**: 199 (1973).
3. BAKER, N. ET AL. *J. Virol.*, **11**: 137 (1973).
4. *Bull. World Health Organ.*, **45**: 119 (1971).
5. COLEMAN, M. T. ET AL. *Lancet*, **2**: 1384 (1968).
6. DARLINGTON, R. W. ET AL. *J. gen Virol.*, **9**: 169 (1970).

7. FAZEKAS DE ST. GROTH, S. & WEBSTER, R. G. *J. exp. Med.*, **124**: 331 (1966).
8. ISACHENKO, V. A. ET AL. *Ecol. Vir.*, **2**: (1974).
9. LAVER, W. G. *J. mol. Biol.*, **9**: 109 (1964).
10. LAVER, W. G. & WEBSTER, R. G. *Virology*, **48**: 445 (1972).
11. LAVER, W. G. & WEBSTER, R. G. *Virology*, **51**: 383 (1973).
12. LVOV, D. K. ET AL. *Ecol. Vir.*, **2** (1974).
13. TUMOVA, B. & EASTERDAY, B. C. *Bull. World Health Organ.*, **41**: 429 (1969).
14. WARREN, L. *J. biol. Chem.*, **234**: 1971 (1959).
15. WEBSTER, R. G. *Virology*, **42**: 633 (1970).
16. WEBSTER, R. G. & CAMPBELL, C. H. *Virology*, **62**: 404 (1974).
17. WEBSTER, R. G. & LAVER, W. G. *Virology*, **48**: 433 (1972).
18. WEBSTER, R. G. ET AL. *Virology*, **44**: 317 (1971).
19. WEBSTER, R. G. ET AL. *Virology*, **51**: 149 (1973).
20. ZAKSTELSKAYA, L. Ya. ET AL. *Am. J. Epidemiol.*, **90**: 400 (1969).
21. ZAKSTELSKAYA, L. Ya. ET AL. *Ecol. Vir.*, **2** (1974).