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Antibodies to Influenza Viruses (including the Human A2/Asian/57 Strain) in Sera from Australian Shearwaters *(Puffinus pacificus)* *

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*Sera were collected from 320 shearwaters (*Puffinus pacificus chlororhynchus*) nesting on two islands off the east coast of northern Australia in December 1969. About 10 % of these sera specifically inhibited the neuraminidase of the 1957 strain of human influenza (A2/Asian/57), some to high titre.*

The neuraminidase of A2/Hong Kong/68, which has shown considerable drift in man since 1957, was inhibited to a lesser extent by the shearwater sera while the neuraminidase of influenza A0 (Bel strain) was not inhibited at all. The sera showed no evidence of short-lived antibody to the internal antigen of influenza type A, suggesting that the birds had been infected a considerable time previously with an influenza virus possessing neuraminidase identical to that of the virus causing the human 1957 Asian pandemic. On the other hand, about 10 % of the sera from one of the islands was found to be positive in immunodiffusion tests with influenza type A soluble internal antigen (ribonucleoprotein), suggesting that another, much more recent, epidemic of avian influenza had occurred on this island only. The surface antigens of the virus responsible for this epidemic have not so far been identified.

Observations suggest that avian influenza may be common in wild birds and the finding of antibody to an antigen of human influenza virus in shearwaters is consistent with the idea that human influenza pandemics may originate from avian or animal reservoirs.

Pandemics of influenza type A occur in man at irregular intervals but the origin of the virus strains causing these pandemics is not known. It has been suggested (Pereira, 1969) that the new strains arise from animal or avian influenza viruses either by direct mutation or by a process of genetic recombination with human strains, producing viruses with virulence for man but possessing the surface antigens of the animal or avian strains. Evidence for the existence of animal or avian viruses with surface antigens similar to those of viruses infecting man has therefore been sought. Webster & Pereira (1968) have described 4 avian influenza viruses having a neuraminidase which was immunologically similar to that of the Asian strain of human influenza (A2/Sing./1/57) and Schild et al. (1969) have described a duck influenza virus with a neuraminidase similar to that of human A0 influenza virus strains. Production in the laboratory of recombinant viruses (antigenic hybrids) from avian or equine strains and human influenza viruses has been described (Easterday et al., 1969; Kilbourne, 1968).

In this report, the presence of antibodies against the internal antigen of type-A influenza virus and against the neuraminidase of human influenza A2/Asian/57 in the sera of wild birds found off the coast of northern Australia is described.

MATERIALS AND METHODS

Sera were collected from shearwaters (mutton birds) (*Puffinus pacificus chlororhynchus*) nesting on Heron and Tryon islands. These are coral cays situated in the Capricorn group of islands at the southern end of the Great Barrier Reef about 45 miles (72 km) off the coast of Queensland. Heron Island lies at 23°27′S. latitude and 151°55′E. longitude while the position of Tryon Island is 23°15′S. latitude, 151°47′E. longitude. Many thousands of

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shearwaters inhabit the islands during the breeding season and their behaviour and nesting habits have been described by Gross et al. (1963).

Sera from 201 birds on Tryon Island and 119 birds on Heron Island were collected between 15 December 1969 and 3 January 1970. The sera were stored without refrigeration for periods of up to 3 weeks before they were tested, sodium azide being added to prevent bacterial growth. The sera were then tested against different strains of influenza virus for the presence of neuraminidase-inhibiting antibodies and, in immunodiffusion tests, for the presence of antibodies to the type-specific internal ribonucleoprotein (RNP) antigen of influenza type A.

Neuraminidase-inhibition tests were carried out by mixing 0.01-ml samples of serum with 0.1 ml of virus suspended in calcium magnesium saline (Fazekas de St. Groth et al., 1958) and leaving the mixture to stand at 4°C for 5 hours. Fetuin was prepared by the method of Graham (1961); 2.5 mg were dissolved in 0.2 M sodium phosphate buffer, pH 5.9, and 0.1 ml was then added to the serum–virus mixture which was incubated at 37°C for 16 hours. The N-acetyl neuraminic acid liberated was assayed by the method of Warren (1959) modified so that the colour was extracted into *n*-butanol containing 5% (v/v) of concentrated hydrochloric acid (Aminoff, 1961). The concentration of virus was adjusted so that the amount used in each test was sufficient to give an optical density of 0.900 in a 1-cm cell at 549 nm after 16 hours' incubation with fetuin, as described above, in the absence of serum. By using such small amounts of neuraminidase, very low levels of antibody to the enzyme could be detected (Downie, 1970).

Double immunodiffusion tests were done in 1% agar gels (containing 5% sodium chloride) using a preparation of influenza type A soluble RNP antigen, prepared from infected allantoic fluid by precipitation at pH 4.5, and samples of the undiluted sera.

Sucrose-gradient centrifugation of sera to test for inhibitory activity was carried out in the following way. A sample of serum (0.1 ml) was layered on to a 5-ml linear sucrose gradient (10%–40% sucrose in 0.15 M sodium chloride) and centrifuged in a Spinco SW65 rotor [1] at 45 000 rev/min for 16 hours. Fractions were collected through an aperture at the bottom of the tube, dialysed against 0.15 M sodium chloride to remove sucrose (which interfered in the neuraminidase assay) and tested for inhibition of A2/Asian/57 neuraminidase, using X-7Fl virus as an enzyme source.

RESULTS

When tested by neuraminidase-inhibition tests, 18 out of the 320 sera inhibited 70% or more of the activity of A2/Asian/57 neuraminidase at an initial serum dilution of 1/10. Of these 18 sera, 5 inhibited more than 90% of the enzyme activity; 47 sera inhibited between 30% and 69% of the enzyme activity and the remainder (255 sera) had little (less than 30%) or no inhibitory activity for type-A2 neuraminidase (Table 1). Sera from each of the two islands contained about the same proportion of positives.

TABLE 1

SCREENING OF SERA [a] FOR ANTIBODY TO A2/ASIAN/57 NEURAMINIDASE

Inhibition of A2/Asian/57 neuraminidase (%)	No. of inhibiting sera
90–100	5
70–89	13
50–69	15
30–49	32
<30	255

[a] A total of 320 sera was tested at an initial dilution of 1/10. A recombinant virus, X-7F1, possessing A0 (NWS) type haemagglutinin and A2/Asian/57 type neuraminidase (Webster et al., 1968), was used as the enzyme source.

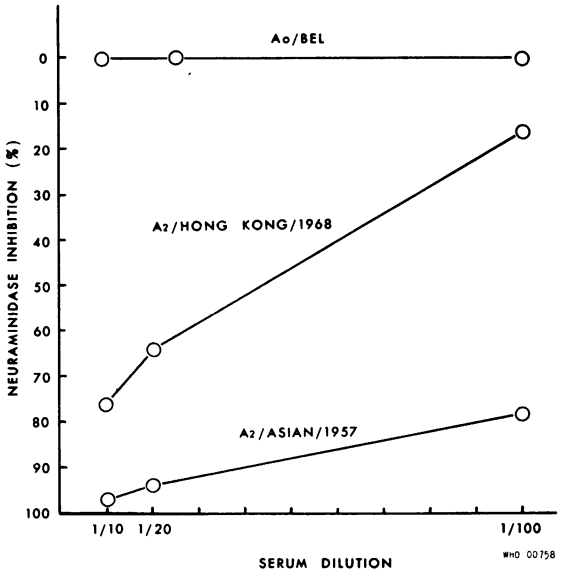
Titres of the 5 sera with the highest inhibitory activity for type A2/Asian/57 neuraminidase were determined in neuraminidase-inhibition tests using the enzymes of A2/Asian/57, A2/Hong Kong/68 and A0/Bel viruses. A sample of serum from a rabbit hyperimmunized with A2/Asian/57 neuraminidase isolated from a recombinant virus, X-7Fl (Kilbourne et al., 1968), was included for comparison. A titration curve for one serum is given in Fig. 1 and titres of all 5 sera are given in Table 2. Titres are expressed as the dilution of serum in the serum–virus mixture inhibiting 50% of the enzyme activity when measured under the conditions described above.

The serum (No. 179) with the highest titre (1/234) for A2/Asian/57 neuraminidase had a much lower titre when tested against the neuraminidase of the A2/Hong Kong/68 strain. This serum failed to inhibit the enzyme of the A0/Bel strain of influenza

[1] Supplied by Beckman Instruments Ltd, Palo Alto, Calif. USA.

FIG. 1

INHIBITION OF THE NEURAMINIDASE ACTIVITY OF VARIOUS STRAINS OF INFLUENZA VIRUS BY DIFFERENT DILUTIONS OF ANTISERUM FROM SHEARWATER No. 179 [a]



[a] Samples of serum, 10 μl, 5 μl and 1 μl, were mixed with 0.1 ml of each virus; the mixtures were kept at 4°C for 5 hours and then assayed for neuraminidase activity as described in the text.

TABLE 2

NEURAMINIDASE-INHIBITION TITRES OF THE SHEARWATER SERA [a] WITH VARIOUS INFLUENZA A VIRUSES

Serum	Viruses		
	X-7F1 (A2/Asian/57 neuraminidase)	A2/ Hong Kong/ 68	A0/Bel
59	1/190	1/50	<1/10
179	1/234	1/43	<1/10
185	1/130	1/13	<1/10
188	1/100	1/55	<1/10
233	1/80	1/33	<1/10
Rabbit antiserum [b]	1/10 000	1/2 000	<1/10

[a] The 5 sera giving 90 %–100 % inhibition of A2/Asian/57 neuraminidase (see Table 1) were tested. Titres are expressed as the serum dilution producing 50 % inhibition of the neuraminidase activity of the virus tested under the conditions described in the text.

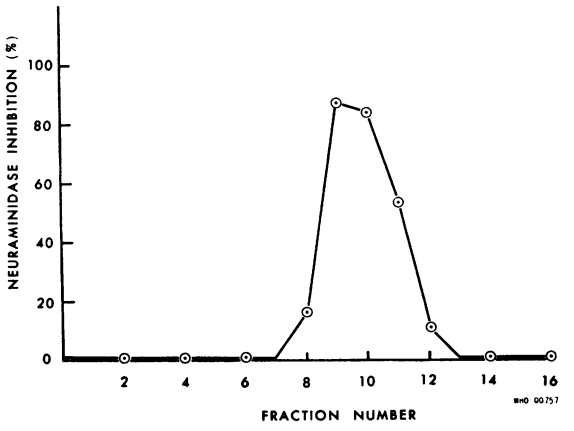
[b] Hyperimmune rabbit antiserum prepared against purified A2/Asian/57 neuraminidase isolated from X-7F1 virus (Kilbourne et al., 1968).

virus. Similar results were obtained with the other high-titre shearwater sera and with the hyperimmune rabbit antiserum prepared against purified A2/Asian/57 neuraminidase (Table 2). The rabbit serum had a very much higher titre than the shearwater sera but the pattern of cross-reaction was the same and was similar to that found previously with the neuraminidases of the A2/Asian/57 and the A2/Hong Kong/68 strains of influenza virus (Coleman et al., 1968).

These findings suggest that the inhibitory activity in the shearwater sera was due to antibody directed specifically against the neuraminidase of the A2/Asian/57 strain of human influenza virus. Further evidence that the inhibitory effect of the shearwater sera for type A2 neuraminidase was due to specific antibody was obtained by sucrose-gradient centrifugation and by absorption experiments. Samples of the sera were centrifuged on sucrose gradients and a sharp peak of inhibitory activity was obtained (Fig. 2). When compared with the rate of sedimentation of a marker protein in a companion tube (horse haemoglobin, 4.4S) the inhibitor in the shearwater sera was estimated to have a sedimentation coefficient of approximately 6S. Chicken immunoglobulin, IgG, has a sedimentation coefficient of 7S (Hawkes & Lafferty, 1967). Absorption experiments showed that the inhibitory activity could be removed completely from the sera by virus particles containing A2/Asian/57 neuraminidase but not at all by A0/Bel virus.

FIG. 2

SUCROSE-GRADIENT CENTRIFUGATION OF A SHEARWATER SERUM (No. 99)



When tested in immunodiffusion tests, 23 out of the 201 sera from Tryon Island gave a strong precipitin line with the preparation of type A soluble antigen, whereas none of the 119 sera from Heron Island was positive in this test. No correlation was found between the sera positive in the neuraminidase-inhibition and immunodiffusion tests. It was confirmed by Dr G. C. Schild at the World Influenza Centre, London, using detergent-disrupted virus parcles as the source of antigen, that the precipitin lines were indeed due to antibody to the type-specific soluble (RNP) antigen of influenza type A virus (Schild & Pereira, 1969).

Avian influenza viruses have not been reported to occur in Australian domestic birds; 100 sera collected from chickens running free on farms in New South Wales were tested for the presence of antibody to A2/Asian/57 neuraminidase but none was found to be positive.

DISCUSSION

The results of these experiments suggest that the shearwaters on Tryon Island had experienced at least two infections with different influenza viruses. Antibody to the internal (RNP) antigen found in convalescent sera following infection with influenza is generally short-lived, suggesting that the birds on Tryon Island which were positive in immunodiffusion tests with influenza type A soluble antigen had experienced a fairly recent epidemic of avian influenza. These birds would also be expected to have high levels of antibody to the haemagglutinin and neuraminidase of the virus which caused this epidemic, but so far tests with a number of known haemagglutinin and neuraminidase antigens have been negative. Sera positive in neuraminidase-inhibition from both Tryon and Heron islands showed no evidence of antibody to type A internal antigen, suggesting that the infection which gave rise to the antibody inhibiting human A2/Asian/57 influenza virus neuraminidase had occurred a much longer time previously.

How the shearwaters acquired these antibodies is not known. These birds are thought not to be migratory (Gross et al., 1963) but it is possible that avian viruses, including those possessing human A2/Asian/57 neuraminidase, are common in South-East Asia and in areas further north and that these viruses were brought south by migratory birds. In this case, infection of the shearwaters could have occurred during the breeding season when the birds are crowded closely together on the islands.

The A2/Asian/57 strain of human influenza which was first detected in China in 1957, may have arisen from such an avian influenza virus, either by mutation or by genetic recombination. It is also possible that the hypothetical avian virus may have acquired its type A2/Asian/57 neuraminidase from the human strain and it is now impossible to decide between these two alternatives.

Our observations suggest that avian influenza may be a common infection of wild birds and the finding in the shearwaters of antibody to an antigen of a human influenza virus is consistent with the idea that human influenza pandemics may originate from avian or animal reservoirs.

It seems important, however, to try to isolate and characterize, as soon as possible, all the avian and animal strains of influenza, so that when another influenza pandemic occurs it may be possible to determine if animals or birds played any role in its origin.

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RÉSUMÉ

ANTICORPS DIRIGÉS CONTRE DES VIRUS GRIPPAUX (DONT LA SOUCHE HUMAINE A2/ASIAN/57) DANS DES SÉRUMS PRÉLEVÉS CHEZ DES PUFFINS (*PUFFINUS PACIFICUS CHLORORHYNCHUS*) D'AUSTRALIE

En décembre 1969, on a prélevé des échantillons de sérum chez 320 puffins nichant sur les îles Heron et Tryon, au large de la côte est de l'Australie septentrionale. Environ 10% de ces sérums inhibaient de manière spécifique la neuraminidase de la souche A2/Asian/57 responsable de l'épidémie de grippe humaine de 1957, dans certains cas à des titres élevés. La neuraminidase de la souche A2/Hong Kong/68 était inhibée dans une

mesure moindre et aucune inhibition n'était décelée vis-à-vis de la neuraminidase de la souche A0/Bel. On n'a mis en évidence aucun anticorps, à activité limitée dans le temps, dirigé contre l'antigène interne des virus grippaux A, ce qui donne à croire que les oiseaux ont été infectés, longtemps auparavant, par un virus grippal renfermant une neuraminidase identique à celle du virus responsable de la pandémie de grippe asiatique de 1957.

D'autre part, en épreuve d'immunodiffusion, plus de 10% des sérums prélevés dans l'île Tryon donnaient des lignes de précipitation en présence de l'antigène soluble spécifique des virus grippaux A. On peut donc supposer qu'une épizootie de grippe aviaire a sévi très récemment sur le territoire de cette île. Les antigènes de surface du virus responsable de cette épizootie n'ont pas encore été identifiés.

Il semble, d'après ces observations, que la grippe aviaire soit une infection courante chez les oiseaux sauvages. La mise en évidence chez des puffins d'anticorps actifs contre un antigène d'un virus grippal humain appuie l'hypothèse selon laquelle les épidémies de grippe humaine pourraient avoir pour origine des réservoirs animaux, et notamment aviaires, de l'infection.

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