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Antibodies to human influenzavirus neuraminidase (the A/Asian/57 H2N2 strain) in sera from Australian pelagic birds*

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*Sera collected from Australian pelagic birds specifically inhibited the neuraminidase of the Asian/57 (H2N2) strain of human influenzavirus. Neuraminidase inhibition titres of some sera were high and the avidity of the inhibitor was comparable to that of specific antibody. The neuraminidase of A/Hong Kong/1/68 (H3N2), which has undergone considerable antigenic drift in man since 1957, was inhibited to a lesser extent by the bird sera, while the neuraminidases of the A/BEL/42 (H0N1) and A/FM/1/47 (H1N1) strains were not inhibited at all. The inhibitor could be removed from the sera by adsorption with A/57 virus particles, but not by particles of A/BEL or A/FM1 viruses. These results suggested that the inhibitor in the bird sera was specific antibody. The antibodies to A/57 neuraminidase were found in sera from wedge-tailed shearwaters (*Puffinus pacificus*) and noddy terns (*Anous minutus*) nesting on islands off the north-east coast of Australia. They were not found in sera from bridled terns (*Sterna anaetheta*) or brown gannets (*Sula leucogaster*) nesting on the same islands. Antibodies to A/57 neuraminidase were also detected in sera from short-tailed shearwaters (*Puffinus tenuirostris*), which migrate around the Pacific Ocean, suggesting that these birds may be responsible for bringing avian influenzaviruses from areas in the Northern Hemisphere into Australian coastal waters.*

Pandemics of type A influenza are caused by "new" strains of virus that appear suddenly in the human population, but the origin of these strains is not known. There are two main theories to explain the origin of the "new" viruses: that they are derived by direct mutation from existing human strains or that they arise by mutation or genetic recombination from mammalian or avian influenzaviruses. There is an increasing body of evidence to support the second explanation. Genetic interaction between influenza A viruses occurs frequently (Hirst & Gotlieb, 1955) and antigenic hybrids (recombinants) of type A influenzaviruses can be readily made *in vitro* (Laver & Kilbourne, 1966; Webster, 1970). Production of recombinant viruses (stable antigenic hybrids) in the laboratory between avian or equine strains and human influenzaviruses has been described (Kilbourne, 1968; Easterday et al., 1969). Stable antigenic hybrids have also been isolated from mammals and birds following their mixed infection with different type A influenzaviruses (Webster et al., 1971). Influenzaviruses isolated from mammals and birds have been found whose surface antigens were similar to, if not identical with, the same antigens on human influenzaviruses (Webster & Pereira, 1968; Schild et al., 1969; Tumová & Easterday, 1969; Schild & Newman, 1969). Serological evidence from animals and birds (Kaplan & Payne, 1959; Tumová & Easterday, 1969; Schild & Newman, 1969; Kasel & Couch, 1969; Dasen & Laver, 1970) suggested that influenzaviruses isolated from man had either infected these hosts directly or shared antigenic determinants with influenzaviruses of mammalian or avian origin.

Dasen & Laver (1970) found that sera from one kind of Australian shearwater (*Puffinus pacificus*) inhibited the neuraminidase of the A/57 (H2N2) strain of human influenzavirus and that this inhibitor had the properties of specific antibody to the enzyme. The present report confirms these findings and demonstrates the presence of high levels of antibody to A/57 neuraminidase in sera from other Australian pelagic birds, including those that migrate around

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the Pacific Ocean. It was demonstrated that the inhibitor present in the sera was specific for A/57 neuraminidase and measurement of the equilibrium constant of the neuraminidase inhibition reaction showed that it had the avidity characteristic of antibody.

MATERIALS AND METHODS

Sera were collected from 172 short-tailed shearwaters (Tasmanian mutton birds, *Puffinus tenuirostris*) nesting on Philip Island in Westernport Bay near Melbourne, Victoria, on 2 and 3 October 1970 and from pelagic birds nesting on coral cays in the Bunker group of islands off the north-east coast of Australia during the second week in December 1970. Samples were also collected from Masthead Island. Sera were collected from 189 wedge-tailed shearwaters (*Puffinus pacificus*), 88 noddy terns (*Anous minutus*), 33 bridled terns (*Sterna anaetheta*) and 11 brown gannets (*Sula leucogaster*). 148 tracheal swabs and 32 sets of lungs were collected from the birds at the same time as the sera.

The sera were stored without refrigeration for up to 2 weeks before they were tested, sodium azide being added to prevent bacterial growth. Tracheal swabs and lungs were immediately placed in liquid nitrogen in the field and were maintained at this temperature until tested.

Haemagglutinin (HA) titrations and haemagglutinin inhibition (HI) tests were carried out in plastic trays as previously described (Fazekas & Webster, 1966). Neuraminidase inhibition (NI) tests were performed as described by Dasen & Laver (1970), fetuin being used as substrate. The tests were made more sensitive by using very small amounts of neuraminidase and overnight incubation of the serum, enzyme, and substrate mixtures (Downie, 1970). The influenza viruses used in the tests A/BEL/42 (H0N1), A/FM/1/47 (H1N1), A/Japan/305/57 (H2N2), A/Aichi/68 (H3N2), and the recombinant virus X-7 (F1) described by Kilbourne et al., (1967) (having A/NWS haemagglutinin and A/57 neuraminidase) were grown in the allantoic sac of 11-day-old chick embryos and concentrated by adsorption–elution on chicken red blood cells or by high-speed centrifugation.

The equilibrium constant of the reaction between A/57 neuraminidase and the inhibitor present in the bird sera was determined by equilibrium filtration using the methods of Fazekas & Webster (1961). The calculations were performed according to the methods of Fazekas (1961) and the results gave a measure of the avidity of the inhibitor in the sera. The X-7F1 virus was used as the source of A/57 neuraminidase and in the calculations it was assumed that the number of neuraminidase antigenic sites on the virus was equal to the number of haemagglutinin antigenic sites.

Attempts to isolate influenzavirus from the lungs and tracheal swabs were made in the following way. Lung samples were ground in a cold mortar and pestle with standard medium (Fazekas & White, 1958) to make a 10% suspension. The tracheal swabs, stored frozen in standard medium, were thawed. Approximately 1 000 IU of penicillin and streptomycin were added per ml of sample and 0.05-ml aliquots were inoculated into 11-day-old embryonated chicken eggs. All the lung samples were passaged twice in the amniotic cavity of the chick embryo. Half of the tracheal swabs received two amniotic passages and half were passaged twice in the allantoic sac of embryos. Fluids were assayed for haemagglutinin activity using chicken red blood cells.

Double immunodiffusion tests were carried out in 1% agar gel (containing 5% sodium chloride) with 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.

RESULTS

The sera from the different birds were tested (at an initial serum dilution of 1/10) for their ability to inhibit A/57 neuraminidase and the results are given in Table 1. A/57 neuraminidase was inhibited by 30% or more by 16 (9%) of 172 sera from the migratory short-tailed shearwaters (Tasmanian mutton birds, *Puffinus tenuirostris*), 52 (28%) of 189 sera from wedge-tailed shearwaters (*Puffinus pacificus*) and 43 (49%) of 88 sera from noddy terns (*Anous minutus*). Some of the sera were strongly inhibitory—a total of 28 inhibited the enzyme by 90% or more (Table 1). None of the sera from 33 bridled terns (*Sterna anaetheta*) or 11 brown gannets (*Sula leucogaster*) had any inhibitory activity for A/57 neuraminidase.

The distribution of antibodies to A/57 neuraminidase among birds nesting on different islands off the coast of north-eastern Australia is given in Table 2. Most striking was the complete absence of any antibody from sera of the bridled terns and brown gannets that were nesting on the islands in

Table 1. Screening of sera for antibody to A/57 neuraminidase [a]

		Short-tailed shearwater	Wedge-tailed shearwater	Noddy tern	Bridled tern	Brown gannet
no. of sera tested		172	189	88	33	11
no. of positive sera [b]		16 (9 %)	52 (28 %)	43 (49 %)	0	0
no. of sera inhibiting A/57 neuraminidase by:	90–100 %	1	18	9	0	0
	70–89 %	4	14	29	0	0
	50–69 %	5	18	5	0	0
	30–49 %	6	2	0	0	0
	<30 %	156	137	45	33	11

[a] The sera were tested at an initial dilution of 1/10. A recombinant virus, X-7 (F1), possessing A/57-type neuraminidase (Webster et al., 1968), was used as the enzyme source.
[b] Sera that inhibited the neuraminidase by 30 % or more were taken as positive.

close proximity to the noddy terns and shearwaters, both of which yielded a high proportion of positive sera.

The titres of the positive sera are given in Table 3. Some of the sera had high levels of antibody: 32 sera gave titres greater than 100 per 0.1 ml while 2 sera from the wedge-tailed shearwaters had neuraminidase inhibition titres as high as 1 200 per 0.1 ml. The sera that inhibited A/57 neuraminidase also inhibited neuraminidase of the A/Hong Kong/68 (H3N2) strain of influenzavirus, but the titres were only about one-fifth of the above figures (Table 4). This corresponds to the degree of cross-reaction found previously between the enzymes of the A/57 and A/HK/68 strains (Coleman et al., 1968). The same degree of cross-reaction was obtained with hyperimmune rabbit serum prepared against purified A/57 neuraminidase (Dasen & Laver, 1970). The sera that had neuraminidase inhibition titres greater than 100 per 0.1 ml for A/57 neuraminidase were also tested in neuraminidase inhibition tests against the neuraminidases of A/BEL and A/FM1 influenzaviruses (the enzymes of these strains are immunologically unrelated to N2 neuraminidase). None of the sera gave any inhibition at all with the enzymes of the A/BEL or A/FM1 strains (Table 4).

Table 2. Distribution of antibodies to A/57 influenzavirus neuraminidase in the sera of pelagic birds nesting on islands off the north-east coast of Australia

Island	Bird type	No. tested	No. positive [a]	Percentage positive
Lady Musgrave	wedge-tailed shearwater	175	47	27
	noddy tern	36	18	50
	bridled tern	6	0	0
Fairfax	noddy tern	22	12	55
	brown gannet	11	0	0
Hoskyn	noddy tern	15	6	40
	bridled tern	22	0	0
Masthead	wedge-tailed shearwater	14	5	26
	noddy tern	15	7	47
	bridled tern	5	0	0

[a] Positive sera are defined as those that inhibited the neuraminidase by 30 % or more at a dilution of 1/10 in the neuraminidase inhibition test described previously (Dasen & Laver, 1970).

Table 3. Neuraminidase inhibition titres of the positive bird sera against A/57 neuraminidase [a]

Type of bird		Neuraminidase inhibition titres of sera 5-50	51-100	101-500	501-1 000	>1 000
wedge-tailed shearwater	no. within range	23	9	13	5	2
	mean titre/0.1 ml	23	79	235	620	1 200
noddy tern	no. within range	13	17	8	4	0
	mean titre/0.1 ml	38	69	281	640	0

[a] Titres are expressed as the reciprocals of the serum dilution giving 50 % inhibition of A/57 neuraminidase activity under the conditions of the test.

The inhibitor for A/57 neuraminidase present in the bird sera was specifically adsorbed by A/57 influenzavirus particles, but not by particles of the A/FM1 strain. Some of the sera did inhibit A/FM1 neuraminidase to a low titre (about 10). This inhibitor, however, was removed by centrifugation at 50 000 *g* for 2 hours unlike the inhibitor to A/57 neuraminidase, which was unaffected by this treatment.

Measurement of the equilibrium constant of the reaction between A/57 neuraminidase and its inhibitor in the bird sera gave a value of 1.3×10^8 cgs units, which is comparable with that of high avidity antibody reacting with its homologous antigen. The inhibitor in the bird sera had an avidity similar to (or greater than) that found previously for anti-neuraminidase antibody prepared in rabbits (Webster et al., 1968).

Haemagglutinin inhibition tests with the positive bird sera and different human influenzaviruses (A/BEL, A/FM1, A/Japan/305/57 and A/Aichi/68 were completely negative. Immunodiffusion tests with a preparation of influenza type A soluble antigen from allantoic fluid infected with X-7(F1) gave inconclusive results. Some sera yielded a precipitin line but this failed to join completely with the line obtained with a hyperimmune chicken antiserum to the RNP. Furthermore, preparations of RNP from allantoic fluid infected with A/BEL and A/57 that gave strong precipitin lines with the hyperimmune chicken serum failed to give any lines with the bird sera.

Attempts to isolate an influenzavirus from the tracheal swabs or the lungs of the birds were completely negative. A total of 25 tracheal swabs and 3 lung samples collected from the short-tailed shearwaters were passaged twice allantoically; 33 tracheal swabs and 23 sets of lungs from noddy terns, 24 tracheal swabs and 4 lungs from bridled terns, and tracheal swabs from 62 wedge-tailed shearwaters, 11 adult brown gannets, and 13 baby brown gannets (less than 2 months old) were passaged twice both amniotically and allantoically in 11-day-old chick embryos but none yielded any influenzavirus.

DISCUSSION

The experiments reported in this paper have confirmed the findings of Dasen & Laver (1970) that sera from Australian pelagic birds contained an

Table 4. Neuraminidase inhibition titres of the bird sera with different influenza A viruses [a]

Type of bird	No. tested [b]	Neuraminidase inhibition titres [c] with the strains of virus shown A/BEL	A/FM1	A/Japan/305/57	A/Aichi/68
wedge-tailed shearwater	15	<5	<5	525	130
noddy tern	9	<5	<5	430	80

[a] The concentrations of the different strains of virus were adjusted before testing so that all had equivalent enzyme activity.

[b] Sera giving neuraminidase inhibition titres of 100 or greater with A/57 neuraminidase were tested.

[c] Mean titres are given. Titres are expressed as the reciprocal of the dilution of serum giving 50 % inhibition of the neuraminidase of the virus tested.

inhibitor specifically directed against the neuraminidase of the A/57 strain of human influenzavirus. This inhibitor cross-reacted with the neuraminidase of A/HK/68 to the same extent (about 20%) as hyperimmune rabbit serum did to A/57 neuraminidase, but did not react at all with the immunologically unrelated neuraminidases of A/BEL or A/FM1 influenzaviruses. The inhibitor was removed from the sera by adsorption with A/57 virus particles, but not by particles of A/BEL or A/FM1 viruses. The inhibitor had the same sedimentation coefficient as IgG immunoglobulin and had an avidity for A/57 neuraminidase that was characteristic of antibody reacting with its homologous antigen. Thus the inhibitor in the bird sera had the properties of specific antibody. However, the nature of the antigen responsible for the induction of this antibody is not known. There appear to be two possibilities. Either the birds were infected with an avian influenzavirus possessing A/57 neuraminidase or they had experienced an antigen quite unrelated to influenzavirus (e.g., a bacterial antigen) that possessed antigenic determinants identical with those of A/57 neuraminidase. This question may not be resolved unless an influenzavirus possessing A/57 neuraminidase can be isolated from the birds, and attempts to isolate any virus have been unsuccessful. However, avian influenzaviruses possessing a neuraminidase immunologically identical with that of the A/57 strain have been isolated from domestic birds (Webster & Pereira, 1968) and a similar virus infecting pelagic birds may ultimately be found.

Such avian influenzaviruses—if they exist—might be dispersed by migrating birds. The short-tailed shearwaters (*Puffinus tenuirostris*) that possessed antibody against A/57 neuraminidase are known to migrate around the Pacific Ocean basin in a "figure of eight" flight path (Serventy, 1958). The birds leave the southern Australian coast in April and fly around the Pacific via the coastal waters of New Zealand and Fiji, then up the coast of Viet-Nam to Japan, Korea, and the USSR, past the Bering Strait islands and down the coast of North America. From there the birds fly across the Pacific, reaching the eastern Australian coast during the last week in September. The other two kinds of pelagic bird found to have antibodies to A/57 neuraminidase, the wedge-tailed shearwaters (*Puffinus pacificus*) and the noddy terns (*Anous minutus*), are thought to be sedentary, dispersing out into the Pacific Ocean and the Coral Sea but not following any long migratory flight path. They could, however, come into contact with the migrating short-tailed shearwaters during their journey down the Australian coast each spring.

Little is known about the migratory habits of the other two kinds of pelagic bird tested, the bridled terns (*Sterna anaetheta*) and the brown gannets (*Sula leucogaster*). Surprisingly, none of these birds had any detectable antibody to A/57 neuraminidase although both were nesting on the islands in close proximity to the wedge-tailed shearwaters and noddy terns.

The relatively high levels of antibody activity to A/57 neuraminidase in some of the bird sera suggested that, if the birds had been infected with an influenzavirus, the infection had occurred fairly recently. A/57 neuraminidase has undergone considerable antigenic drift in man since 1957, but the results obtained above suggest that, in birds, antigenic drift (in the neuraminidase) might not occur or might occur much more slowly than in man. Again, this question may not be resolved unless virus can be isolated from the birds.

The importance of investigating animal and avian influenzaviruses has been stressed by Kaplan (1969) and by others and evidence is accumulating that suggests that influenzaviruses of mammals and birds play an important role in the emergence of new human pandemic viruses. Thus, Webster et al. (1971) have obtained recombination *in vivo* between mammalian and avian influenzaviruses and it is reasonable to suppose that similar recombination between mammalian (or avian) viruses and human strains could also occur. It therefore seems to be important to try to isolate and characterize now as many mammalian and avian influenzaviruses as possible so that when another influenza pandemic occurs we may be in a better position to decide whether the pandemic strain did in fact arise from a mammalian or an avian source.

ADDENDUM

Recently a type A influenzavirus has been isolated from a tracheal swab taken from an apparently healthy shearwater (*Puffinus pacificus*) nesting on Tryon island off the east coast of Australia (Downie & Laver, in press). This virus was not responsible, however, for inducing the antibodies described above.

The haemagglutinin subunits of the shearwater virus were of antigenic subtype Hav6, but the neuraminidase subunits were not related antigenically to those of any known virus and represent a new neuraminidase subtype, Nav5. These findings support the suggestion that there may be many avian influenza viruses with novel haemagglutinin or neuraminidase subunits, from which human pandemic strains could arise by genetic recombination.

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RÉSUMÉ

ANTICORPS ACTIFS CONTRE LA NEURAMINIDASE D'UN VIRUS GRIPPAL HUMAIN (SOUCHE A/ASIAN/57 H2N2) DANS LES SÉRUMS D'OISEAUX MARINS D'AUSTRALIE

Des pandémies de grippe de type A atteignent à intervalles irréguliers les populations humaines. Elles sont causées par de « nouvelles » souches de virus grippaux d'origine inconnue mais qui sont peut-être issues, par mutation ou recombinaison génétique, de virus grippaux infectant des mammifères ou des oiseaux; d'où l'intérêt des recherches visant à découvrir des virus grippaux animaux possédant des antigènes de surface similaires à ceux des virus humains.

Des sérums prélevés chez des oiseaux marins d'Australie renfermaient un facteur inhibant spécifiquement la neuraminidase de la souche grippale humaine A/Asian/57. Les titres de certains sérums étaient élevés et l'activité de l'inhibiteur était comparable à celle d'un anticorps spécifique. La neuraminidase du virus A/Hong Kong/68 était aussi inhibée, mais dans une mesure moindre, par des sérums d'oiseaux, alors qu'on ne notait aucune inhibition des neuraminidases des souches A/BEL/42 et A/FM/1/47. L'inhibiteur a pu être éliminé des sérums par adsorption avec des particules de virus A/Asian/57, mais non par des particules de virus A/BEL/42 ou A/FM/1/47. Ces résultats montrent que l'inhibiteur présent dans les sérums d'oiseaux est un anticorps spécifique.

Les essais d'isolement d'un virus grippal à partir d'échantillons de mucosités trachéales ou de poumons des oiseaux sont restés complètement infructueux.

Des anticorps anti-neuraminidase A/Asian/57 ont été trouvés dans des sérums de puffins à queue pointue (*Puffinus pacificus*) et de noddis à cape blanche (*Anous minutus*) nichant sur des îles près de la côte nord-est de l'Australie, ainsi que dans des sérums de puffins à queue courte (*Puffinus tenuirostris*). Les sérums de sternes bridées (*Sterna anaetheta*) et de fous bruns (*Sula leucogaster*) étaient négatifs.

Il semble d'après ces observations que la grippe soit fréquente chez les oiseaux sauvages. La découverte chez certains d'entre eux d'anticorps dirigés contre un antigène de virus grippal humain vient à l'appui de l'hypothèse selon laquelle les pandémies de grippe humaine auraient pour origine des réservoirs de virus chez des mammifères ou des oiseaux.

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