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Immunological Relationships between the Neuraminidases of Human and Animal Influenza Viruses

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The neuraminidases of 3 human A2 virus strains—A2/Singapore/1/57, A2/England/76/66 and A2/Hong Kong/1/68—were compared immunologically with those of a number of influenza A virus strains of animal origin (14 avian, 2 equine and 1 porcine). The methods used were enzyme-inhibition tests and immuno-double-diffusion. In enzyme-inhibition tests, the neuraminidases of the A2/57 and A2/66 viruses cross-reacted with those of Turkey/Wisconsin/66, Turkey/Massachusetts/65 and Duck/Italy/574/66 viruses. The enzyme of the Hong Kong variant had a wider spectrum of cross-reactions than did those of the A2/57 and A2/66 viruses. No cross-reaction was detected with Turkey/Massachusetts/65 but the A2/Hong Kong enzyme showed major antigenic similarities with the enzymes of Turkey/Wisconsin/66 and Duck/Italy/574/66 and Duck/Germany/1998/68 viruses. Minor cross-reactions were also detected between the A2/Hong Kong enzyme and those of fowl plague virus, virus " N ", Duck/England/56 and Swine/Cambridge/39.

The cross-reactions between the enzymes of A2/Hong Kong and Turkey/Wisconsin/66 were confirmed by immunoprecipitin tests. Those between A2/Hong Kong and fowl plague virus, virus " N ", Duck/England/56 and Swine/Cambridge/39 could not be confirmed by immunoprecipitation.

The finding of antigenic similarities between the enzymes of human and avian influenza A viruses may be of importance in considering the origin of the new antigenic variants of influenza A virus which appear in man.

Influenza type A viruses have been isolated from human, porcine, equine and avian sources and are related by possessing a common (type-specific) ribonucleoprotein (RNP) antigen located internally in the virion. The outer envelope of the influenza virus contains two further antigens, the haemagglutinin and neuraminidase which are immunologically independent components of the virus (Seto & Rott, 1966; Webster & Laver, 1967). Serological tests have indicated a wide range of antigenic variation in the surface (subtype-specific) antigens of human and animal influenza A viruses (see Pereira, 1969) and until recently attempts to demonstrate close antigenic similarities between the envelope antigens of human and animal influenza strains have produced largely negative results (Tumova & Pereira, 1968). However, there is now strong evidence that the neuraminidases of certain avian influenza A virus strains are antigenically closely related to those of human influenza viruses. Certain strains of avian influenza, including A/Turkey/Massachusetts/65, were found in neuraminidase-inhibition tests (Pereira, Tumova & Webster, 1967; Webster & Pereira, 1968) and immunoprecipitin tests (Easterday et al., 1969) to contain enzymes antigenically closely related to that of human A2/57 virus. In addition, Schild, Pereira & Schettler (1969) found antigenic similarities between the neuraminidase of A/Duck/Germany/1868/68 virus and that of human A0 and A1 virus strains. It appeared that the avian and human viruses with cross-reacting enzymes did not also contain antigenically related haemagglutinins.

Apart from the above findings, little is known about the antigenic relationships between the neuraminidases of the large number of animal (avian and mammalian) influenza A viruses which are available

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for study. In addition, the full spectrum of antigenic relationships between the enzymes of animal and human viruses is relatively unexplored. The appearance in 1968 of the new Hong Kong variant of human influenza A2 provided an opportunity to investigate further the relationships between human and animal influenza A viruses. The present report describes the results of immunological comparisons between the neuraminidases of 3 examples of human A2 viruses, isolated in 1957, 1966 and 1968, and those of a collection of animal influenza A virus strains.

MATERIALS AND METHODS

Virus strains

The human A2 virus strains A2/Singapore/1/57, A2/England/52/64, A2/England/76/66 and A2/Hong Kong/1/68 were from the collection of this laboratory. The following animal influenza A viruses were also from the collection held at the World Influenza Centre, London:

(1) Turkey/Wisconsin/66
(2) Turkey/Massachusetts/3740/65
(3) Turkey/Canada/63 (Wilmot strain)
(4) Virus " N "
(5) Quail/Italy/1117/65
(6) FPV (Dutch strain)
(7) Turkey/England/63 (Langham strain)
(8) Duck/England/56
(9) Duck/Czechoslovakia/56
(10) Tern/South Africa/61
(11) Chicken/Scotland/59 (Smith strain)
(12) Duck/Germany/210/67
(13) Duck/Germany/833/67
(14) Duck/Germany/1998/68
(15) Equi-1/Prague/56
(16) Equi-2/Miami/63
(17) Swine/Cambridge/39

The origins of all these strains except 12, 13, 14, and 17 have been described previously (Pereira et al., 1966; Pereira, Rinaldi & Nardelli, 1967; Tumova & Pereira, 1968). Duck/Germany/210/67, Duck/Germany/833/67 and Duck/Germany/1998/68 were received from Professor Glystorff and Dr C. H. Schettler, Ludwig-Maximilians-Universität, Munich, Federal Republic of Germany. Swine/Cambridge/39 was a virus described by Blakemore & Gledhill (1941). Viruses bracketed together have been shown to cross-react in haemagglutination-inhibition tests (see Pereira, 1969).

FPV–A2/HK was a recombinant virus strain obtained from FPV (fowl plague virus) and A2/Hong Kong/68 as parent viruses. Antigenic analysis (McCahon & Schild, unpublished observation) revealed that this virus contained FPV haemagglutinin and A2/Hong Kong enzyme.

Virus purification and concentration

Virus used in neuraminidase-inhibition tests, for the preparation of rabbit antisera and for immunodiffusion tests was grown in the allantoic cavity of 11-day-old chick embryos and purified by adsorption on to and elution from chick erythrocytes followed by differential centrifugation and sedimentation through a sucrose gradient (10%–40% sucrose in 0.15 M NaCl as described by Laver & Webster, 1966, and Schild & Pereira, 1969). Concentrates contained between 2.5×10^5 and 5×10^6 HA units per 0.25 ml.

Antisera

A hyperimmune rabbit antiserum against purified influenza A2/57 neuraminidase was prepared as described by Laver & Webster (1966), using a recombinant virus X-7 (F1) (Kilbourne et al., 1967) as the source of enzyme. Immune antiviral rabbit sera were prepared against purified, inactivated influenza viruses as described by Webster & Laver (1967). The first injection of virus was given intramuscularly with Freund's complete adjuvant, and the second intravenously.

Neuraminidase-inhibition tests

Neuraminidase-inhibiting antibody was assayed by a modification of the technique described by Webster & Pereira (1968). Dilutions of purified, concentrated influenza virus (treated with pronase where appropriate) containing 1–2 units of enzyme (Webster & Laver, 1967) in 0.05-ml volumes were incubated at 4°C for 18 hours with 10-fold or 3.3-fold serum dilutions (0.05 ml). Fetuin solution (0.1 ml) in buffered saline (pH 5.9) was added as substrate and the mixture incubated for 60 minutes at 37°C. Residual enzyme activity was assayed by the method of Warren. The neuraminidase-inhibition titre of an antiserum was expressed as the reciprocal of the serum dilution just producing 50% inhibition of enzyme activity. In some cases the percentage of enzyme activity inhibited by a 1 : 10 serum dilution was determined. As a control, samples of enzyme were incubated with dilutions of normal rabbit serum.

Virus used as a source of enzyme was treated with pronase to destroy virus haemagglutinin. Virus concentrates were incubated with 0.05% pronase

(British Drug Houses Ltd) in 0.01 M phosphate buffer, pH 7.2, for at least 1 hour at 37°C or until no residual haemagglutinin activity could be detected. By this treatment non-specific inhibition of neuraminidase by anti-haemagglutinin antibody was avoided (Easterday et al., 1969).

Immunodiffusion studies

A micro double-immunodiffusion technique (Crowle, 1958) was used. The tests were carried out with virus concentrates disrupted with sodium dodecyl sulfate (1%) as described by Schild & Pereira (1969).

RESULTS

Comparisons of the enzymes of human A2 viruses

The results of neuraminidase-inhibition tests to compare the enzymes of 5 different human influenza A2 viruses are shown in Table 1. The tests were performed using hyperimmune rabbit sera. Virus concentrates used as the source of enzyme were treated with pronase to destroy haemagglutinin, thus avoiding non-specific inhibition of enzyme activity by anti-haemagglutinin antibody (Easterday et al., 1969). The results are expressed as the serum dilution which just inhibited 50% of enzyme activity and also as the percentage reduction in enzyme activity produced by a 1 : 10 dilution of antiserum. There was evidence of cross-reactions between all the A2 virus strains tested except that antiserum against A2/Hong Kong/1/68 failed to inhibit A2/57 enzyme. No cross-reactions were detected between the enzymes of A0/Bel., and any of the A2 viruses. The degree of cross-reaction between the A2 viruses provided strong evidence of antigenic " drift " among their enzymes. Thus, antiserum against purified A2/57 enzyme reacted with a high titre with the homologous enzyme and produced complete enzyme neutralization (95% inhibition) but this antiserum reacted with a low titre with A2/Hong Kong/1/68 enzyme producing incomplete neutralization. In contrast, potent antiserum against A2/Hong Kong/1/68 failed to react with A2/57 enzyme. The A2 virus strains of 1964, 1966 and 1967 gave reactions intermediate between those of the A2/57 and A2/68 viruses. An antiserum prepared against a recombinant influenza virus, FPV–A2/HK, known to contain the haemagglutinin of its FPV parent and the enzyme of its A2/Hong Kong/68 parent (McCahon & Schild, unpublished observation) gave reactions which were similar to those of anti-A2/Hong Kong/1/68 antiserum, providing evidence of the specificity of the neutralization reaction for anti-neuraminidase antibody.

As a result of these tests, 3 A2 strains—namely, A2/Singapore/1/57, A2/England/76/66 and A2/Hong Kong/1/68—which contained immunologically distinguishable enzymes representative of the group of A2 strains were selected to compare with a collection of animal influenza viruses in enzyme-inhibition tests.

TABLE 1

IMMUNOLOGICAL RELATIONSHIPS BETWEEN THE NEURAMINIDASES OF HUMAN INFLUENZA A2 VIRUSES IN ENZYME-INHIBITION TESTS [a]

Source of enzyme [b]	Antisera from hyperimmunized rabbits					Anti-A0/Bel. (1939) virus
	Anti-purified A2/57 enzyme	Anti-A2/England/12/64 virus	Anti-A2/England/76/66 virus	Anti-A2/Hong Kong/1/68 virus	Anti-FPV–A2 HK (rec.) virus	
A2/Singapore/1/57	2 500 (>95%)	30 (65%)	10 (50%)	<10 (<5%)	<10 (<5%)	<10
A2/England/12/64	500 (>95%)	1 500 (>95%)	450 (>95%)	75 (85%)	Not tested	<10
A2/England/76/66	75 (75%)	750 (>95%)	1 200 (>95%)	120 (>95%)	320 (>95%)	<10
A2/Tokyo/3/67	25 (75%)	750 (>95%)	1 200 (>95%)	150 (>95%)	Not tested	<10
A2/Hong Kong/1/68	15 (60%)	450 (>95%)	1 000 (>95%)	320 (>95%)	750 (>95%)	<10
FPV-A2/HK (rec.)	10 (50%)	300 (>95%)	750 (>95%)	250 (>95%)	750 (>95%)	<10
A0/Bel.	<10	<10	<10	<10	<10	3 200

[a] Results expressed as the serum dilution inhibiting 50% enzyme activity (with the percentage inhibition of enzyme activity with 1 : 10 serum dilution in parentheses).

[b] Enzyme was pronase-treated, purified influenza virus concentrate diluted to contain 1–2 units of enzyme activity per 0.05 ml.

TABLE 2

CROSS-REACTIONS OF ANTISERA AGAINST HUMAN A2 VIRUSES IN ENZYME-INHIBITION TESTS WITH ANIMAL INFLUENZA A VIRUS STRAINS [a]

Source of enzyme	Rabbit antisera		
	Anti-purified A2/57 enzyme	Anti-A2/England/ 76/66 virus	Anti-A2/Hong Kong/ 68 virus
Homologous human A2 virus	1 500	750	750
FPV (Dutch strain)			10
Virus " N "			10
A/Turkey/Canada/63			
A/Quail/Italy/1117/65			
A/Turkey/Wisconsin/66	1 200	150	150
A/Turkey/Massachusetts/65	3 200	20	
A/Duck/Czechoslovakia/56			
A/Duck/England/56			
A/Duck/Germany/210/67			
A/Tern/South Africa/61			
A/Turkey/England/63			
A/Duck/Italy/574/66	750	350	250
A/Duck/Germany/1998/68			350
A/Chick/Scotland/59			
A/Duck/Germany/833/67			
A/Equi-1/Prague/56			
A/Equi-2/Miami/63			10
A/Swine/Cambridge/39			25

[a] Results expressed as the reciprocal of the serum dilution inhibiting 50% of enzyme activity Blank spaces represent enzyme-inhibition titres less than 1 : 10.

Immunological similarities between the enzymes of human A2 and animal influenza A viruses

Rabbit antisera aga nst purified A2/57 enzyme and against A2/England/76/66 and A2/Hong Kong/1/68 viruses were tested against members of a collection of avian, equine and porcine influenza A viruses in enzyme-inhibition tests. The animal viruses were selected on the basis of exhibiting a wide variety of different antigenic variation in haemagglutination-inhibition tests (see Pereira, 1969). The results are shown in Table 2. The reactions of anti-purified A2/57 neuraminidase antiserum against A/Turkey/Massachusetts/65, A/Turkey/Wisconsin/66 and A/Duck/Italy/574/66 confirmed the results of previous studies (Webster & Pereira, 1968). In tests with anti-A2/66 antiserum the same spectrum of cross-reactions was observed as with purified anti-A2/57 euraminidase although the titre of the A2/66 antiserum against A/Turkey/Massachusetts virus was considerably lower than with A2/57 antiserum. In contrast, antiserum against A2/Hong Kong/68 had a wider spectrum of reactions. Anti-A2/68 antiserum, like anti-A2/57 and anti-A2/66 antisera, strongly neutralized the enzyme of A/Turkey/Wisconsin/66 and A/Duck/Italy/574/66 and in addition had high titres against A/Duck/Germany/1998/68 virus. The anti-A2/68 antiserum also neutralized the enzymes of FPV, Equi-2 and Swine/Cambridge/39 virus but with low titres. It was of interest that there was no cross-reaction between anti-A2/68 antiserum and A/Turkey/Massachusetts/64 although anti-A2/57 antiserum reacted strongly with this virus.

The results of the reciprocal cross-reactions, i.e., antisera prepared against purified, concentrated

TABLE 3

CROSS-REACTIONS OF ANTISERA AGAINST ANIMAL INFLUENZA A VIRUS IN ENZYME-INHIBITION TESTS WITH HUMAN A2 VIRUS STRAINS [a]

Rabbit antiserum (antiviral)	Source of enzyme [b]				
	Homologous avian or porcine virus	A2/Singapore/1/57	A2/England/76/66	A2/Hong Kong/68	FPV–A2/HK (rec.)
FPV (Dutch)	750	20		10	10
Virus " N "	>1 000	25		50	
A/Turkey/Canada/63	1 000				
A/Quail/Italy/1117/65	500				
A/Turkey/Wisconsin/66	>1 000	500	75	500	300
A/Turkey/Massachusetts/65	850	750	20		
A/Duck/Czechoslovakia/56	>1 000				
A/Duck/England/56	>1 000			100	30
A/Duck/Germany/210/67	750				
A/Tern/South Africa/61	750				
A/Swine/Cambridge/39	>1 000			30	20

[a] Results expressed as the reciprocal of the serum dilution inhibiting 50% of enzyme activity. Blank spaces represent enzyme-inhibition titres less than 1 : 10.

[b] Concentrated, purified virus treated with pronase.

suspensions of animal influenza A viruses tested against the enzymes of the human A2 viruses, are shown in Table 3. For practical reasons, because of the large number of animal viruses involved, suitable rabbit antisera were not available for all the animal influenza viruses mentioned in Table 2. Thus the present results are somewhat preliminary since the complete range of reciprocal reactions was not investigated. However, the tests confirmed the close relationship between the enzymes of A/Turkey/Wisconsin/66 and those of the 3 A2 variants and also the absence of cross-reaction between A2/Hong Kong/1/68 and A/Turkey/Massachusetts/65. Also, the minor cross-reactions of A2/Hong Kong/1/68 with A/Swine/Cambridge/39 and FPV were confirmed in these tests. In tests using FPV–A2/HK recombinant as a source of enzyme, the enzyme-inhibition reactions detected were similar to those of A2/Hong Kong/1/68 virus with the exception that a minor cross-reaction detected between antiserum for virus " N " virus and A2/Hong Kong enzyme was not apparent with the enzyme of the recombinant strain. A further cross-reaction detected in these tests was the neutralization of A2/Hong Kong and the recombinant enzymes by Duck/England/56 antiserum.

To test for similarities in their haemagglutinins, the virus strains showing cross-reactions with A2/Hong Kong/1/68 in enzyme-inhibition tests were tested against post-infection ferret antiserum for the Hong Kong variant in haemagglutination-inhibition tests. A/Turkey/Wisconsin/66, FPV, virus " N ", Duck/Germany/1998/68, Duck/England/56 and A/Swine/Cambridge/39 viruses failed to react with this antiserum.

Antigenic relationships between the neuraminidases of animal influenza A viruses

The cross-reactions in enzyme-inhibition tests were investigated for the group of 14 avian, 2 equine and 1 porcine influenza A viruses. Where suitable rabbit antisera were available (see Table 3) the reciprocal relationships were investigated. An extremely complex pattern of cross-reactions was observed between the members of this group of viruses. The detailed description of these findings is beyond the scope of the present paper. However, the major cross-reactions, i.e., those in which the enzyme-inhibiting titre of an antiserum with a heterologous virus was not less than 5% of its titre with the homologous virus, are indicated in Table 4. It is clear from the results that cross-reactions occurred

TABLE 4

MAJOR CROSS-REACTIONS [a] BETWEEN ANIMAL INFLUENZA A VIRUSES IN NEURAMINIDASE-INHIBITION TESTS

Reciprocal cross-reactions	One-way cross-reactions	
	Antiserum	Enzyme
FPV – " N "	Duck/England/56	– " N "
Quail/Italy/1117/65 – " N " – Turkey/Canada/63	Duck/England/56	– Turkey/Wisconsin/66
Du♀/Czechoslovakia/56 – Duck/England/56	FPV	– Equi-1/Prague/56 [b]
Duck/Czechoslovakia/56 – " N "	" N "	– Equi-1/Prague/56 [b]
Duck/Czechoslovakia/56 – Duck/Germany/210/67	Duck/England/56	– Equi-1/Prague/56 [b]
Swine/Cambridge/39 – Duck/Germany/210/67	Turkey/Canada/63	– Equi-2/Miami/63 [b]
Turkey/Wisconsin/66 – Turkey/Massachusetts/65 – Duck/Italy/574/66	Quail/Italy/1117/65	– Equi-2/Miami/63 [b]
	Quail/Italy/1117/65	– Duck/Germany/1998/68 [b]
	Quail/Italy/1117/65	– Duck/Italy/574/66
	Turkey/Canada/63	– Duck/Germany/1998/68 [b]
	Turkey/Germany/210/67	– Chicken/Scotland/59 [b]
	Swine/Cambridge/39	– Chicken/Scotland/59 [b]
	Tern/South Africa/61	– Turkey/England/63 [b]

[a] Cross-reactions are recorded only if the titre of the antiserum against the heterologous virus is not less than 5% of the homologous titre.

[b] Potent rabbit antisera were not available for these agents, thus reciprocal reactions were not investigated.

between strains irrespective of their avian, equine or porcine origins. The cross-reaction between these viruses in enzyme-inhibition tests differed in many cases from their known relationships as indicated in haemagglutination-inhibition tests. Details of the relationships between these viruses in haemagglutination-inhibition tests have been published elsewhere (see Pereira, Tumova & Law, 1965; Pereira et al., 1966; Tumova & Pereira, 1968; Pereira, 1969).

Cross-reactions in immunoprecipitin tests

Schild & Pereira (1969) used immuno-double-diffusion techniques to study influenza virus antigens. Using purified and concentrated suspensions of virus disrupted by a detergent (sodium dodecyl sulfate), they were able to detect precipitin lines corresponding to influenza-A RNP antigen and to influenza-A2 neuraminidase. In the present study the immunological relationships between the enzymes of A2/Singapore/1/57,A2/England/12/64,A2/England/76/66 and A2/Hong Kong/1/68 virus were compared using similar techniques. Detergent-disrupted virus concentrates were added to the peripheral wells in the agar and tested against anti-purified A2/57 neuraminidase antiserum placed in the central well. This antiserum gave a single, well-defined precipitin line which was continuous for all 4 A2 virus strains (Fig. 1). Even when the A2/57 and A2/68 viruses, which cross-reacted poorly in enzyme-inhibition tests, were placed in adjacent wells in the agar they shared an identical precipitin reaction. As a control A0/Bel. virus was included in the tests since its enzyme does not cross-react with that of A2 viruses in enzyme-inhibition tests. This virus produced no precipitin reaction. The results of the immuno-diffusion tests thus suggested that the enzymes of the A2 viruses were immunologically closely related and did not reveal any antigenic differences. In contrast the results of enzyme-inhibition tests made possible the detection of immunological differences between these enzymes.

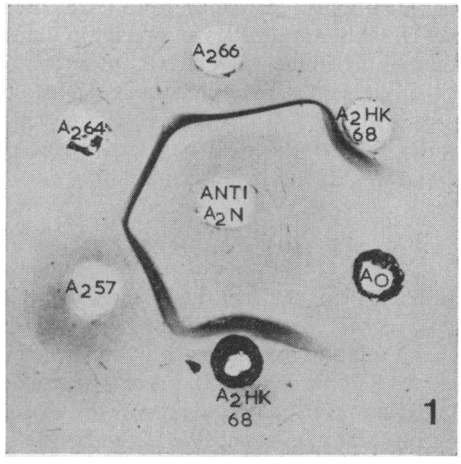
Concentrates of a number of avian influenza A viruses were similarly tested against the anti-A2/57 neuraminidase antiserum (Fig. 2). A/Turkey/Massachusetts/65 and A/Turkey/Wisconsin/66 virus gave precipitin lines which were continuous with those produced by human A2 virus strains, confirming that their enzymes were antigenically related to those of the human A2 viruses. A/Turkey/Canada/63, A/Tern/South Africa/61, A/Quail/Italy/1117/65 and A/Duck/Czechoslovakia/56, which failed to cross-react with human A2 viruses in enzyme-inhibition

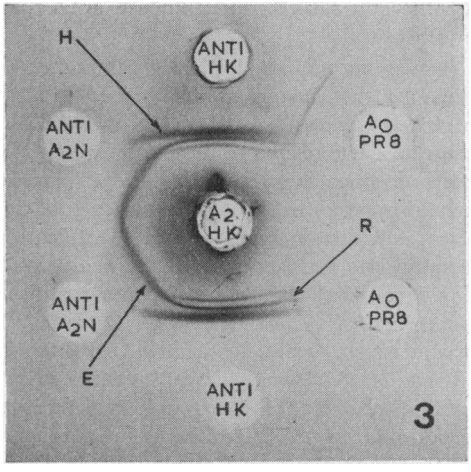
FIG. 1-5

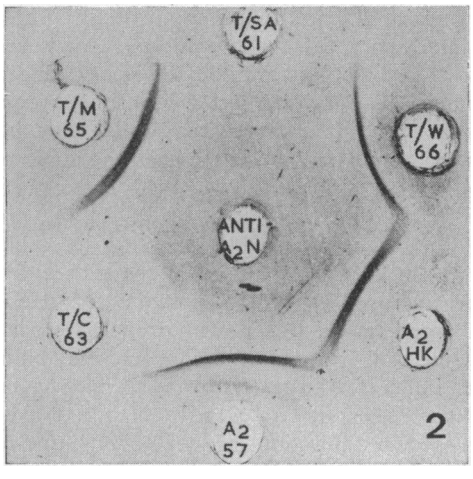
IMMUNOPRECIPITIN REACTIONS OF HUMAN AND ANIMAL INFLUENZA A VIRUS STRAINS

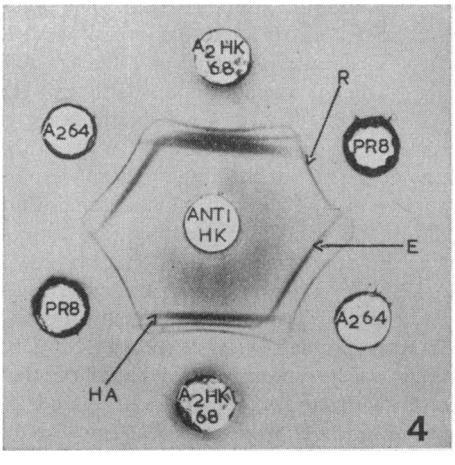
Antisera: Throughout these figures wells marked anti-A2N contain rabbit antiserum against purified A2/57 neuraminidase, those marked anti-HK, rabbit antiserum against purified A2/Hong Kong/1/68 virus; those marked anti-T/W/66, rabbit antiserum against Turkey/Wisconsin/66 virus; and those marked anti-T/SA/61, rabbit antiserum against Tern/South Africa/61 virus.

Antigens: A2/57, A2/64, A2/66, A2/HK and A0 represent wells containing purified, concentrated viruses A2/Singapore/1/57, A2/England/12/64, A2/England/76/66, A2/Hong Kong/1/68 and A0/Bel. respectively. Wells marked T/M/65, T/W/66, T/C/63, T/SA/61 contain purified, concentrated viruses Turkey/Massachusetts/65, Turkey/Wisconsin/66, Turkey/Canada/63 and Tern/South Africa/61. All viruses were disrupted by the addition of 1% sodium dodecyl sulfate.









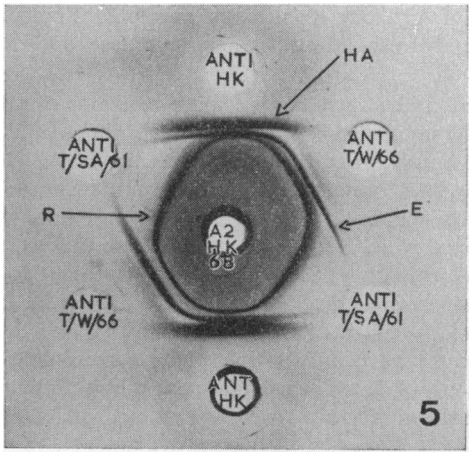


FIG. 1. Reactions of antiserum against purified A2/57 enzyme with human influenza A2 viruses. The A2 viruses, but not A0/Bel. virus, showed production of a continuous precipitin line corresponding to A2 enzyme.

FIG. 2. Reactions of antiserum against purified A2/57 enzyme with avian influenza A viruses. Turkey/Massachusetts/65 and Turkey/Wisconsin/66 gave precipitin lines corresponding to A2 enzyme. Tern/South Africa/61 and Turkey/Canada/63 did not react.

FIG. 3. Identification of multiple precipitin lines (see text). R represents ribonucleoprotein, E represents A2 enzyme and H represents A2/HK haemagglutinin.

FIG. 4. Identification of multiple precipitin lines (see text). All 3 virus strains share a common ribonucleoprotein line. The 2 A2 strain viruses share a common enzyme line. The haemagglutinin line HA is specific for A2/Hong Kong virus.

FIG. 5. Reactions of antisera against avian influenza strains in tests with A2/Hong Kong virus. Anti-Turkey/Wisconsin/66 produced an enzyme and ribonucleoprotein line but anti-Tern/South Africa/61 antiserum gave only 1 precipitin line corresponding to influenza A ribonucleoprotein.

tests, also failed to do so in precipitin tests. Virus " N " and FPV showed minor cross-reactions with A2 viruses in enzyme-inhibition tests but failed to react with anti-A2/57 neuraminidase antiserum in precipitin tests.

In further immunoprecipitin tests immune rabbit sera against the various animal influenza A virus were tested against A2/Hong Kong/1/68 virus concentrate. The results of these tests were difficult to interpret since the antisera frequently produced more than 1 precipitin line and contained antibody to influenza-A RNP antigen. However, identification of a precipitin line corresponding to A2 neuraminidase was made possible by using a reference antiserum prepared against A2/Hong Kong/1/68 virus. This antiserum gave 3 well-defined precipitin lines when tested against A2/Hong Kong/1/68 virus (Fig. 3). The central one of these lines could be identified as corresponding to A2 neuraminidase since it was continuous with the single precipitin line given by anti-A2/57 neuraminidase antiserum. The precipitin line forming nearest to the well containing A2/Hong Kong/1/68 virus was identified as influenza-A RNP (see Fig. 3) since it was continuous with a single line given by A0/PR/8 virus (which contains neuraminidase and haemagglutinin distinct from that of A2/Hong Kong). That this line might correspond to host antigen seems unlikely since preparations of B/England/5/66 virus obtained by the same techniques as the influenza-A2 virus failed to react with the anti-A2/Hong Kong antiserum. The evidence that the third and outermost precipitin line (see Fig. 3) corresponded with A2/Hong Kong haemagglutinin was presumptive and based on the fact that tests with an A2 virus (A2/England/12/64), which contains an enzyme cross-reacting with that of A2/Hong Kong but a haemagglutinin distinct from that of A2/Hong Kong virus, gave only 2 precipitin lines, corresponding to neuraminidase and RNP (Fig. 4).

Fig. 5 illustrates the precipitin reactions of rabbit antiserum against A/Turkey/Wisconsin/66 and A/Tern/South Africa/61 virus in tests against A2/Hong Kong/1/68 virus. With antiserum against the Turkey/Wisconsin/66 virus, 2 precipitin lines were detected which could be identified as corresponding to A2 neuraminidase and RNP by reference to the precipitin lines given by anti-A2/Hong Kong antiserum. In contrast, antiserum against A/Tern/South Africa/61 gave only 1 line which corresponded to influenza-A RNP. In similar tests rabbit antisera to the following viruses were tested against A2/Hong Kong virus: A/Turkey/Wisconsin/66, virus " N ", FPV, A/Quail/Italy/1117/65, A/Duck/Czechoslovakia/56, A/Duck/England/56, A/Duck/Germany/210/67, A/Tern/South Africa/61 and A/Swine/Cambridge/39. All antisera gave precipitin lines corresponding to influenza-A RNP, thus confirming that the viruses were influenza type A strains. However, only A/Turkey/Wisconsin/66 gave a precipitin line which could be identified as corresponding to A2/Hong Kong neuraminidase.

DISCUSSION

Antibodies to the neuraminidase of influenza viruses react in enzyme-inhibition tests but do not in general inhibit virus haemagglutination (Webster, Laver & Kilbourne, 1968). Anti-haemagglutinin antibody, however, may cause low levels of enzyme-inhibition (Easterday et al., 1969; Schild & McCahon, 1970). In comparing the antigenic character of the enzymes of different influenza viruses it is therefore of importance to take precautions to ensure that anti-haemagglutinin antibody does not affect the results. This may be effected by using appropriate recombinant strains of virus which contain the enzyme of one parent and the haemagglutinin of the other parent (Kilbourne et al., 1967; Easterday et al., 1969; Schild & McCahon, 1970). In the present study it was impracticable to obtain recombinants of all the virus strains used in the tests and these were employed on only a limited scale. However, virus preparations treated with pronase were used as a source of neuraminidase for enzyme-inhibition tests. Such treatment destroys virus haemagglutinin and has been found an effective method of avoiding non-specific inhibition of enzyme by anti-haemagglutinin antibody (Easterday et al., 1969). It is possible that antibody directed against host components present in the influenza virus envelope might have a role in inhibiting enzyme activity. However, tests in this laboratory (Schild—personal observation) with antibody against purified host component (Haukenes, Harboe & Mortensson-Egnund, 1966) failed to detect enzyme-inhibition activity. Immunoprecipitin tests were used as an alternative method of comparing virus enzymes. Schild & Pereira (1969), using detergent-disrupted influenza viruses, were able to detect precipitin lines corresponding to A2 virus neuraminidase.

In the present study enzyme-inhibition tests and immunoprecipitin tests both provided evidence of a close antigenic relationship between the enzyme of

the A2/Hong Kong virus and that of Turkey/Wisconsin/66. The enzyme of this turkey virus was also related to that of the A2/57 and A2/66 virus. It is of interest that, while Turkey/Massachusetts/65 enzyme is closely related to that of A2/57 virus, it did not cross-react with A2/Hong Kong enzyme. In contrast, the enzyme of a recently isolated avian virus (Duck/Germany/1998/68) showed cross-reactions with A2/Hong Kong enzyme but not with that of the A2/57 or A2/66 strains. Although minor cross-reactions between the enzymes of FPV, virus "N", Duck/England/56 and Swine/Cambridge/39 and that of A2/Hong Kong were detected in enzyme-inhibition tests, these reactions could not be confirmed by precipitin tests and their significance requires further investigation.

A number of hypotheses can be made to explain the occurrence in avian viruses of neuraminidase antigenically related to that of human virus strains. One explanation is that human subtypes may be derived from avian virus strains which are able to overcome host-range barriers. However, this does not explain why the avian and human viruses with related enzymes do not also share related haemagglutinins. Another possibility is that the enzymes of both avian and human viruses have arisen as a result of independent changes in antigenic structure and their antigenic similarity is a chance phenomenon. A further possibility is that genetic interaction may occur in nature between influenza A viruses of different hosts and may give rise to viruses sharing the envelope antigens of both parent virus strains. It has been clearly demonstrated in laboratory studies (Tumova & Pereira, 1965; Easterday et al., 1969) that such genetic interactions occur with a high frequency between avian and human influenza A viruses and may result in the appearance of recombinant virus strains having an enzyme derived from one parent and haemagglutinin derived from the other.

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