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Antibody against influenza A2 virus neuraminidase in human sera

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Surveys of antibody to influenza A viruses in the human population have frequently relied upon the haemagglutination-inhibition technique and this method has also been widely used in the serological diagnosis of influenza infections. Haemagglutination-inhibition tests have the advantage of rapidity and simplicity and they make it possible to detect antibody reacting specifically with different subtypes of influenza virus. In addition the titre of haemagglutination-inhibiting antibody is related to the capacity of a serum to neutralize virus infectivity and the presence of such antibody has been correlated with the susceptibility of the individual to influenza virus infection (Bell *et al.* 1957; Tyrrell, 1967). However, it is now well established that haemagglutinin is but one of the two principal viral antigens present on the surface envelope of the influenza A virus; the other virus-coded antigen being the enzyme neuraminidase. The neuraminidase of influenza A virus has been purified and separated from haemagglutinin (Laver, 1964; Laver & Kilbourne, 1966). Anti-neuraminidase antisera have enzyme-inhibiting capacity but do not inhibit haemagglutination and anti-haemagglutinin antisera do not inhibit enzyme activity (Webster & Laver, 1967; Seto & Rott, 1966). Because of the immunological independence of haemagglutinin and neuraminidase it seems likely that the measurement of haemagglutination-inhibiting antibody alone might give an incomplete picture of the antibody response in human influenza infections. We have therefore studied the development of neuraminidase-inhibiting antibody for influenza A2 viruses in man following natural influenza A2 infections and following vaccination with killed influenza A2 virus.

MATERIALS AND METHODS

Virus strains

Human influenza viruses A1/FM1, A2/Singapore/1/57, A2/England/2/64, A2/England/76/66 and B/England/5/66 were from the stocks of this laboratory. A0/BEL was kindly supplied by Drs W. G. Laver and R. G. Webster of the John Curtin School of Medical Research, Canberra, Australia.

FPV-A2 (R4) was a laboratory recombinant virus derived from fowl plague and A2/Singapore/1/57 (Tumova & Pereira, 1965). The neuraminidase of this strain has been shown to be immunologically identical with that of the A2 parent whilst the haemagglutinin is identical with that of fowl-plague virus (Easterday, Laver,

Pereira & Schild, in preparation). Influenza A/Turkey/Massachusetts/65 (Pereira *et al.* 1966) is a virus of avian origin which contains a neuraminidase immunologically identical with that of A2/Singapore/1/57 and a haemagglutinin unrelated to that of human A2 virus (Pereira, Tumova & Webster, 1967; Webster & Pereira, 1968).

Virus purification and concentration

The viruses used in neuraminidase inhibition tests were grown in the allantoic cavity of 10-day embryonated eggs. The allantoic fluids were harvested 1–3 days after infection and the virus was purified by adsorption onto and elution from fowl erythrocytes followed by differential centrifugation and one or two cycles of rate zonal centrifugation on a linear 10–40 % sucrose gradient. The final yield of purified virus from 100 eggs was suspended in 0·5 ml. saline. These preparations contained between 500 and 5000 units of neuraminidase activity per 0·1 ml.

Human sera

Acute and convalescent serum samples taken on the first or second day of illness and three to four weeks later were obtained from adults with clinical influenza between December 1967 and February 1968. These sera were collected during the course of a clinical trial of an antiviral compound carried out under the auspices of the College of General Practitioners, London. However, in the present study the sera used were from donors who received an inactive placebo.

Pre- and post-immunization serum specimens were obtained from adult volunteers who received formalin-inactivated influenza virus vaccines containing A2/Singapore/1/57 virus. Sera were tested from six volunteers who received a vaccine incorporating a vegetable-oil adjuvant (A65) in a vaccine trial carried out by the Medical Research Council at R.A.F. Halton. (Unpublished report to the M.R.C. Committee on Influenza and other Respiratory Virus Vaccines, 1967). A further seven serum pairs from volunteers receiving a mineral-oil adjuvant (drakeol-arlacel) vaccine in a trial carried out by Evans Medical Ltd., Liverpool, were used.

Rabbit antisera

The preparation of a rabbit antiserum against purified A neuraminidase has been described (Webster & Laver, 1967) The source of the purified neuraminidase was X7 (F1) virus (Kilbourne *et al.* 1967), a recombinant virus which contains neuraminidase derived from its A2(R1/5) (1957) parent. This serum had no haemagglutinin-inhibiting antibody for A2 virus. For use in neuraminidase inhibition tests antisera against ultraviolet-inactivated, purified influenza virus preparations with Freund's complete adjuvant were prepared in rabbits as described by Webster & Laver (1967). All sera were stored at $-20°$ C.

Haemagglutinin estimations

Influenza virus haemagglutinin and the measurements of haemagglutinin-inhibiting antibody in serum specimens were carried out by standard methods (W.H.O. Expert Committee on Influenza, 1953). Virus and antiserum were left in contact for 1 hr. before addition of erythrocytes.

Preparation of purified neuraminidase.

Purified and concentrated FPV-A2(R4) virus (Tumova & Pereira, 1965) was disrupted by sodium dodecyl sulphate (1 %) and the proteins separated by electrophoresis on cellulose acetate strips (Laver, 1964). The protein band containing the enzymic activity was eluted with water and the enzyme freed of sodium dodecyl sulphate by precipitation of the detergent with saturated potassium chloride solution.

Estimation of neuraminidase activity and neuraminidase-inhibiting antibody

The neuraminidase activity of virus preparations was estimated by incubating serial dilutions of virus (0·05 ml.) for 1 hr. at 37° C. with 0·1 ml. volumes of buffered fetuin solution (12 mg./ml. in phosphate buffer, pH 5·9). The release of *N*-acetyl neuraminic acid was assayed by a modification of the Warren method (Webster & Laver, 1967) employing optical density determinations on the Unicam SP500 at 549 μ.

For the estimation of neuraminidase-inhibiting antibody a modification of the technique described by Laver & Kilbourne (1966) was used. Dilutions of purified influenza virus containing 1–2 units of enzyme (Webster & Laver, 1967) in 0·05 ml were incubated at 4° C. for 15 hr. with serial 10–fold or 3·3-fold dilutions of serum. Fetuin solution (0·1 ml.) in buffered saline (pH 5·9) was added and the mixture incubated at 37° C. for 1 hr. Control samples of enzyme were incubated with equivalent dilutions of normal human or rabbit serum. The neuraminidase-inhibition titre of a serum was expressed as the reciprocal of the serum dilution which produced 50 % inhibition of enzyme activity. This value was obtained from the plot of serum dilution against the percentage inhibition of enzyme activity.

RESULTS

Neuraminidase antibody in natural A2 infections

The development of neuraminidase-inhibiting antibody was studied in paired sera from twenty adults with serologically confirmed influenza A2 infections. For each serum pair, serological evidence of influenza A2 infection was obtained both in complement-fixation tests with influenza A soluble antigen and in haemagglutination-inhibition tests with A2/Singapore/1/57 and A2/England/10/67. The latter is a virus strain isolated at the time of the epidemic during which the paired sera were collected. Representative results are shown in Table 1. Neuraminidase-inhibiting antibody for A2/Singapore/1/57 virus was detected in only three of 20 (15 %) of the acute serum samples and the titres were low, ranging from 1/10 to 1/50. In 18 of the 20 serum pairs studied neuraminidase-inhibiting antibody for A2/Singapore/1/57 was acquired during the course of the infection or showed an increase in titre. The neuraminidase-inhibiting antibody titres in the convalescent sera ranged from 1/50 to 1/3000 and the arithmetic mean titre in the convalescent sera was 1/420. In the remaining two serum pairs no neuraminidase-inhibiting antibody was detected in the acute or convalescent serum. When A0/BEL and

Table 1. *Development of neuraminidase-inhibiting antibody in adults with serologically confirmed influenza A 2 infections**

Serum pair		Complement-fixation antibody titres (PR 8-S antigen)	Haemagglutination-inhibition antibody titres with		Neuraminidase-inhibiting antibody titres† with various influenza virus strains:			
			A 2/Singapore/1/57	A 2/England/10/67	A 0/BEL	A 1/FM 1	A 2/Singapore/1/57	B/England/5/66
2B	*a*‡	10	480	48	<	<	<	<
	c	320	1280	192	<	<	450	<
3B	*a*	<§	160	<	<	<	<	15
	c	> 320	> 1280	96	<	<	75	15
5A	*a*	<	30	<	<	<	30	<
	c	320	1280	192	<	<	500	<
14A	*a*	<	<	<	<	<	<	<
	c	320	36	24	<	<	130	<
16A	*a*	20	10	40	<	<	<	<
	c	320	640	96	<	<	3000	<
18D	*a*	<	240	24	<	<	<	<
	c	> 320	1280	> 384	<	<	750	<
26A	*a*	<	240	24	<	<	<	<
	c	> 320	960	96	<	<	450	<
20A	*a*	20	60	<	<	<	<	10
	c	240	> 1280	192	<	<	50	120

* Infections occurred in the period December–February 1968 in England. † Reciprocal of serum dilution producing 50 % inhibition of neuraminidase activity. ‡ *a*, Acute serum specimen; *c*, convalescent serum specimen 2–4 weeks after onset of illness. § < = titres less than 1/10.

A1/FM1 viruses were used, none of the sera showed detectable neuraminidase-inhibiting antibody. In one of the 20 serum pairs (20A) there were simultaneous increases in neuraminidase-inhibiting antibody against influenza A2 and influenza B/England/5/66. It seems likely that these sera were from an individual with simultaneously occurring influenza A2 and B infections, although haemagglutination-inhibiting tests indicated an antibody response to A2 virus but not B/Engand/5/66.

Table 2. *Development of neuraminidase-inhibiting antibody in individuals with influenza B infections**

Serum pair	Haemagglutination-inhibiting antibody titres	Neuraminidase-inhibiting antibody titres with	
	B/England/5/66	B/England/5/66	A2/Singapore/1/57
767 *a*†	<‡	<	20
c	320	75	20
769 *a*	<	<	10
c	160	10	10
770 *a*	<	<	<
c	160	30	<

* Infections occurred in 1966 in England.

† *a*, Acute serum specimen;
c, denotes convalescent serum taken 2–4 weeks after onset of illness.

‡ < = titres less than 1/10.

Table 2 shows the results of similar studies with paired sera from individuals with serologically confirmed influenza B infections. In all three serum-pairs neuraminidase-inhibiting antibody to influenza B/England/5/66 virus was acquired during the infection. However, the antibody titres for influenza B neuraminidase in the convalescent sera were much lower than those detected for A2 virus in convalescent sera from individuals with influenza A2 infections. There was no simultaneous rise in antibody for the neuraminidase of influenza A2/Singapore/1/57 virus.

To extend these observations, a random collection of sera from adults obtained in the period January to November 1967, before the onset of the 1967–8 influenza A2 epidemic, was tested for frequency and titre of neuraminidase-inhibiting and haemagglutination-inhibiting antibody. In Table 3 the relationship between the antibody titres of the random sera in both types of test is compared with that found in a collection of convalescent sera. Neuraminidase-inhibiting antibody was detected in 30 % of the random serum samples and the titres were low, in no case exceeding 1/50. This neuraminidase antibody was more frequent in sera which had high titres of haemagglutination-inhibiting antibody. Thus, 77 % of random serum samples with haemagglutination-inhibition titres of 1/640 or greater had neuraminidase antibody whilst only 8 % of those with haemagglutination-inhibition titres of 1/160 or less had antibody for neuraminidase. For the collection of random serum samples the ratio of the mean neuraminidase-inhibiting antibody titre and the mean haemagglutination-inhibiting antibody titres were low ($< 0{\cdot}05$). However,

Table 3. *Relationship between the neuraminidase-inhibition and haemagglutination-inhibition antibody titres for influenza A 2/Singapore/1/57 virus in pre-epidemic and convalescent sera from adults*

	Haemagglutination-inhibiting antibody titre	No. of sera with the stated haemagglutination inhibition titre	No. and % of sera with neuraminidase-inhibiting antibody at 1/5 or greater	Mean neuraminidase-inhibiting antibody titre in positive sera	Ratio: mean neuraminidase-inhibition titre / mean haemagglutination-inhibition titre
Random (pre-epidemic) serum specimens	< 10	24	0 (0%)	—	—
	80–160	24	2 (8·3%)	1/5	0·04
	⩾640	26	20 (77%)	1/17	0·02
	All pre-epidemic sera	74	22 (30%)	< 1/17	0·05
Convalescent serum specimens	⩾640	15	15 (100%)	1/520	0·54

for the collection of convalescent sera (Table 3) neuraminidase-inhibiting antibody was detected in all samples and the ratio of the mean neuraminidase-inhibition to haemagglutination-inhibition antibody titres was at least ten-fold higher (0·54). Indeed for four of 15 convalescent serum samples the neuraminidase-inhibiting antibody titre was higher than the haemagglutination-inhibition antibody titre with A2/Singapore/1/57.

Table 4. *Persistence of neuraminidase-inhibiting antibody after human influenza A2 infections*

Patient		Pre-infection serum*	Post-infection serum samples† 1st	2nd	3rd	4th
11/1	NI‡	<§	200	10	<	<
	HI	<	1280	160	120	80
	CF	<	> 320	> 80	> 80	⩾ 80
11/2	NI	<	250	<	<	<
	HI	<	1280	320	80	160
	CF	<	> 320	⩾ 80	⩾ 80	⩾ 80
15/1	NI	<	75	<	<	<
	HI	<	20	20	15	10
	CF	<	80	> 80	> 80	> 80
19/2	NI	<	150	20	<	<
	HI	<	480	640	320	320
	CF	<	⩾ 320	⩾ 80	⩾ 80	⩾ 80

* Pre-infection serum samples were obtained in June 1957. † 1st post infection serum samples were obtained in November 1957; 2nd in March 1958; 3rd in June 1958; 4th in June 1959. ‡ NI = Antibody titres detected in neuraminidase-inhibition tests using A2/Singapore/1/57 virus. HI = Haemagglutination-inhibition antibody titres using A2/Singapore/1/57 virus. CF = Antibody titres in complement-fixation tests with influenza A soluble antigen. § < denotes titres of less than 1/10.

Persistence of neuraminidase antibody after influenza infections

One interpretation of the above findings is that antibody to neuraminidase, as detected in neuraminidase-inhibition tests, persists for shorter periods of time after influenza A2 infection than does antibody to haemagglutinin. To test this possibility, serial serum samples obtained from four individuals at intervals after they were infected with influenza A2 in 1957 were tested for neuraminidase-inhibiting antibody (Table 4). For two of the four persons antibody could no longer be detected 5 months after infection and for two individuals it was present in much reduced titres. None of the four individuals had detectable antibody 9 months after infection. In two of the four individuals a decrease in haemagglutination-inhibiting antibody was detected in the first 5 months after infection but thereafter the antibody levels remained relatively constant over a 2-year period. In two individuals the haemagglutination-inhibiting antibody titres did not decrease significantly.

Table 5. *Specificity of the neuraminidase-inhibition antibody response in human influenza A 2 infections*

Human serum pair*	Haemagglutination-inhibiting antibody titres with: A2/Singapore/1/57	Haemagglutination-inhibiting antibody titres with: A/turkey/Mass/65	Neuraminidase-inhibiting antibody titres with influenza A2 neuraminidase from various sources: Purified A2 enzyme	A/turkey/Mass/65 virus	FPV-A2 (R4) virus	A2/Sing/1/57 virus	A2/Eng/12/64 virus	A2/Eng/76/66 virus
3A *a*	480	<†	<	<	<	<	<	<
c	> 1280	<	300	750	200	250	50	100
5A *a*	30	<	10	40	20	30	5	5
c	1280	<	600	1200	500	500	150	75
16A *a*	10	<	<	<	<	<	<	<
c	640	<	1500	4500	700	3000	750	500
18C *a*	240	<	5	20	5	20	<	<
c	1280	<	200	750	250	350	75	25
20B *a*	48	<	<	<	<	<	<	<
c	960	<	100	120	50	50	350	400

* *a* denotes acute serum specimen, *c* denotes convalescent serum 2–4 weeks after onset of illness. † < denotes titres less than 1/10.

Table 6. *Serological relationships between the neuraminidases of various influenza A 2 virus strains and A/turkey/Massachusetts/65 in neuraminidase-inhibition tests using rabbit antisera*

Source of neuraminidase	Rabbit antisera ... Anti purified A2/57 neuraminidase	Anti A2/Singapore/1/57	Anti A2/England/12/64	Anti A/turkey/Massachusetts/65	Anti A0/BEL
Purified A2 neuraminidase (source A2/57)	**3,000***	250	20	400	<
A2/Singapore/1/57 virus	2,500	**300**	30	350	<
A2/England/12/64 virus	1,500	<	**3,500**	40	<
A2/England/76/66 virus	1,680	<	**3,500**	20	<
A/turkey/Massachusetts/65 virus	10,000	120	800	**2,510**	<
A0/BEL virus	<	<	<	<	**3,000**
A1/FM1 virus	<	<	<	<	450

* Reciprocal of serum dilution producing 50 % inhibition of neuraminidase activity.

Specificity of neuraminidase antibody

It was important to establish that the neuraminidase-inhibiting activity detected in convalescent sera was due to antibody reacting specifically with neuraminidase. Neuraminidase-inhibition tests carried out with sera from hyperimmunized rabbits (Paniker, 1968; Easterday, Laver, Pereira & Schild, in preparation) have indicated that high titres of antibody to neuraminidase may produce apparent neutralization of neuraminidase when intact virus particles are used as source of neuraminidase. Such effects are probably dependent upon antibody to haemagglutinin coating the virus surface and producing steric hindrance of the access of substrate to the enzyme. Thus, a number of acute and convalescent human sera were retested against a purified enzyme preparation obtained by the electrophoretic separation of influenza A2 virus after sodium dodecyl-sulphate treatment. In addition, influenza A/turkey/Massachusetts/65 virus and FPV-A2 (R4) virus were used since these viruses are known to contain influenza A2 neuraminidase but not influenza A2 haemagglutinin. It was also of interest to compare neuraminidase-inhibiting antibody titres with A2 strains other than A2/Singapore/1/57.

In five serum-pairs tested against purified A2 neuraminidase and FPV-A2 (R4) the antibody rises observed were of the same order as those found using intact A2/Singapore/1/57 (Table 5). When A/turkey/Massachusetts/65 was used neuraminidase-inhibiting antibody titres in the convalescent sera were in some cases higher than with A2/Singapore/1/57 although there was no haemagglutination-inhibiting antibody for the turkey influenza virus in these sera. It was concluded from these results that the neuraminidase-inhibiting activity of the human convalescent sera was due to antibody directed against virus neuraminidase.

When virus strains A2/England/12/64 and A2/England/76/66 were used as sources of neuraminidase all five serum pairs showed significant rises in neuraminidase-inhibiting antibody. Four of the five convalescent sera had titres lower than those detected with A2/Singapore/1/57 and for the fifth convalescent serum the neuraminidase-inhibition antibody titres with the A2/64 and A2/66 viruses were higher than with the A2/57 virus.

Studies with rabbit antisera

The results obtained with the convalescent human sera suggested that the neuraminidases present in the A2/64 and A2/66 viruses might differ immunologically from that present in A2/Singapore/1/57. This possibility was investigated in tests with immune animal sera (Table 6). A rabbit antiserum prepared against purified A2/57 neuraminidase reacted with all A2 virus strains tested and with A/turkey/Massachusetts/65. However, rabbit antiserum prepared against intact A2/Singapore/1/57 virus reacted only with purified A2 neuraminidase and with the neuraminidases of intact A2/Singapore/1/57 and A/turkey/Massachusetts/65 virus but not with A2/England/12/64 or A2/England/76/66. The failure of the antiserum prepared against A2/Singapore/1/57 virus to react with the A2/64 and A2/66 viruses probably reflects the fact that this antiserum had a lower homologous neuraminidase-inhibition titre than did the antiserum against purified A2/57

neuraminidase. Antiserum against A/turkey/Massachusetts/65 virus reacted with higher titres to A2/Singapore/1/57 than with the A2/64 and A2/66 virus strains. Antiserum for A2/England/12/64 virus reacted to high titres with the neuramini-

Table 7. *Neuraminidase antibody response in man after immunization with killed influenza A2 virus*

Serum sample	Haemagglutination-inhibition antibody titre, A2/Singapore/1/57	Neuraminidase-inhibiting antibody titre using: A2/Singapore/1/57	Neuraminidase-inhibiting antibody titre using: A/turkey/Massachusetts/65
1 *a**	384	10	< 5
b	384	10	< 5
c	1536	50	30
4 *a*	12	< 5	< 5
b	768	120	75
c	1536	175	150
6 *a*	192	10	< 5
b	1536	30	50
c	1536	120	150
10 *a*	192	5	< 5
b	1536	35	30
c	1536	200	150
13 *a*	24	< 5	< 5
b	96	< 5	< 5
c	1536	< 5	< 5
17 *a*	48	< 5	< 5
b	1536	50	75
c	1536	150	200
21 *a*	96	< 5	< 5
b	1536	20	35
c	1536	20	50
3 *a*†	24	< 5	< 5
b	576	20	10
12 *a*	48	< 5	< 5
b	384	< 5	< 5
55 *a*	192	15	< 5
b	768	40	30
65 *a*	36	< 5	< 5
b	768	75	50
66 *a*	9	< 5	< 5
b	768	30	50
72 *a*	9	< 5	< 5
b	192	< 5	10

* Persons receiving drakeol-arlacel adjuvant vaccine containing 3500 haemagglutinating units of A2/Singapore/1/57 and A2/England/1/66 virus inactivated with 1/10,000 formalin. *a*, pre-immunization serum; *b*, 1 month post-immunization; *c*, 3 months post-immunization.

† Persons receiving A65 vegetable-oil adjuvant vaccine containing 3500 haemagglutinating units of A2/Singapore/1/57 inactivated with 1/10,000 formalin. *a*, Pre-immunization serum; *b*, 5–6 weeks post-immunization.

dase of the homologous virus and with A2/England/76/66 but poorly with A2/Singapore/1/57 neuraminidase.

These results indicate that amongst the influenza A2 viruses there are two immunologically distinguishable, but related types of neuraminidase. These are represented by the neuraminidases present in A2/Singapore/1/57 and in A2/England/12/64 virus. The neuraminidase of A/turkey/Massachusetts/65 seemed to resemble that of A2/Singapore/1/57 more than that of A2/England/12/64. This latter finding is in agreement with the conclusions of Pereira *et al.* (1967), who compared the turkey virus with human A2 strains in strain specific complement fixation tests.

No cross-reactions were detected between the neuraminidases of the A2 viruses and examples of the A0 or A1 subtypes of human influenza virus, although the neuraminidases of the A0 and A1 viruses were themselves immunologically related. Similar findings in regard to the cross-reactions between the neuraminidases of A0 and A1 virus have been reported (Paniker, 1968).

Neuraminidase antibody induced by vaccination

We wondered whether neuraminidase-inhibiting antibody appeared after immunization with killed influenza virus. The sera from individuals who had received a single dose of two different influenza virus vaccines were therefore titrated using A2/Singapore/1/57 and A/turkey/Massachusetts/65 (Table 7). One vaccine preparation contained formalin-inactivated influenza virus with vegetable oil (A65) adjuvant (Woodhour *et al.* 1964), the other contained a preparation of formalinized virus with mineral-oil adjuvant. Neuraminidase-inhibiting antibody was detected in only a proportion of individuals who received the vegetable-oil adjuvant vaccine and the titres were low although the same vaccines stimulated high titres of haemagglutination-inhibiting antibody. In individuals receiving the mineral-oil adjuvant vaccine the titres of neuraminidase-inhibiting antibody (and also of haemagglutination-inhibiting antibody) were higher than with the vegetable-oil adjuvant vaccine but not as high as those developing after natural influenza A2 infections.

DISCUSSION

The biological properties of antibody prepared specifically against influenza neuraminidase have been studied in *in vitro* systems by a number of workers. Such antibody has potent enzyme-inhibiting activity but it does not inhibit virus haemagglutination (Seto & Rott, 1966; Laver & Kilbourne, 1966; Webster & Laver 1967). Another activity of antineuraminidase antiserum is to reduce virus plaque size (Seto & Rott, 1966; Jahiel & Kilbourne, 1966) and to cause apparent virus neutralization by inhibiting virus release from infected cells (Webster & Laver 1967). Elution of virus after adsorption to erythrocytes is also inhibited (Brown & Laver, 1968). These biological properties of antineuraminidase antibody suggest that such antibody if stimulated by natural influenza infections, might play a role in the development of immunity to influenza in man.

In the present investigation 18 of 20 individuals studied developed neuramini-

dase-inhibiting antibody after natural influenza A2 infections. However, it should be emphasized that the paired sera used in the study were from individuals with clinical influenza in which the diagnosis was confirmed by the demonstration of significant antibody rises both in complement fixation tests with influenza A soluble antigen and in haemagglutination-inhibition tests. It is possible that the frequency and titre of neuraminidase-inhibiting antibody would have been lower had the study included individuals for which the serological evidence of infection was less complete, or if individuals with subclinical infections were included in the study.

The relevance of our findings in the development of immunity to influenza infections in man requires further study. A suitable experimental model might be the study of experimental influenza virus infections in animals specifically immunized against neuraminidase.

It is of interest that the studies with rabbit antisera indicated two immunologically distinguishable types of neuraminidase among the influenza A2 viruses which were compared. The strains A2/Singapore/1/57 and A2/England/12/64 are examples of viruses containing the two different types of enzyme. These findings suggest that antigenic variation among human influenza A virus strains of a single subtype may reflect changes in neuraminidase as well as in the haemagglutinin. However, both the studies of Paniker (1968) and those reported here indicate that the neuraminidase of the human influenza viruses of A0 and A1 subtypes are closely related immunologically although their haemagglutinins are different. It seems therefore that antigenic variation in neuraminidase and in haemagglutinin occur independently. The human convalescent sera were in general more broadly reactive than the immune rabbit sera and frequently these sera had similar neuraminidase-inhibition titres irrespective of whether A2/Singapore/1/57 or A2/England/12/64 was used as a source of neuraminidase.

The failure of neuraminidase-inhibiting antibody to persist longer than 5 months after infection in the four individuals from whom serial specimens were available is of interest. Before the significance of this finding can be determined further studies are required on the nature of neuraminidase-inhibiting antibodies in convalescent sera.

SUMMARY

1. High titres of neuraminidase-inhibiting antibody were detected in convalescent human sera following natural influenza A2 infections.

2. Such antibody was encountered infrequently in acute serum samples. Antibody persisted only 5–6 months after infection in the four individuals from whom serial serum specimens were available.

3. Following immunization with killed influenza virus vaccines (with adjuvant) neuraminidase inhibiting antibody was detected in human sera. The titres were in general lower than those detected in convalescent human sera.

4. The specificity of the neuraminidase-inhibiting antibody in human and animal antisera was studied. Tests with convalescent human sera using purified neuraminidase preparations and with a recombinant virus containing A2 neuraminidase

and haemagglutinin distinct from that of human influenza A viruses enabled the conclusion that the antibody detected was specific for influenza A 2 neuraminidase.

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