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Natural infection with influenza A (H3N2). The development, persistence and effect of antibodies to the surface antigens

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SUMMARY

A technique for estimating antibodies to the neuraminidase antigens of influenza A is described.

The antibody response to the haemagglutinin and neuraminidase antigens of influenza A (H3N2) was studied in a boys' public school over the four-year period 1970–4. During this time there were two outbreaks of influenza A and the effect of antibody on the result of natural challenge was investigated. No boy who had homotypic neuraminidase antibody had clinical influenza.

INTRODUCTION

A controlled trial of influenza vaccines in a boys' boarding school provided an opportunity to study the serology of natural infection with influenza A. Some results from this trial have already been reported (Hoskins *et al.* 1973, 1976). Fifty-six boys, who were present in the school when the trial started in 1970, received only influenza B vaccine and were bled each year from 1970 to 1974. During this period there were two outbreaks of influenza A – one due to a strain similar to A/England/42/72 in December 1972 and another due to a strain similar to A/Port Chalmers/1/73 in March 1974. The results presented here show the relation of circulating antibody to infection.

Since the advent of influenza A/Hong Kong (H3N2) in 1968 techniques for investigating the antigenic structure of the influenza viruses have become more refined and small differences in the surface antigens are now recognized (Schild, Oxford & Virelizier, 1975). Although the strains of influenza A in circulation since 1968 are all of the same sub-type with common components in both the haemagglutinin (HA) and the neuraminidase (NA), there have been progressive changes in both these surface antigens. Sera raised against early strains of the H3N2 series cross-react with recent strains only to the extent that the tests used detect the common antigenic components. The antigenic drift of the surface antigens of the H3N2 series is shown schematically in Table 1.

The examination of human sera for antibodies to the surface antigens of current strains may be undertaken to assess the state of immunity of a population or to evaluate a vaccine. The test most commonly used for these purposes is the haemagglutination-inhibition test (HI). Since antibodies to the neuraminidase antigens

Table 1. *Antigenic drift of Influenza A*

(after Schild *et al.* 1975)

	Asian era	Hong Kong era						
	1957–67	1968	1969	1970	1971	1972	1973	1974
Antigen HA	H2 (A/Eng/64)	H3 (A/HK and Rec HK) →	→	→	H3′ →	H3″ (A/Eng/72) →	H3‴ (A/PC) →	H3⁗ (A/Scot)
NA	N2 (X15) →	→	N2′ (X38) →	→	→	→	N2″ (X42) →	→

Strains used shown in parenthesis (for abbreviations see text).

are known to play a part in immunity to influenza (Couch *et al.* 1974) and as it has been suggested that their presence is related to resistance to clinical symptoms (Murphy, Kasel & Chanock, 1972) it is clearly relevant to measure these also. The two techniques most commonly used are the enzyme inhibition test using fetuin as a substrate (Aymard-Henry *et al.* 1973) and single radial diffusion (Schild, Henry-Aymard & Pereira, 1972). The enzyme inhibition test is technically extremely cumbersome to perform and uses a relatively large amount of serum. Single radial diffusion, once set up, is simple to perform with small volumes of serum, but requires high titre purified antigen. Dowdle, Sarateanu & Reimer (1972) showed that, under certain conditions, it is possible to use a haemagglutination inhibition test to detect neuraminidase antibody. Since HI tests were already in use in our laboratory and had been carefully standardized, it seemed appropriate to use the same system adapted to anti-neuraminidase assay.

MATERIALS AND METHODS

The boys

The trial started in October 1970 when boys whose parents had consented were allocated by date of birth to receive either influenza A or B vaccine. A blood sample was collected before vaccination. In the two following years a special group of boys, all born in 1957 or 1958, were re-vaccinated with the same vaccine that they had received previously and a further blood sample was obtained. These boys were bled again in the autumn of 1973 and in May 1974. Each autumn the new entrants to the school (age 11–12 years) joined the trial.

All boys who reported to the school medical officer with an influenza-like illness were investigated. Throat swabs were examined for pathogenic bacteria and viruses and paired sera were collected.

All sera were stored at −25° C.

Viruses for haemagglutinin antibody estimations

A/England/12/64 (H2N2)	A/Eng/64
A/Hong Kong/1/68 (H3N2)	A/HK/68
A/England/42/72 (H3N2)	A/Eng/72
A/Port Chalmers/1/73 (H3N2)	A/PC/73

were obtained from Dr M. S. Pereira, Virus Reference Laboratory, Colindale, England.

A/Scotland/840/74 (H3N2) — A/Scot/74
Recombinant virus:
A/Hong Kong/1/68 (H3) X A/equine/Prague/1/56 (Neq 1) — Rec HK

were obtained from Dr G. C. Schild, W.H.O. World Influenza Centre, London.

Recombinant viruses for neuraminidase antibody estimations

A/equine/Prague/1/56 (Heq 1) X A/Hong Kong/1/68 (N2) — X15
A/equine/Prague/1/56 (Heq 1) X A/England/42/72 (N2) — X38
A/equine/Prague/1/56 (Heq 1) X A/Port Chalmers/1/73 (N2) — X42

were obtained from Dr G. C. Schild.

Viruses were grown in embryonated eggs and pooled harvests of allantoic fluids were used as antigen.

Treatment to remove non-specific inhibitors

Receptor destroying enzyme (RDE) was obtained from Dr C. M. P. Bradstreet, Standards Laboratory, Colindale, London. Sera were diluted, one part of serum to four parts of RDE in microtitre trays and incubated in a moist chamber at 37° C. for 17 hr. followed by 1 hr. at 56° C. Evaporation loss was low (3 %); 96 sera could be treated on one microtitre tray.

Standardization of red cell suspension.

Human group O Rh-positive cells were washed three times in saline, counted on a Coulter model S and diluted to give 60,000 cells per μl. Buffered saline (CFT diluent Oxoid Ltd) with the addition of bovine plasma albumin to a final concentration of 0·1 % was used as diluent throughout.

Haemagglutination-inhibition tests (HI)

These were carried out in disposable microtitre trays using 0·025 ml. unit volumes. Doubling dilutions of sera were made from 1/10 to 1/1280. Serum controls were included on a separate microtitre tray. One volume of antigen (6HA 50) was added to each serum dilution, mixed and held at room temperature for 1 hr. One unit volume of red cells was added, mixed and left at 4° C. for 90 min. before reading. The HI titre of the sera was taken to be the highest dilution showing complete inhibition. All sera from one individual were examined at the same time. Positive and negative control sera were put up with each batch of tests and results were only accepted when these gave the expected titres. In the tables that follow those sera with an HI antibody titre of 1/20 or more are recorded as 'with antibody' and those with a titre of less than 1/20 as 'without antibody'. (The vast majority of the latter had no detectable HI antibody at a dilution of 1/10.)

It has been suggested (Slepushkin *et al.* 1971) that low titre HI antibody to A/HK may result from infection with Asian (H2N2) strains presumably due to the common neuraminidase. Sera showing HI antibodies to A/Eng/64 and A/HK/68 only were titrated against Rec HK virus to ensure that the HI titres were not

Table 2. *Virus-fetuin chessboard*

Virus dilution	Fetuin dilution					
	1/5	1/10	1/20	1/40	1/80	No fetuin
1/20	−	−	+	+	+	+
1/40	−	−	+	+	+	+
1/80	−	−	±	+	+	+
1/160	−	−	−	±	+	+
1/320	−	−	−	−	±	+
1/640	−	−	−	−	−	±
1/1280	−	−	−	−	−	−

+ = haemagglutination; − = no haemagglutination; ± = 50% haemagglutination.

Table 3. *Inhibition titre of convalescent serum in the presence of varying dilutions of virus and fetuin*

Virus dilution	Fetuin dilution			
	1/20	1/30	1/40	1/50
1/40 (16 HA units)	20	10	10	< 10
1/60 (12 HA units)	80	40	20	< 10
1/80 (8 HA units)	80	40	20	< 10
1/120 (6 HA units)	160	80	40	< 10
1/160 (4 HA units)	Unreadable	Unreadable	40	10
1/320 (2 HA units)	Unreadable	Unreadable	Unreadable	Unreadable

influenced by the presence of N2 neuraminidase antibody. Those sera showing no HI activity against the Rec HK virus were considered to be without HI antibody to the H3 subtype.

Neuraminidase-haemagglutination inhibition tests

Dowdle *et al.* (1972) showed that haemagglutination by influenza viruses could be inhibited by antibodies to neuraminidase. This technique requires the use of recombinant influenza viruses having irrelevant (equine) haemagglutinin and relevant (human) neuraminidase surface antigens. The anti-neuraminidase antibody titre can then be enhanced by the addition of protein to the test system. Dowdle, Galphin & Noble (1975) suggested fetuin as the enhancing protein.

Treatment of sera, red blood cell suspension, unit volumes and diluent were as described for HI tests. Antigens X15, X38 and X42 were used at 12 HA 50. Fetuin was prepared by ammonium sulphate precipitation of fetal calf serum by Fisher's method as described by Ham & Puck (1962). The final preparation contained 28 g. protein per litre.

Standardization of virus and fetuin

Careful cross standardization of virus and fetuin was essential to obtain enhancement of neuraminidase–haemagglutination inhibition (N–HI). Doubling dilutions of virus and fetuin were tested in a chessboard as shown in Table 2. It can be seen that fetuin reduces the virus titre. In the example shown the most

suitable combination for use would lie between virus dilutions 1/40 and 1/80 and fetuin dilutions 1/20 and 1/40. Dilutions of virus and fetuin in the appropriate range were tested against convalescent sera from patients known to have been infected with the virus concerned. An example of the results obtained is shown in Table 3.

It will be seen that both virus and fetuin doses are critical. Excess of virus reduces the apparent antibody titre and leads to difficulty in reading the end-point. Reduction of the virus dose while increasing the apparent antibody titre also increases the cross-reaction with different strains.

Excess of fetuin relative to the virus reduces haemagglutination to such an extent that the test is unreadable. Optional proportions of virus and fetuin give a relatively sensitive and specific test for neuraminidase antibodies.

N–HI test

Doubling dilutions of RDE-treated sera were prepared as described for HI tests and one volume of antigen was added. The serum-antigen mixtures were left at room temperature for 30 min. One volume of 1/20 fetuin was added to each well, mixed and left for a further 30 min. at room temperature. One volume of red blood cells was added to each well, mixed and left at 4° C. for one hour before reading. The N–HI titre of the sera was taken to be the highest dilution showing complete inhibition. A titre of 1/10 (the starting dilution) was regarded as indicating the presence of antibody. Serum controls to confirm the absence of non-specific haemagglutination and A/equine haemagglutinin antibodies were included for each serum.

Positive and negative serum controls and antigen titrations with and without fetuin were also included.

Once the technique had been standardized it was possible for two people to titrate 140 sera a day against three antigens.

RESULTS

Evidence of previous infection

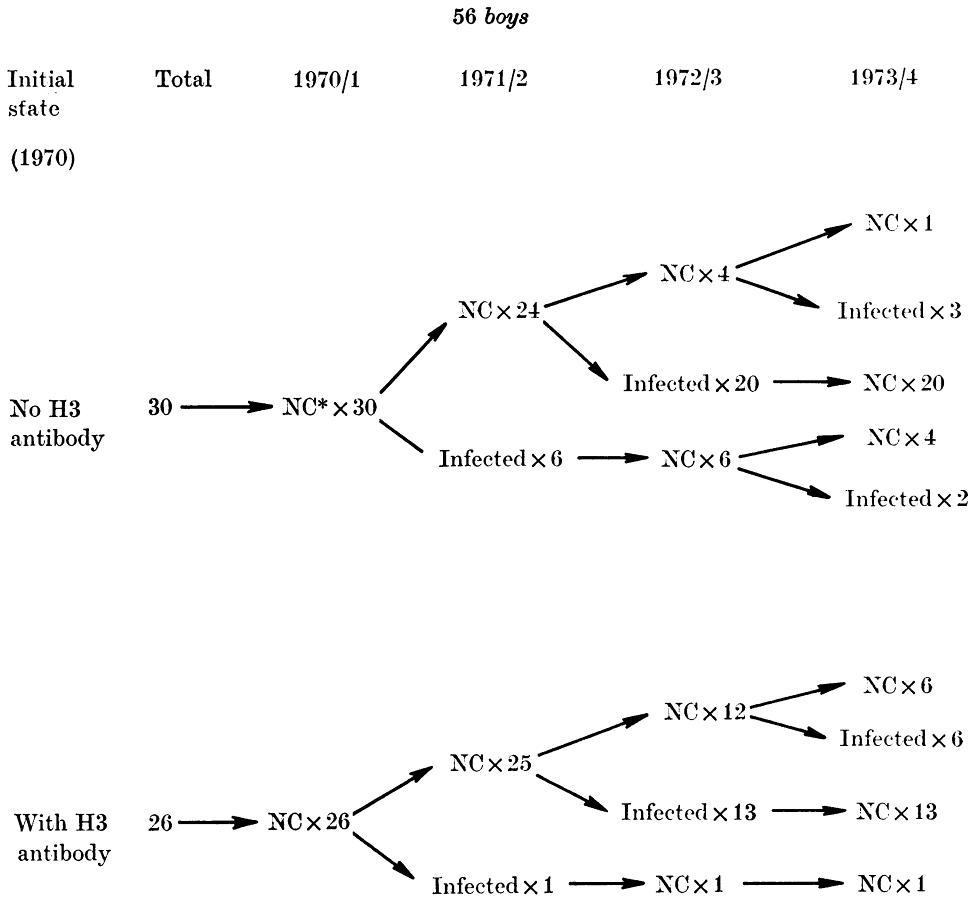
The baseline sera, collected when the boys entered the trial in November 1970, showed evidence of their previous experience of influenza A. Three patterns of antibody were observed:

(1) No detectable HI or N antibodies were found in five boys. This was interpreted as no previous experience of influenza A.

(2) HI antibody to A/Eng/64 and N antibody to X15. This was observed in 25 boys and was interpreted as previous infection with influenza A (H2N2) but no experience of influenza A (H3N2).

(3) HI antibody to A/Eng/64 and to A/KH/68 and N antibody to X15.
Twenty-six boys showed this pattern; 17 of them had cross-reacting HI or N antibodies to later members of the H3N2 series. These boys were considered to have had previous infection with influenza A H2N2 and H3N2. (The actual pattern of antibody in these boys is shown in Table 7, col. 1.)

Table 4. *Influenza A infections over a 4-year period*



* NC = No change in antibody titre (three boys showed a fall in titre–see text).

Influenza A infections 1970–4

Table 4 shows the incidence of infection over the 4-year period of observation. Boys who showed a fourfold or greater rise in titre to one or more of the antigens used between two successive annual sera were regarded as having been infected during the year. The boys were divided into those who had or had not evidence of previous infection with H3N2 virus in 1970.

During the first year no infections were observed. In the second year seven boys were infected and an A/HK-like strain was isolated from one of them who was ill in March 1972.

In December 1972 an outbreak of influenza caused by A/Eng/72 occurred in the school. Of the 24 boys remaining with no experience of H3N2 virus, 20 were infected. A further 13 infections occurred in the 32 boys who had HI antibodies to A/HK/68. A more detailed analysis of the effect of antibody on infection is given later.

In March 1974 there was an outbreak of influenza caused by A/Port Chalmers/73. None of the 33 boys who had been infected in 1972 was re-infected in this outbreak. Of the 23 boys who escaped infection in 1972, 11 were infected with A/PC/73.

It will be seen from Table 4 that only 7 of the 56 boys were not infected at any time during the 4 years.

Persistence of antibody

It became clear from the examination of these successive annual sera that antibody to influenza A once acquired is remarkably persistent. Only three boys showed any fall in antibody titre; the HI antibodies only were affected and falls in titre were in most cases minimal (twofold). The antibodies detected in successive annual sera from the remaining 53 boys were unchanged in titre throughout the period of observation, or until they became infected in 1972 or 1974. Some information on the relation of the resting titre in annual sera to the titres achieved shortly after infection was available for 13 boys infected in 1972. Convalescent sera were collected about 6 weeks after the December outbreak and the boys were bled again in October 1973. In no case did the antibody developed in convalescence fall to an undetectable level 9 months later. Four boys showed a fourfold fall in antibody titre to one or more of the haemagglutinins and two boys a fourfold fall in titre to one or more of the neuraminidase antigens. The rest showed only twofold falls in titre or no change. Seven of these boys were tested again 18 months later and showed no change in antibody titre.

Effect of antibody on infection and the result of infection

The A/Eng/72 outbreak, December 1972

The boys were bled in late October 1972 about 5 weeks before the first cases of influenza occurred. In addition to the 56 boys who were in school in 1970 and who had been bled annually, an assessment of infection in 1972 could be made on a further 44 boys who entered the school in the Autumn of 1972 and were bled in October and again in the Autumn of 1973. These boys had had very different previous experience of influenza A compared with the 1970 entry. (See Table 7, col. 2.)

One had no detectable HI or N antibodies.

Thirteen had evidence of H2N2 infection only.

Thirty had evidence of infection with H3N2. Two of these boys had no detectable HI antibody to A/Eng/64. Twenty-nine of them had HI or N antibodies to A/Eng/72.

Table 5 shows the fate of all 100 boys in the 1972 outbreak. The infection rate in boys with no previous experience of H3N2 virus was very high (83 %). Sixty per cent of those infected had symptoms of influenza. At the other end of the scale none of the boys who entered the outbreak with homotypic HI and N antibodies had clinical influenza; only one of the 25 boys in this group was infected. The infection rates were similar in those who had either homotypic HI antibody or homotypic N antibody, although the numbers are very small. None of those with homotypic N antibody had clinical influenza.

Table 5. *Effect of antibody on infection with A/Eng/42/72*

Antibody state in October 1972:				Number of boys			
HI*		N		Infected			
HK	72	X15	X38	Symptoms	No symptoms	Not Infected	Total
–	–	+/–	–	23	10	5	38
+	–	+	–	4	3	3	10
+	+	+	–	4	2	9	15
+	–	+	+	0	5	7	12
+	+	+	+	0	1	24	25
			Total	31	21	48	100

+ = antibody present; – = antibody not detected.
* The antigens were as described in the text.

Table 6. *Post-infection antibody titre*

(52 boys assessed in October 1973 after infection in December 1972.)

Antigen*		Reciprocal of titre < 10	10	20	40	80	160	320	640	GMT
HI	HK	.	.	8	14	15	12	3	.	68
	42/72	.	.	9	23	15	4	1	.	50
	PC	.	.	14	20	13	3	1	1	47
N	X15	.	.	1	19	24	8	.	.	67
	X38	5	12	28	6	1	.	.	.	16
	X42	7	15	16	10	4	.	.	.	17

* Antigens as described in text.

*The A/PC/*73 *outbreak, March* 1974

Assessment of the effect of natural antibody on infection in 1974 is possible only for the 56 boys who had been bled annually since 1970 and who had received influenza B vaccine. They were the only boys then in the school who had never had influenza A vaccine at any time and who were bled before and after the outbreak whether or not they had had symptoms of influenza. Of the 16 boys with no HI or N antibody to A/PC/73, 10 were infected, 5 with clinical influenza. One symptomless infection occurred in the remaining 40 boys. All of them had some homotypic antibody; 10 had homotypic HI antibody only, one (who was infected) had homotypic N antibody only and 20 had both HI and N antibody to A/PC/73. Thirty-three of these 40 boys had been infected in the 1972 outbreak.

Response to infection

The 1972 *outbreak*

HI antibody response. The annual sera, collected 10 months after the A/Eng/42/72 outbreak, showed that all of those who had been infected produced homotypic antibody (Table 6 and Table 7, col. 3). They all had antibody reacting with the A/HK/68 antigen and also with the A/PC/73 antigen. Those making a primary

Table 7. *Antibody state in baseline sera and post-infection sera*

Antibody state*						Baseline sera			
HI			N			1970 (56 boys)	1972 (44 boys)	Infected Dec. 1972 (52 boys)	Infected March 1974 (11 boys)
HK	72	PC	X15	X38	X42	(1)	(2)	(3)	(4)
–	–	–	+/–	–	–	30	14	.	.
+	–	–	+	–	–	9	1	.	.
+	+	–	+	–	–	5	2	.	.
+	+	+	+	–	–	5	3	5	.
+	–	–	+	+	–	3	8	.	.
+	+	–	+	+	–	.	6	.	.
+	+	+	+	+	–	4	4	2	1
+	+	–	+	+	+	.	1	.	.
+	+	+	+	+	+	.	5	45	10

* + = antibody present; – = antibody not detected.

response to the H3 haemagglutinin tended to produce a similar titre of antibody to all three antigens; those who had pre-existing A/HK antibody generally produced a higher titre to A/HK/68 than to the homotypic A/Eng/72 and a lower titre to A/PC/73.

N antibody response. The response to the neuraminidase antigens followed a similar pattern to the HI response, most boys producing antibody to all three antigens. However, 5 of the 52 boys infected had no detectable homotypic N antibody in October 1973 and no N antibody to X42. An additional two boys made a homotypic response but this did not extend to X42. All infected boys had antibody to X15; the higher titres detected with the X15 antigen probably reflected a boost to pre-existing N2 antibody in boys previously infected with H2N2.

Neither the antibody titre in October 1973 nor the breadth of response was related to whether or not infection had been accompanied by symptoms of influenza.

The 1974 *outbreak*

Fifty-six boys who had been bled annually since 1970 were bled again in May 1974 about 6 weeks after the outbreak caused by influenza A/PC/73. The 11 shown to be infected produced antibody to all three haemagglutinins and, with one exception, to all three neuraminidase antigens (Table 7, col. 4).

*Antibody to A/Scot/*74

It was relevant to determine the extent to which antibody produced against early strains of the H3N2 subtype cross-reacted with more recent strains. Sera from all boys in the group bled annually from 1970 who had HI antibody to A/PC/73 were examined for HI antibody to A/Scot/74. No such antibody was found before the A/Eng/72 outbreak. Of those infected in this outbreak 24 out of 33 had antibody reacting with the A/Scot/74 antigen, 12 of these to the same titre as their HI antibody to A/PC/73. All 11 of those infected with A/PC/73 in 1974 developed HI antibody to A/Scot/74.

DISCUSSION

It was not surprising to find that 51 of the 56 boys first bled in November 1970 showed evidence of previous infection with influenza H2N2. All the boys were born in 1957 or 1958 and were therefore alive during the whole of the Asian influenza era. It was interesting that nearly half of these boys also had evidence of previous infection with influenza A/Hong Kong. The H3N2 virus had only been in circulation in this country for 2 years, although a large number of cases occurred in the winter of 1969–70. After a further 2 years, when the boys who entered the school in the Autumn of 1972 were bled, two thirds of them showed evidence of H3N2 infection. It seems that children acquire experience of new antigenic variants of influenza A very rapidly.

Following infection both HI and N antibodies were produced. These often fell in titre over a period of months but they remained detectable at least for a few years. In children of this age re-infection is likely to occur at intervals and boost the antibody titres. The HI antibody produced in response to infection with one strain of the H3N2 series often cross-reacted with future antigenic variants. This is seen in the baseline sera of the 1970 entry (Table 7, col. 1) where half of the boys showing evidence of infection with H3N2 virus had cross-reacting antibody to A/Eng/72 and nine of them had antibody cross-reacting with A/PC/73. In boys of the 1972 entry two-thirds of those with H3N2 antibody had HI antibody reacting with the A/Eng/72 antigen. A few of them might have acquired this as a result of infection with A/Eng/72 itself since this variant was first isolated in this country in the Spring of 1972. It seems likely that most of them had been infected with Hong Kong strains circulating before 1972. Infection with A/Eng/72 always resulted in the production of HI antibody cross-reacting with A/PC/73; and infection with A/PC/73 produced antibody cross-reacting with A/Scot/74.

The response to the neuraminidase antigen also produced antibody cross-reacting with future strains. This is seen most clearly in the boys infected with A/Eng/72; 45 out of 52 produced antibody cross-reacting with the X42 strain (A/PC/73 neuraminidase). It is not possible to determine how much of the X38 antibody resulted from a homotypic stimulus since this antigenic variant appeared in 1969.

The occurrence of two outbreaks of influenza A in the school made it possible to assess, in those boys who were bled before and after the outbreaks whether they had symptoms or not, who was infected and what was the outcome of infection. The first outbreak in 1972 had its main impact on those boys who had had no experience of the A/Hong Kong subtype. Even in this presumably susceptible group 40% of infections were not accompanied by symptoms of influenza. Those 25 boys in whom previous infection with H3N2 virus had resulted in the production of antibodies reacting with A/Eng/72 haemagglutinin and X38 neuraminidase were spared altogether from clinical influenza and only one of them was infected. Perhaps the most interesting groups were the boys who had dissociated homotypic HI or N antibodies. About a third of them were infected but clinical influenza did not occur in those with homotypic neuraminidase antibody. This

lends some support to the suggestion (Couch *et al.* 1974; Murphy *et al.* 1972) that neuraminidase antibody protects not so much against infection as against symptoms and by so doing permits the individual to 'up-date' his antibody status from time to time without suffering clinical influenza. The significance of antibody to the neuraminidase antigen in immunity to influenza has also been investigated by Rott, Becht & Orlich (1974). They showed that chickens immunized with swine influenza virus were fully protected against challenge with virulent fowl plague virus. The viruses used had serologically distinct haemagglutinins but related neuraminidases.

Although only 56 boys were available for assessment in the A/PC/73 outbreak the effect of homotypic antibody was similar to that in 1972. All 29 boys with homotypic HI or N antibody and 10 boys with homotypic HI antibody only escaped infection; one, with homotypic N antibody only, had a symptomless infection. There were five cases and five symptomless infections in the 16 boys without homotypic antibody.

Characterization of the neuraminidase antigen of influenza A and of the antibody response to it increases our understanding of the epidemiology of influenza and may permit a more accurate prediction to be made of the likely state of immunity of an individual or a community than can be achieved by consideration of the haemagglutinin alone.

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