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Influenza A neuraminidase antibodies in children and young adults studied by serum absorption

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SUMMARY

A study is described of influenza A anti-neuraminidase antibodies in the sera of young people of three different age groups. Each serum was individually absorbed with viruses containing the N2 neuraminidases of 1957, 1968 and 1972. Rabbit antisera prepared against the viruses were similarly absorbed. Results obtained with the animal sera suggested that these neuraminidases were antigenically distinct, but the human sera had a broader range of anti-neuraminidase activity and gave indication of asymmetric antigenic relationships. Earlier workers who surveyed anti-haemagglutinin antibodies reported that the virus of primary infection absorbed all antibodies, and the virus of secondary infection only those directed against itself. We too found that the virus of secondary infection absorbed only homologous anti-neuraminidase antibody. However, although the primary infecting virus did absorb some secondary antibody, this absorption was incomplete and it lessened with the lengthening of the time interval between the primary and secondary infecting viruses. A similar pattern was seen with anti-haemagglutinin antibodies.

Absorption of anti-neuraminidase antibodies from human sera proved much more difficult than absorption of anti-haemagglutinin antibodies particularly after repeated influenza virus infections. The relative rarity of antigenic shift in the neuraminidase subunit also creates problems in the interpretation of results of serum neuraminidase antibody surveys.

INTRODUCTION

The term 'original antigenic sin' (Francis, Davenport & Hennessy, 1953) has been used to describe the phenomenon of recall of haemagglutination-inhibiting (HI) antibody to the influenza A virus first encountered in childhood by subsequent heterotypic infections. This implied mysticism has, however, been largely refuted by Morita, Suto & Ishida (1972), who showed that there was no recall of anti-H0 and anti-H1 antibodies in older people infected with H3 viruses, and hence the phenomenon was probably dependent upon shared antigenic determinants in the viruses of primary and secondary infection. In absorption studies in which viruses were complexed to red blood cells, Jensen, Davenport, Hennessy & Francis (1956) showed that the virus of primary infection removed both primary and secondary HI antibodies from ferret and human sera, while the virus of secondary infection

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removed secondary antibodies alone and merely reduced the titres of primary antibodies. Fazekas de St Groth & Webster (1966), working in the same laboratory, concluded that the secondary antibodies were homogeneous but had greater avidity for the primary infecting virus than for the secondary infecting virus.

Kendal *et al.* (1973) have now performed a related study on anti-neuraminidase (NI) antibodies in human sera and have found that they too show evidence of recall. Their technique consisted of direct antibody measurement in serum pools taken from people of different age groups. They did not absorb any antibodies. Since a degree of error may be inherent in this method we have now carried out a supporting investigation on individual specimens with a different technique. Sera collected from children whose ages would suggest that their initial influenza A infection was likely to have been with viruses containing the N2 neuraminidase serotypes, A/Singapore/57, A/Hong Kong/68 or A/England/72, were absorbed with each of the 3 viruses. They were then tested for residual content of both HI and NI antibodies. Rabbit antisera produced by intravenous inoculation were treated in parallel to obtain a standard for comparison and to assist in the interpretation of results.

MATERIALS AND METHODS

Viruses

A/PR/8/34 (H0 N1) had an unknown passage history with numerous passes in ferrets, mice and eggs; A/Singapore/1/57 (H2 N2) had multiple unrecorded egg passes; A/Hong Kong/1/68 (H3 N2) had received 2 passes in monkey kidney tissue culture and 6 egg passes; and A/England/42/72 (H3 N2) a total of 7 egg passes.

Recombinant strains were A/PR/8/34–Aichi/2/68 (H3 N2) (X-31) (Kilbourne 1969), A/RI/5+/57 (H2)–NWS (N1) (X-9) and A/equine/Prague/1/56 (Heq1)– Hong Kong/1/68 (N2) (X-15 HK). These had all been originally selected at the Mount Sinai Hospital, New York. A/PR/8/34 (H0)–England/42/72 (N2) (MRC 3) and A/England/42/72 (H3)–PR/8/34 (N1) (MRC 8) (Beare, Schild & Craig, 1975) were provided by Dr G. C. Schild. Recombinants were passed a few times in eggs in this laboratory before use.

All viruses were propagated in the allantoic cavity of 11-day fertile hens' eggs by standard methods.

Virus concentrates for absorption of sera

Pooled allantoic fluids (HA titre about 1000) were clarified by low-speed centrifugation and then centrifuged at 50000 *g* for $1\frac{1}{2}$ h in an MSE 8×50 angled rotor. Pellets were resuspended in phosphate-buffered saline (PBS) to 1/10 the original volume.

Human sera for antibody studies.

Because initial infection with influenza virus is likely to occur at school (Fry, 1958), sera were obtained from people who would have been about 5 years old at the times of the first epidemics of A/Singapore/1/57, A/Hong Kong/1/68 and A/England/42/72 respectively. They were therefore drawn from individuals born in 1952, 1963

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Table 1. *Efficiency of the procedure used for the absorption of HI and NI antibodies from sera*

(A standard dilution was made of A/PR/8/34 (H0 N1) rabbit hyperimmune serum and 1·0 ml was absorbed with varying amounts of a concentrate of homologous virus. To calculate % NI, a 1/20 dilution of the absorbed serum was used (see Methods)).

Quantity of virus concentrate used for serum absorption (ml)	Residual HI antibodies in serum (reciprocal titres against A/PR/8/34)	Residual NI antibodies in serum (% NI obtained with MRC 8 (H3 N1))
0	2560	90
0·25	<20	34
0·5	<20	7
1·0	<20	0

and 1967. The sera of the 1952 group (5 specimens) were obtained from people who had attended the Common Cold Unit in 1974 and who had been inoculated with rhinoviruses or control material. Sera for the 1963 and 1967 groups (7 and 5 specimens, respectively) were kindly provided by Dr J. V. T. Gosling, Public Health Laboratory, Portsmouth and had been taken from hospital patients with a variety of complaints.

Standardization of virus concentrates and absorption of sera

Rabbit antisera to A/Singapore/1/57, A/Hong Kong/1/68 and A/England/42/72 prepared as previously described (Callow & Beare, 1976) were heated at 56 °C for 30 min and diluted with PBS until the NI titres with the homologous viruses were the same. Small volumes (usually 1·0 ml or less) were mixed with varying volumes of concentrate (0·25–1·0 ml) and kept for 30 min at room temperature. Mixtures were then centrifuged at 50000 ***g*** for 30 min and supernatants were assayed for NI activity. The least amount of concentrate required to remove all NI activity from 1·0 ml of diluted serum was defined as 1 absorbing dose. This amount was also found to remove all HI antibody (Table 1).

The absorption of sera was then performed by adding 1 absorbing dose to each ml of serum and keeping the mixture at room temperature for 30 min. The mixture was centrifuged at 50000 ***g*** for 30 min and the residual NI activity of the supernatant measured as above. An unabsorbed serum control was diluted with PBS to the same degree as the absorbed serum.

NI tests

The technique used was similar to that described previously (Callow & Beare, 1976). Neuraminidase activity (Aminoff, 1961) was determined on a substrate of human serum glycoprotein, Cohn Fraction VII-7 provided by J. Watt, Protein Fractionation Centre, Edinburgh Royal Infirmary. The virus dose in the NI test

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was that giving an absorbance of about 0·6 at 549 nm. NI activity of sera (calculated as a percentage) was determined from a single serum dilution (final 1/45). As a control a human serum was used which had been collected in 1957 before the arrival of the Asian virus and which contained no anti-N2 antibodies. It did, however, have a slightly inhibitory effect on the recombinant MRC 3 (H0 N2) and was therefore absorbed initially with the A/PR/8/34 (H0 N1) concentrate to remove traces of anti-H0 antibodies. When tests were made with rabbit antisera, a normal rabbit serum was used as a control. Significant NI activity, or significant reduction of activity through absorption, in sera was considered to be 18% or more. This figure was derived from the standard deviation calculated for the test (Callow, 1976).

For the measurement of serum antibodies against the three N2 neuraminidases, antigenic hybrid viruses were used whose haemagglutinins were unlikely to interact with any HI antibodies that might be present. X-15 possessed the Asian neuraminidase and the A/equine/1/56 haemagglutinin, while X-15 HK had the Hong Kong neuraminidase and the A/equine/1/56 haemagglutinin. The H0 haemagglutinin of MRC 3 encountered no anti-H0 antibodies since all our subjects were born in 1952 or after.

HI tests

A micro-technique (Takatsy, 1955) with 0·05 ml volumes was used throughout. Fifty per cent haemagglutination with 0·5% fowl red blood cells was taken as the end-point. Absence of haemagglutination was signified by tear-shaped streaming of red cells during tilting of the plates. Fourfold or greater reduction of HI antibodies after absorption was considered significant.

RESULTS

Absorption of rabbit antisera

A pilot study of the absorption of a rabbit anti-PR8 antiserum is shown in Table 1. All significant amounts of HI and NI antibody (as defined in Materials and Methods) were removed by 0·5 ml of a concentrate of homologous virus. Concentrates of the virus A/Singapore/57, X-31 (antigenically equivalent to A/Hong Kong/68 but with a better growth capacity), and A/England/72 were prepared so that neuraminidase antibodies could be completely absorbed from homologous antisera. Each serum was then separately absorbed with each virus. As shown in Table 2 the unabsorbed serum produced maximum neuraminidase inhibition with the homologous virus and relatively little with the other strains, although A/Singapore/57 antiserum did produce appreciable inhibition of A/Hong Kong/68 neuraminidase. After absorption with homologous virus, only insignificant (< 18%) activity remained against the neuraminidase that it contained. Although absorption with either of the heterologous strains did sometimes reduce homologous NI titres this was always less than 18%. Minor degrees of cross reaction were abolished by absorption with any of the viruses.

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Table 2. *Antigenic relation between N2 neuraminidases of A/Singapore/57, A/Hong Kong/68 and A/England/72 as shown by antibody absorption*

(Before absorption rabbit antisera were diluted to give approximately the same inhibition titres with their homologous viruses.)

Hybrid recombinant virus	N2 component	Unabsorbed rabbit sera (% NI*)			Rabbit sera absorbed with A/Sing./57			Rabbit sera absorbed with A/HK/68			Rabbit sera absorbed with A/Eng/72		
		A/Sing/57	A/HK/68	A/Eng/72	A/Sing/57	A/HK/68	A/Eng/72	A/Sing/57	A/HK/68	A/Eng/72	A/Sing/57	A/HK/68	A/Eng/72
X-15	A/Sing/57	**86**	10	18	**13**	0	0	**74**	0	0	**75**	10	0
X-15 HK	A/HK/68	39	**83**	5	0	**67**	0	0	**17**	0	0	**85**	0
MRC 3	A/Eng./72	17	2	**70**	0	0	**53**	0	0	**69**	0	0	**10**

* % NI in all cases were measured at a final serum dilution of 1/45 and were compared with a normal rabbit serum control.

Table 3. *Antigenic relations between the haemagglutinins of the three viruses used in the study*

(Before absorption of the rabbit antisera they were diluted to give approximately the same titres with the homologous viruses. Figures are reciprocal HI titres.)

Virus used for testing	Unabsorbed rabbit sera			Rabbit sera absorbed with A/Sing/57			Rabbit sera absorbed with X-31*			Rabbit sera absorbed with A/Eng/72		
	A/Sing/57	A/HK/68	A/Eng/72	A/Sing/57	A/HK/68	A/Eng/72	A/Sing/57	A/HK/68	A/Eng/72	A/Sing/57	A/HK/68	A/Eng/72
A/RI+/57 (H2) −NWS (N1) X-9	**864**	< 18	< 18	**< 18**	< 18	< 18	**432**	< 18	< 18	**432**	< 18	< 18
A/HK/68	36	**1152**	576	18	**1152**	432	< 18	**18**	18	< 18	**216**	18
A/England/72	216	864	**864**	72	432	**576**	72	108	**144**	48	108	**18**

* H3 N2 antigens of A/HK/68.

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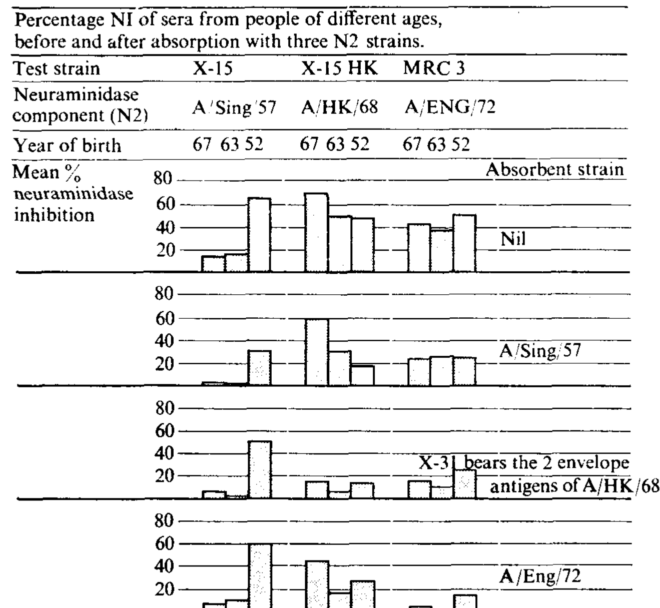

Fig. 1. Titres of neuraminidase antibodies in sera, using recombinants of three different N2 serotypes. Heights of histograms are the arithmetic mean titres of the sera tested in each group.

Resting NI antibodies in human sera

Sera from human subjects generally had high NI titres (Fig. 1), and those from children born in 1963 and 1967 also inhibited the 1957 neuraminidase. Presumably this was due to antigenic crossing between the 1957, 1968 and 1972 neuraminidases and not because these children had been infected with Asian viruses. In the two oldest groups maximum anti-neuraminidase activity was seen against the predicted primary infecting agent (cf Kendal *et al.* 1973). High titres to A/Hong Kong/68 neuraminidase in children born in 1967 might have been due to infection with A/England/72 virus and to the common neuraminidase antigens in these two viruses. In general, high titres to one neuraminidase correlated well with high titres to another (see Fig. 2, subject 12 born in 1963).

Absorption of neuraminidase antibodies from human sera with A/Singapore/57

A/Singapore/57 did not absorb all homologous NI antibody from sera of people born in 1952 (Fig. 1), but did apparently absorb out just as much 1968 and 1972 antibody. In subjects 3 and 5 (Fig. 2), this virus removed all significant NI antibody to the three neuraminidases. However, a more usual effect was a simple reduction in titre to the two heterologous neuraminidases. A/Singapore/57 also removed some antibody to the 1968 and 1972 neuraminidases in the youngest age groups, and this was more likely due to common antigenic determinants in the three enzymes, rather than to early infection with a virus containing the 1957 neuraminidase. Anomalous findings in absorption experiments are, however, not always explicable, and Morita *et al.* (1972) reported that swine virus absorbed HI

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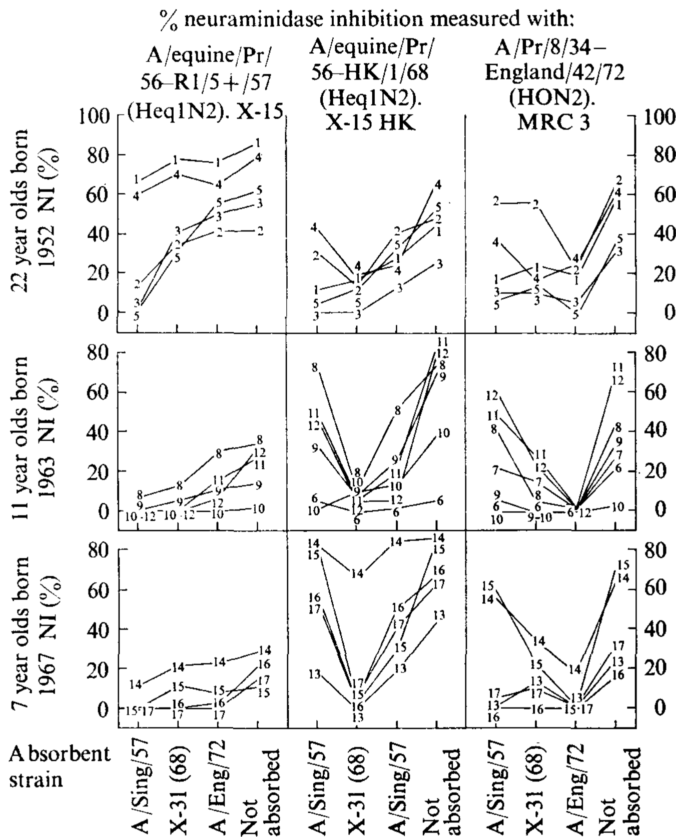

Fig. 2. Percentage NI titres of sera of young people of different ages before and after absorption with the three N2 serotypes, using the same recombinants as in Fig. 1. Numbers identify individual subjects. Omission of individuals from parts of the figure signifies absence of antibody before absorption.

antibody to later influenza serotypes from sera of people who could not have experienced it.

Absorption of neuraminidase antibodies from human sera with A/Hong Kong/68

A/Hong Kong/68 did slightly reduce A/Singapore/57 neuraminidase antibodies in the sera of all groups, but in only one serum from the oldest group (Fig. 2, subject 5) was this significant. This virus was more effective in the removal of A/England/72 antibodies however, particularly from the children's sera.

Absorption of neuraminidase antibodies from human sera with A/England/72

A/England/72 absorbed all homologous antibody but very little A/Singapore/57 antibody. In all groups, it absorbed A/Hong Kong/68 antibody but not as well as A/Hong Kong/68 absorbed A/England/72 antibody.

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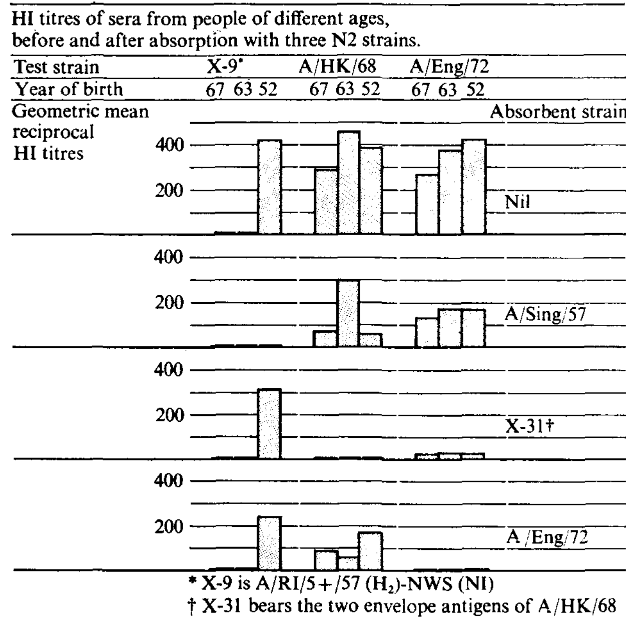

Fig. 3. Titres of haemagglutination-inhibiting antibodies. Heights of histograms are the geometric mean titres of the sera tested in each group.

General findings with HI and NI antibodies

The antigenic relation between the haemagglutinins of the three viruses with our rabbit antisera is shown in Table 3. Clearly the system was not perfect either because of interference by host antigen or because of the difficulty of entirely removing non-specific inhibitors from sera with cholera filtrate. The persistent one-way cross between A/Singapore/57 (H2) and A/England/72 (H3) was a particular anomaly. Nevertheless it seemed that the absorbing procedure itself was as effective with A/Singapore/57, A/Hong Kong/68 and A/England/72 as it had been with A/PR/8/34 (Table 1). The evident relation between the haemagglutinins of A/Hong Kong/68 and A/England/72 in Table 3 had no parallel in their neuraminidases (Table 2) which, although both N2, seemed entirely different as shown by both resting antibodies and a lack of significant cross-absorption. In the human sera taken from the two youngest age groups, there was little significant A/Singapore/57 HI antibody but small amounts of A/Singapore/57 NI antibody (Figs. 1, 3), a finding compatible with absence of abrupt antigenic change in the neuraminidase subunit since 1957. Absorption of haemagglutinin antibodies always proved much easier than that of neuraminidase antibodies (Fig. 3).

DISCUSSION

Francis *et al.* (1953) thought that the influenza A virus of first infection imprinted itself on the immunological system in such a way that HI antibodies to this virus were constantly produced much later in life in response to stimuli that were antigenically different. This scientifically inexplicable phenomenon was designated original antigenic sin. The fact that the virus of primary infection absorbed all

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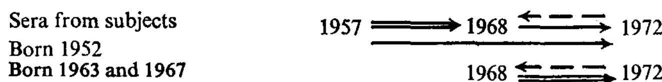
HI antibodies and the virus of secondary infection only homologous HI antibodies (Jensen *et al.* 1956) was believed to confirm the original observation, and it seemed natural therefore for Kendal *et al.* (1973) to suggest that NI antibody formation was subject to a similar law. More recently, however, it has seemed doubtful that the pattern of HI antibody production could have been accurately interpreted by Francis and his colleagues on the basis of simple laboratory techniques. Populations of antibody molecules were evidently complex, generalizations were repeatedly challenged, and some elements of the Francis doctrine could be explained by something as simple as the fact that different influenza virus haemagglutinins often contained similar antigenic determinants (Morita *et al.* 1972; Virelizier, Allison & Schild, 1974*a*; Virelizier, Postlethwaite, Schild & Allison, 1974*b*).

We cannot comment on immunological mechanisms, and shall consider only from our own findings the potential reliability of NI antibody surveys for the study of epidemiological history. Whatever difficulties may exist in the characterization of long-standing anti-haemagglutinin antibodies, they are far less than those of the characterization of anti-neuraminidase antibodies. From time to time the situation in regard to the haemagglutinin is transformed by antigenic shift, and adults are exposed to new influenza viruses as infants are to viruses of first infection. The relative rarity of major shift in the neuraminidase subunit leads to an accumulation in most adults of populations of NI antibodies against the same subtype, and of both recent and remote origin. Patterns which are seen cannot always be confidently explained. The sera which we produced by the intravenous inoculation of rabbits gave little evidence in our test of antigenic relations between the 1957, 1968 and 1972 neuraminidases (Table 2), but those following natural human infections showed clearly that they were all of the N2 subgroup and that the crossing was asymmetrical.

Our findings with the absorption of HI antibodies from human sera can be represented in the following diagram, where direction of the arrow indicates direction of absorption, i.e. absorbing virus is at base of arrow, absorbed antibodies at head of arrow.



A double arrow signified absorption of 60 % or more of initial antibody, a single arrow 50–60 %, a dashed arrow 40–50 %, and no arrow less than 40 %. The strain of probable primary infection strongly absorbed secondary antibody, but as the relation between them became more distant, the degree of absorption declined. Hence the primary strain did not invariably remove all secondary neuraminidase antibody. Secondary strains were also much less effective in the removal of primary neuraminidase antibody. The pattern with haemagglutinin antibodies was similar. In their study of HI antibodies in mice, Virelizier *et al.* (1974*a*, *b*) postulated the existence of three populations of antibodies against the haemagglutinin, two of them strain-specific for the viruses of primary and secondary infection respectively, and the third cross-reactive. Our own findings are consistent with this hypothesis.

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