Neuraminidase and resistance to vaccination with live influenza A2 Hong Kong vaccines*

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SUMMARY

Thirty-seven volunteers were inoculated intranasally with living attenuated influenza A2 viruses. Rising titres of circulating antineuraminidase (AN) were detected in 14 of 17 infected volunteers. AN was also found in nasal secretions. Statistical analysis showed that there was a correlation between the titres of haemagglutination-inhibiting antibody (HI) and AN in nasal washings, and between AN in blood and washings. Resistance to infection could be predicted from antibody titres in 29 of 37 volunteers and blood AN alone predicted the outcome of 25 volunteers.

INTRODUCTION

It has been known for some time that vaccination with living attenuated influenza A virus strains protects against infection with the same serotype of virus either by re-exposure to the vaccine strain or by natural exposure in epidemic conditions. It has also been shown that infection with unattenuated virus stimulates circulating antibodies and also neutralizing (N) and haemagglutination-inhibiting (HI) antibody in the nasal secretion (Mann *et al.* 1968). Antineuraminidase (AN) appears in serum and secretion after vaccination with live influenza B virus (Downie, 1970); but more information is needed about the importance of circulating and local antibody in resistance to influenza virus infections. We have therefore undertaken studies in which influenza A vaccines were administered to normal subjects in isolation and serum and nasal antibodies were measured.

MATERIALS AND METHODS

Volunteers in isolation at the Common Cold Unit, Salisbury, were inoculated with attenuated influenza virus vaccines using methods previously described (Tyrrell, 1963). They were inoculated in two trials, nos. 74 and 76, which took place at the end of January and the beginning of April 1970.

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Specimens

Nasal washings were collected before inoculation and 7 days after, using about 10 ml. of sterile phosphate buffered saline (PBS). These were shown to be free of blood by the Hemastix test, were dialysed against distilled water, freeze-dried, and reconstituted in 1/10 the original volume of PBS. Blood was collected before inoculation and both 7 days and 3 weeks later. Serum was separated aseptically. These specimens were stored at $-20°$ C.

Nasal washings were also collected on the 2nd, 3rd and 4th days after inoculation, for virus isolation by allantoic inoculation of embryonated eggs.

Antibody titrations

Haemagglutination inhibition (HI) tests

To destroy non-specific inhibitors sera were treated with 5 volumes of cholera filtrate and nasal secretions with an equal volume. Sera were inactivated and titrated with virus in the form of allantoic fluid; secretions were titrated against viruses treated with tween 20 (final dilution 1/20,000) and equal parts of ether; versene saline was used as diluent in the titrations to prevent the effect of neuraminidase on cells.

In all cases 4 units of virus were mixed with each dilution of serum or nasal secretion and the mixtures were held at room temperature for 30 min.; 0.5% human red cells were then added.

Antineuraminidase (AN) assays

Neuraminidase activities were assayed by a modification of Warren's method as described by Webster & Laver (1967) using fetuin as substrate. Assays of antineuraminidase activity in sera and nasal washings were performed essentially as described by Schild & Newman (1969), but with certain modifications to increase the sensitivity and specificity of the test:

(a) A reduced amount of viral neuraminidase was used, the concentration of virus used as a source of neuraminidase was adjusted so that after incubation with excess substrate for 16 hr. at $37°$ C. at pH 5.9 the amount of N-acetyl neuraminic acid released per 0.05 ml. of virus was $10–15\,\mu g$.

(b) Virus and serum (or nasal washing) dilutions were incubated at room temperature for 3 hr. during the enzyme neutralization reaction.

(c) The source of neuraminidase was a recombinant influenza virus, FPV-HK (kindly provided by Dr D. McCahon, National Institute for Medical Research, London), between fowl plague virus and A2/Hong Kong/68 containing neuraminidase of the A2 virus and haemagglutinin of the fowl plague virus. The use of the recombinant virus, since it contained haemagglutinin unrelated to that of the human Hong Kong virus, or other human influenza A viruses, avoided the possibility that anti-haemagglutinin antibody might produce non-specific inhibition of enzyme activity by 'steric hindrance' (Easterday, Laver, Pereira & Schild, 1969; Schild, McCahon & Kendal, 1970). The titres of antineuraminidase were expressed as the dilution of serum (or washing) inhibiting 50% of enzyme activity.

Immunodiffusion tests

Immunodiffusion was performed using concentrates of A 2/Hong Kong/68 virus disrupted by sodium dodecyl sulphate as described previously (Schild & Pereira, 1969; Schild, Winters & Brand, 1971). Sera with high titres of antineuraminidase activity were found to produce precipitin lines corresponding to virus neuraminidase. However, this test was too insensitive to detect antineuraminidase antibody at the low levels found in nasal secretions.

Vaccines

The three vaccine virus strains were antigenically identical and were developed at the Common Cold Unit and produced there or else prepared at the State Institute for Viral Preparations in Moscow, U.S.S.R. The strains were derived from A 2/Hong Kong/1/68 and A 2/Istra/10/69. Their effects are described in detail elsewhere (Beare, Maassab, Slepushkin & Hall, 1971), but since their behaviour in human volunteers was generally similar we have combined the results. Volunteers received 10^5 EID 50 of virus as nasal drops.
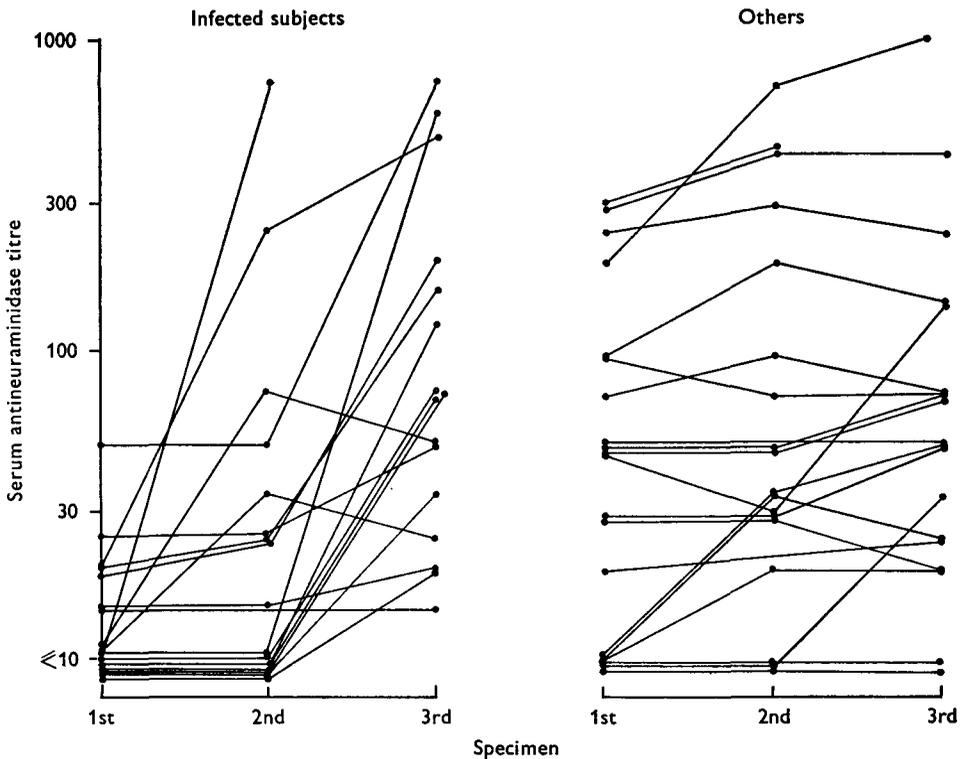
Fig. 1. Titre of serum antineuraminidase antibody. The left panel shows the results in volunteers who were shown to be infected by rising titres of HI antibody with or without virus isolation. The right-hand panel is the results in those who were uninfected by these criteria. The first titre was that immediately before vaccination. The second was collected at the Unit 7 days after vaccination and the third about 3 weeks after the volunteers had returned home.

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RESULTS

For the first analysis volunteers were regarded as infected if virus was isolated or if a rising titre of HI antibody occurred and we considered first the response of circulating antibody and especially AN in infected and uninfected volunteers.

A substantial number of volunteers had circulating HI at the time of challenge - the low titres may have been due to infection with previous Asian strains, but the high titres probably resulted from previous infections with HK strains. As was expected antibody titres rose after infection in many cases, but there was not

Fig. 2. Correlation between the titre of HI antibody in serum and nasal secretion of 36 subjects before vaccination. The open symbols show volunteers who resisted infection and the closed those who became infected as judged by HI antibody rises and virus isolation.

Fig. 3. A similar diagram to Fig. 2, showing the relationship between titres of AN antibody in the two compartments.

complete concordance between the occurrence of rising titres and virus isolation in particular a few subjects yielded virus in one of three specimens and showed no circulating antibody response.

The results of serum AN tests are summarized in Fig. 1. Sera with high AN titres produced precipitin lines corresponding to AN indicating that the AN activity was due to antibody. Fig. 1 shows that the volunteers who became infected had lower titres on the whole than those who were not infected – as judged by HI rises and/or virus recovery. Eleven of 17 who were infected had AN titres of under 20 as against 5 of 20 who were not infected. AN rises occurred in 14 of 17 infected

volunteers but also in 5 of 20 volunteers in the 'uninfected' group – those which occurred early might have represented a response to the antigen in the inoculum but it is more likely that they represented limited infections which were not detected in the other tests used. The titres after vaccination of the subjects who were infected were similar to those of the subjects who resisted infection by the vaccine.

Nasal washing antibodies

The titres of HI in nasal washings were much lower than those in serum and the relation between these and serum HI are shown in Fig. 2. There was no close correlation between the amounts of antibody at the two sites, in particular high titres were found in one and low in the other. In the five days following vaccination there were in the nasal washings rising HI titres in seven subjects and AN titres in three.

In Fig. 3 is shown the relationship between the AN level in serum and the nasal washings and here again the correlation was not particularly close. There seemed, nevertheless, to be a general correlation between the AN and HI titres in the secretions, although a number of specimens with HI titres had no AN activity.

Statistical analysis showed that the titres of each antibody were positively correlated with those of each of the other three; the correlation coefficient between titres of HI and AN in washings was $+0.47$ (significant at 1% level) and between titres of AN in blood and washings was $+0.40$ (significant at 5% level). The other coefficients were not significantly different from zero.

Antibody and resistance to infection

Inspection of Fig. 2 showed that infection occurred in the presence of antibody; however, infection was infrequent (5 of 15) if both nasal and serum HI were detectable (titre of 1/4 or 1/12 or greater) and more frequent (6 of 8) if both were undetectable, and the same applies to AN antibody (Fig. 3). It was striking that only 2 of 13 volunteers with detectable nasal AN antibody were infected whilst 14 of 23 volunteers without such antibody were infected.

The simple analyses used so far were not able to show whether resistance was really related to all the antibodies detected or whether one was more important than others; apparent effects might have been due to correlations between the presence of one type of antibody and another. Therefore a more thorough analysis was performed.

After ascribing a variable with value 1 to those subjects with a positive reaction to the challenge and 0 to those with a negative reaction, and transforming titres to logs, discriminant analyses were carried out by regressing this variable on the titres of each antibody in turn. Titres recorded as 'less than' were given the next lower titre in the dilution series used. The slope was negative for each antibody considered on its own, indicating that those subjects with a low titre of an antibody were more susceptible to influenza than those with a high titre of the same antibody. The separation between the group of subjects who contracted influenza when challenged and the group who did not was most marked when the titre of AN

in the blood was used as the discriminating factor (variance ratio significant at 1 % level). Regressing on the titre of AN in the nose the variance ratio was significant at the 5 % level; the titre of HI in the blood gave a non-significant variance ratio, and that for the titre of HI in the nose was less than 1.

The titres of AN in the blood were lower for trial 76 than they were for trial 74. For trial 74 regression on the titre of AN in the blood gave a variance ratio significant at 0·1 %. When trial 76 was considered separately regression on the titre of AN in the nose gave the best separation between the groups, with the titre of HI in the blood second best.

The extra reduction in the residual sum of squares obtained by regressing on all four variables compared with regressing only on the titre of AN in the blood was not significant. The one missing AN titre was replaced by the mean titre. A prediction of the result of the challenge based on the multiple regression would have classified correctly 29 of the 37 subjects, whereas prediction by the titre of AN in the blood would classify correctly 25 of them.

It was concluded that of the four factors a high titre of AN in the blood contributed most to resistance to infection – there was no evidence that high titres of anti-haemagglutinin, in addition to this, had any effect in increasing the resistance.

The data were also analysed by using the titres of antibody to classify the subjects, and applying the logit transformation ($z = \frac{1}{2} \log p/q$) to the proportion of subjects in each class who contracted influenza. The logit was then used as the dependent variable in a regression on the titres of the antibodies. Maximum likelihood estimates of the parameters led to the same conclusions as the above.

<center>DISCUSSION</center>

The first point of interest in this study is that live influenza vaccine like natural infections and administration of killed vaccine stimulated AN production (Kilbourne, Christenson & Sande, 1968; Schild & Newman, 1969). It has recently been shown in other studies that one of our Salisbury live vaccine strains also stimulates AN in the nasal secretions (Downie & Stuart-Harris, unpublished; Tyrrell *et al.*, unpublished) as did a live influenza B vaccine (Downie, 1970). There is abundant evidence from early work and from our own studies in volunteers that there is a general correlation between the titre of circulating HI antibody and resistance to infection with the same serotype of influenza virus, but it could not be assumed that AN protects.

Evidence from some studies suggests that the neutralizing antibody content of nasal secretion determines almost entirely whether a subject becomes infected or not – examples are the work of Smith, Purcell, Bellanti & Chanock (1966) with parainfluenza 1 and Perkins *et al.* (1969) with a rhinovirus. On the other hand, in other studies, namely with parainfluenza 2 (Tremonti, Lin & Jackson, 1968) and influenza B (Downie & Stuart-Harris, 1970), it seemed that resistance was, to a considerable extent, correlated with high titres of circulating antibody.

The application of discriminant analysis to the results of challenge can, we believe, help to resolve the complicated situation, although it cannot explain the mechanism

of the relationship detected. The poor correlation between resistance to infection and the results of HI tests may partly result from these tests being less reliable than the AN test, rather than that AN itself has more effect. It would now be interesting to test for neutralizing antibody also, but unfortunately many nasal specimens are exhausted and a proper analysis would therefore not be possible. Nevertheless it seems likely that circulating AN mediates immunity to infection – it may well leak out into the respiratory tract and limit the spread of virus there as may be seen in experimentally infected animals and in tissue cultures infected with influenza virus (Kilbourne, Laver, Schulman & Webster, 1968).

The decline in the titre of circulating AN between the first and second trial is probably a reflexion of the rather rapid loss of this type of antibody previously observed after natural infections with influenza (Schild & Newman, 1969). This is a reminder of the fact that the immune status of the population is always changing, and although AN may have an important effect on immunity shortly after an epidemic, it is probable that at other times other types of antibody might make the major contribution.

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