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Influenza B at Christ's Hospital: natural antibody to influenza B estimated by radial haemolysis

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SUMMARY

The technique of radial haemolysis (SRH) was used to assess the response to infection with different strains of influenza B virus, to determine the persistence of antibody following such infection and to examine sera from boys entering school at age 11 years. The technique detected 95% of infections and in primary infection the antibody response was mainly to the infecting strain. Re-infections resulted in a broad response, both to the homotypic strain and to strains more distantly related. Antibody to the homotypic strain persisted for at least 3 years but in some individuals the reaction with heterotypic strains tended to become weaker – resulting in zones of incomplete lysis – or was lost. Examination of the sera collected on entry to the school showed that about 60% of the boys bled before B/Hong Kong became widespread in the United Kingdom had antibody to strains representative of those isolated in the 1960s and few boys had antibody to B/Hong Kong. After 1974 antibody to B/Hong Kong and later strains became more common while antibody to earlier strains was less frequently detected. The significance of the results as an estimate of past experience is discussed.

INTRODUCTION

It is recognized that strains of influenza B virus undergo antigenic changes, in particular in the antigens associated with the haemagglutinin. However, unlike influenza A, there do not appear to be distinct subtypes giving rise to periodic antigenic shifts with associated pandemics of influenza. The changes in the surface antigens are of a less fundamental nature, and all strains which have been analysed cross-react to a greater or lesser extent with sera raised against other strains. Chakraverty (1971) showed that strains isolated in the 1960s from various countries could be grouped by their degree of cross-reactivity. From time to time strains emerge which react poorly with sera raised against previous strains and these may be associated with local or more widespread epidemics. Recent examples of such 'new' strains are B/Hong Kong/5/72 (Schild *et al.* 1973) which achieved a world-wide distribution in the mid 1970s and strains similar to B/Hanover/13/78 which were responsible for a number of outbreaks of influenza in northern Europe in 1979 (W.H.O., 1980).

Epidemiological surveillance of influenza in the United Kingdom (Pereira,

Assaad & Delon, 1978) has shown that in some years there is an increase in the number of reported cases of influenza B, with many outbreaks in schools, and that such a year of activity is usually followed by two or three years when few cases are reported. The spring of 1974 saw an increase in cases associated with strains similar to B/Hong Kong/5/72 or with the strains intermediate between B/Hong Kong and strains circulating in the late 1960s. In the winter of 1975–76 there were a number of local outbreaks caused by the B/Hong Kong/5/72 strain and some associated with a strain which reacted poorly with B/Hong Kong/5/72 antiserum. The latter strain has been shown to be similar to B/Hanover/13/78 (Chakraverty, 1980). In early 1979 strains similar to B/Hanover/13/78 caused school outbreaks and a number of cases in adults.

In 1970 a study of the epidemiology of influenza and the effect of natural infection and vaccination was started at Christ's Hospital, a boarding school for about 800 boys. This paper describes the results of examination of the sera collected when boys first entered the school and the serological response to infection with known strains of influenza B. The single radial haemolysis (SRH) test has been used since Chakraverty (1980) has shown that SRH is more sensitive than haemagglutination inhibition (HI) for detecting antibodies to influenza B.

MATERIAL AND METHODS

A sample of blood was obtained from new boys whose parents had consented when they joined the school at age 11 years in September of each year. Boys experiencing an influenza-like illness had a throat swab examined and were bled when they first became ill. Post-infection sera were collected 1–7 months later. The sera were examined by HI using the method of Smith & Davies (1976), complement fixation and SRH tests. Some boys were bled annually to determine the persistence of antibody. Serum was separated and stored at −20 °C.

The viruses

Four strains of influenza B virus were chosen, representative of those in circulation during the boys' lifetimes. These were B/England/939/59 (B/59), B/England/21/68 (B/68), B/Hong Kong/5/72 (B/Hong Kong) and B/England/2586/76 (B/76). The last strain was similar to B/Hanover/13/78. The England strains were obtained from Dr P. Chakraverty, Virus Reference Laboratory, Colindale, England, and the Hong Kong strain from Dr G. C. Schild, National Institute for Biological Standards and Control, Hampstead, England. In the analysis that follows B/59 and B/68 will be referred to as 'early' strains and B/Hong Kong and B/76 as 'late' strains. Viruses were grown in the allantoic sac of 9–10 day embryonated hens' eggs. The pooled harvest was centrifuged at 3000 r.p.m. in the Heraeus Labofuge 6000 for 20 min to remove debris. The virus was then concentrated by ultracentrifugation. The virus was pelleted at 25000 r.p.m. for 1 h in a Beckman L5-50B ultracentrifuge using a type 50 fixed angle rotor. The deposit was resuspended in saline and treated by ultrasonics at 8 KC for 1 min in an MSE sonicator to break up aggregates of virus. Electron microscopy of the treated virus suspension showed this treatment did not result in damage to intact virions.

The SRH technique

Antibodies to influenza B were detected by SRH using a technique based on that of Oxford *et al.* (1979). An 8% suspension of sheep erythrocytes (Difco) was mixed with an equal volume of virus at a dose selected to give the best zone definition. For the strains used the doses were B/59, B/Hong Kong and B/76, 1000 units/ml: B/68, 2000 units/ml. The unitage is defined as the amount of virus in 1 ml causing 50% agglutination of 0·25 ml of 0·5% chick erythrocytes (i.e. four times the titre using equal volumes of virus and red blood cells).

Buffers, reaction times, chromic chloride treatment and washing procedures were all as described by Oxford *et al.* (1979). Gels were prepared in Hyland immunoplates: 0·3 ml volumes of sensitized or control erythrocytes were mixed in pre-cooled bijoux bottles with 0·1 ml of fresh guinea pig serum stored at −20 °C. This was found to be the best source of complement, being superior to Richardson's preserved complement which generally resulted in zones of haemolysis of reduced quality and size. Induboise A37 agarose (Uniscience) was made up at 1·5% in phosphate buffered saline: 2·6 ml agarose at 70 °C was added to 0·4 ml of cooled virus/cell/complement mixture. This was found to be the most satisfactory method for combining the reagents and resulted in a low temperature mixture (42–45 °C) which poured easily but gelled quickly. Sixteen wells, 2 mm diameter, were cut in each plate – 14 for test sera and two for controls. Plates were prepared on the day before use and stored at +4 °C overnight.

Examination of sera

Sera were inactivated for 30 min at 56 °C and 3 μl volumes were added to the wells in the SRH plates. Positive and negative control sera were included on every plate. Plates were incubated for 6 h at 37 °C. Zones of haemolysis started to appear after 1 h of incubation and continued to increase in size and clarity with longer incubation. Six h incubation was found to be optimal. Longer incubation resulted in loss of clarity at the zone edges and in some cases complete loss of contrast between areas of haemolysis and background. Less than 6 h incubation resulted in smaller zones of haemolysis. Zones of haemolysis were read on a Dynatech Diffusion Zone Reader. A zone greater than 2·5 mm, in the absence of a zone in the control plate, was taken as representing antibody. The antigen–antibody reaction usually resulted in a clear zone of haemolysis. Occasionally zones of incomplete haemolysis ('faint' zones) were observed.

To assess the response to infection all sera from an individual were tested on the same plate. Reproducibility was assessed by testing 14 replicates of each of three sera of different potencies. The range of values for these sera was 0·4 mm or less. Eighty replicates of the positive control serum for each antigen in 14 batches of tests showed a range of values of only 0·6 mm. Therefore a difference between the serial sera of 1·0 mm or more was regarded as indicating a diagnostic difference.

To determine the initial antibody status of the 1092 boys the sera were tested in batches of 84 and each batch contained some sera from each of the ten cohorts who entered the school in the years 1970 to 1979. Of these baseline sera only four had to be excluded because they gave significant zones of lysis on the control plate.

Table 1. *Response to infection with influenza B*

Year	Infection	No. in group	Number (%) with significant change in SRH antibody to: Early strains only	Early and late strains	Late strains only
1971	Primary	19	14 (74)	5 (26)	0 (—)
	Re-infection	26	15 (58)	9 (34)	2 (8)
1974	Primary	29	3 (10)	11 (38)	15 (52)
	Re-infection	43	2 (5)	37 (86)	4 (9)
1979	Primary	30	0 (—)	21 (70)	9 (30)
	Re-infection	34	0 (—)	32 (94)	2 (6)

RESULTS

Response to natural infection

During the period of study three outbreaks of influenza B occurred. The first, in March 1971, was caused by a strain similar to B/England/21/68. In the spring of 1974 another outbreak of influenza B occurred and virus isolation results showed that two strains were involved, a Hong Kong-like strain and one intermediate between Hong Kong and earlier strains. In January and early February 1979 the third outbreak of influenza B occurred, this time caused by a strain similar to B/Hanover/13/78.

The response to infection by SRH in these three outbreaks is shown in Table 1 where boys who had no evidence of previous infection with influenza B (primary infection) are distinguished from those who had such antibody before an outbreak (re-infection). It will be seen that the response to infection in 1971 was largely to the early strains. In 1974 the response was mainly to the late strains and there was no obvious difference between the response to B/Hong Kong infection and that to the intermediate strain. With primary infections 15 out of 29 responded to late strains only but 39 of 43 re-infections showed a response to early strains. In 1979 the response was again largely to the late strains. A response to both early and late strains was a feature of re-infections both in 1974 and 1979. Taking the three outbreaks together the probability of making a response to more remotely related strains was greater for re-infections than for primary infections. ($\chi^2 = 14{\cdot}95$, $P < 0{\cdot}001$). In these three outbreaks, of a total of 189 infections, eight showed no significant response by SRH. Infection in these boys was confirmed by virus isolation, complement fixation or HI tests.

Persistence of antibody following natural infection

The results obtained from the examination of sera collected from boys infected in the 1971 outbreak are shown in Fig. 1. Antibody producing clear zones of lysis by SRH with the two early antigens persisted for the 3 year period of observation.

Although most of the convalescent sera, collected a month after the outbreak, also showed clear zones of lysis with the late antigens, after about 6 months the reaction with late strains resulted in incomplete lysis (faint zones) or disappeared

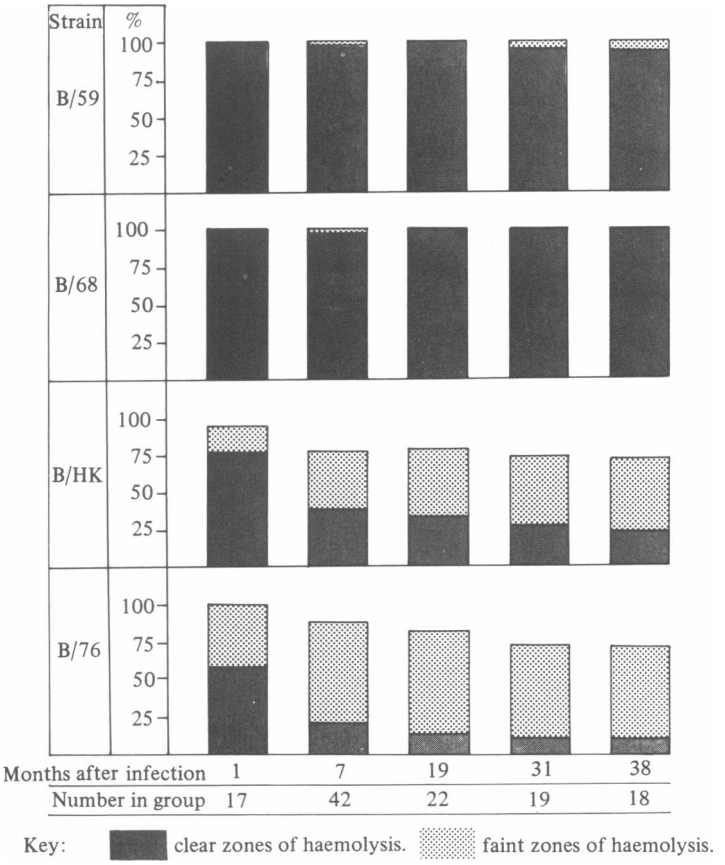

Fig. 1. Persistence of antibody following infection with influenza B in 1971.

completely. The amount of antibody judged by zone diameter to the early antigens dropped by about four-fold between 1 and 6 months, then showed a slow decline over 2½ years to reach approximately half the 6-month value at the end of this period.

Analysis of entry status

The percentage of boys entering the school each year from 1970 to 1979 whose serum contained antibody reacting with the four strains of influenza B in the SRH test is shown in Fig. 2. The boys can be divided into four groups (Table 2) on the basis of their likely exposure to the strains:

Group 1. 1970–73 entries, born between 1958 and 1962 and bled before B/Hong Kong became widespread in the United Kingdom.

Group 2. 1974 and 1975 entries, born between 1962 and 1964 and bled after the first B/Hong Kong outbreak.

Group 3. 1976–78 entries, born between 1964 and 1967 and bled after the second B/Hong Kong outbreak.

Group 4. 1979 entry, born between 1967 and 1968 and bled after the B/Hanover outbreak.

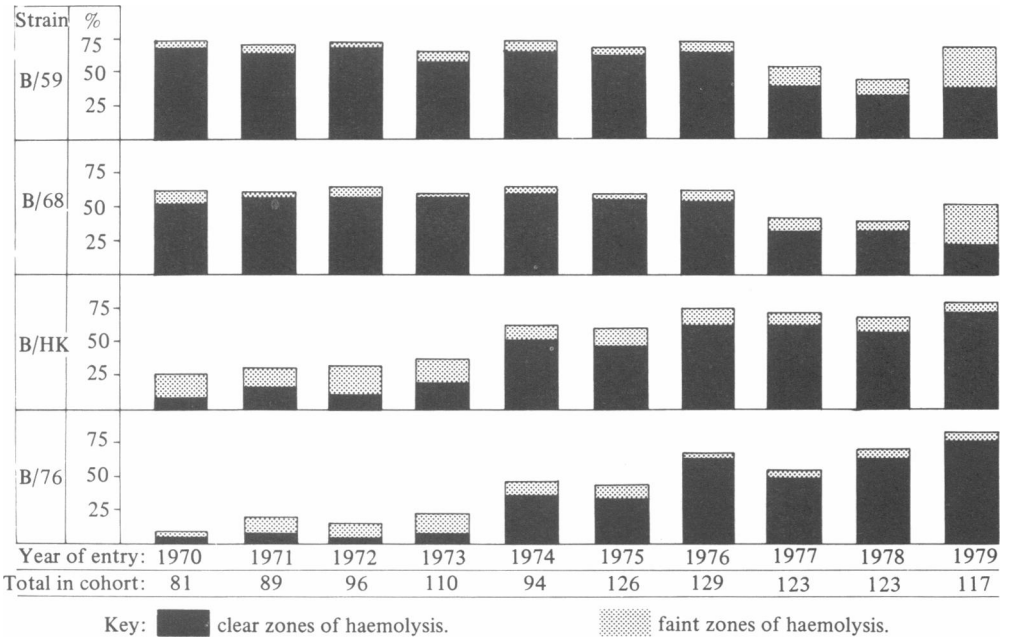

Fig. 2. Antibody to influenza B in entry sera: 1970–79.

Table 2. *Antibody to influenza B in 11-year-olds*

Group	Years of entry	Total in Group	Number (%) reacting with B/59	B/68	B/HK	B/76	None
1	1970–73	376	238 (63)	216 (57)	54 (14)	22 (6)	112 (30)
2	1974–75	220	138 (63)	127 (58)	106 (48)	77 (35)	50 (23)
3	1976–78	375	168 (45)	149 (40)	226 (60)	221 (59)	82 (22)
4	1979	117	44 (38)	28 (24)	83 (71)	89 (76)	13 (11)

Note: The figures in this table refer to clear zones of haemolysis only.

It is clear that boys in Group 1 had had substantial experience of the early strains and few had antibody reacting with the late strains. Those in Group 2 still showed evidence of experience of the early strains but about 50% had antibody to late strains. In Group 3 a diminishing proportion of boys had antibody to early strains while antibody to late strains only was commonly present and this trend continued in Group 4.

Faint zones (Fig. 2) made a significant contribution to the antibody spectrum only when 'real' experience was unlikely, i.e. to the late strains in Group 1 and to the early strains in Group 4. When sera were re-examined using different batches of reagents clear zones of lysis were found to be highly reproducible but some discrepant results were observed. These occurred most frequently with the B/Hong Kong antigen in boys infected before 1974 and with the B/59 antigen in boys in Group 4. Sera giving faint zones with these antigens often gave negative results on re-testing.

DISCUSSION

The antibody response to infection as judged by SRH is best demonstrated by using a closely related strain. In primary infection the response may be limited to such a strain but in re-infection a broad response is common. The homotypic response is well maintained over a period of years but an initial response to more distantly related strains tends to decline. Faint zones may be seen as a late expression of this heterotypic response. Retesting of sera giving faint zones may, if different batches of reagents are used, lead to poor reproducibility. This suggests that antibody resulting from infection with a heterotypic strain is more affected by small changes in the sensitivity of the test than antibody resulting from infection with a closely related strain.

It was expected that boys entering the school in different years would have antibody to influenza B related to the strain or strains with which they had been infected in the past. It appears from the results described here that SRH generally reflected the different experience of different cohorts. Thus children bled before the winter of 1973–74 had antibody to strains typical of those they might have encountered in the late 1960s and early 1970s. After the B/Hong Kong outbreaks in early 1974 and in 1976, there was an increase in the proportion of boys with antibody to this strain and the B/76. There was also a gradual decrease in the proportion of boys with antibody to the earlier strains. Although it is possible that this merely reflects decline of antibody with the passage of time, examination of serial sera from the same individual shows that antibody levels are well maintained. It is more likely that boys who had antibody to late strains only represent those who escaped infection in the first few years of life and experienced a primary infection with B/Hong Kong or later strains.

The antigens used in the SRH test were whole virus particles and it is relevant to consider whether antibody to the neuraminidase played a part in the reactions observed. For influenza A, neuraminidase antibodies can be detected by SRH (Callow & Beare, 1976) but the conditions are critical. When the test is set up to detect antibody to the haemagglutinin, neuraminidase antibodies are unlikely to be detected (Schild, Pereira & Chakraverty, 1975). Patients infected with influenza B are likely to produce antibodies to both surface antigens and, for the purpose of a diagnostic test, any reaction which detects a change in antibody titre will suffice. Schild *et al.* (1973) have shown that there is little apparent difference between the neuraminidase antigens of B/Victoria/98926/70 (similar to B68) and B/Hong Kong/5/72. Five patients infected with a strain similar to B/Victoria showed a response to this strain by HI, but no response to B/Hong Kong. They produced a comparable response to the neuraminidase of both strains. If the SRH test described here were detecting neuraminidase antibody it is probable that sera containing antibody to any strain would react with all the strains used. To estimate past experience a pattern of reactions to the four strains was recorded and a correlation was observed between likely experience and the presence or absence of clear zones of lysis with particular strains. It is possible that 'faint' zones represent a reaction with the neuraminidase antigen under conditions which are suboptimal for its detection since the effect of reading faint zones as positive is to increase the proportion of sera which react with all the antigens.

The SRH technique has many technical advantages over the HI test. It is also more sensitive in detecting antibody to influenza B virus and in diagnosing current infection. In the study reported here the SRH test detected 181 (95 %) of 189 proven infections with influenza B virus. By comparison the HI test detected only 166 (92 %) of the 181 infections examined by this technique.

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