IMMUNITY TO *HAEMOPHILUS INFLUENZAE* TYPE B: THE NATURE OF THE BACTERICIDAL ANTIBODY IN HUMAN BLOOD

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THE well-known high degree of immunity to *Haemophilus influenzae* meningitis enjoyed by subjects under 3 mth of age and by those over 4 yr was correlated by Fothergill and Wright (1933) with the presence in the blood of a bactericidal factor believed to be an antibody (Smith, 1956; Mathies, Hodgman and Ivler, 1965) against the causative organism.

It has been suggested that this antibody is produced against the capsular antigen of *H. influenzae* type b (Turk and May, 1967). In one study (Mpairwe, 1971), however, the age distribution of the type-b capsular antibody in human sera and that of the bactericidal antibody were found to be different. Moreover, the only *H. influenzae* antibodies that have been reported to have an age-distribution similar to that of the bactericidal antibody were those produced against a somatic antigen (Tunevall, 1953).

In view of these discrepancies it seemed worth while to investigate further the nature of the bactericidal antibody in human blood. The purpose of this paper is to report evidence suggesting that the bactericidal antibody develops against a somatic antigen that is present in naturally occurring non-capsulated *H. influenzae* strains.

MATERIALS AND METHODS

A single strain of *H. influenzae* type b isolated from cerebrospinal fluid was used. It formed iridescent mucoid colonies on Levinthal's agar. It was promptly agglutinated into firm, coarse irregular-sized clumps by type-b antiserum (Burroughs, Wellcome and Co.) and was not agglutinated by antisera against any other types.

Non-capsulated strains were isolated from the upper respiratory tract. Their colonies on Levinthal's agar were smooth, but not mucoid or iridescent. They were not specifically agglutinated by any of the six typing antisera.

Human sera were obtained from the blood of Mulago Hospital patients who were not on antibiotic treatment and whose ages ranged from birth (cord blood samples) to adulthood. Sera were stored at -20°C and used within 8 wk of collection.

Rabbit antisera. For immunising rabbits, antigen suspensions of appropriate strains were made from 6-hr cultures at 37°C on Levinthal's agar. The suspensions were adjusted to opacity 30 against distilled water in the MRC grey wedge spectrometer with filter no. 2. The suspensions were used immediately. Three groups of rabbits (five per group) were used. One group was immunised with *H. influenzae* type b, and the other two with non-capsulated strains 1 and 2 respectively. Rabbits were bled to obtain pre-immunisation sera, and 0.2, 0.5, 1 and 1 ml of appropriate antigen suspensions were then inoculated intravenously on the

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1st, 4th, 7th and 10th day respectively. Final bleeding for post-immunisation sera was done on the 15th day.

Titre of capsular antibody in absorbed and unabsorbed portions of type-b rabbit anti-serum was determined by the haemagglutination technique of Turk and Green (1964).

Comparison of pre- and post-immunisation rabbit sera for their bactericidal antibody content against H. influenzae type b. Throughout the tests, drops (0.02 ml) were delivered from a calibrated pasteur pipette. The test sera were inactivated at 56°C for 30 min. To 0.1 ml of each of these, or their ten-fold dilutions in saline, 0.1 ml of fresh normal rabbit serum (complement) was added. A drop of "suspension — 4" of *H. influenzae* type b (a 10^{-4} dilution of an original suspension of two colonies in 5 ml of 10 per cent. heated serum in saline: Mpairwe, 1971) was added. The mixture was shaken gently and incubated in a waterbath at 37°C for 45 min. At the end of this period the mixture was shaken again and a drop was plated on chocolate agar. The plates were incubated at 37°C and colonies counted 18–24 hr later.

TABLE I
The age-distribution of agglutinins against non-capsulated strains of Haemophilus influenzae, 1 and 2

Strain	Age of serum donor	Number of sera examined	Number (and percentage) with agglutinating antibody against the strain
1	newborn	22	20 (91)
	2 mth–1 yr	20	0 (0)
	>1–2 yr	20	5 (25)
	>2–5 yr	22	18 (82)
	>5 yr	29	28 (97)
2	newborn	15	15 (100)
	2 mth–1 yr	15	0 (0)
	>1–2 yr	15	5 (33)
	>2–5 yr	15	11 (73)
	>5 yr	15	14 (93)

Absorption of bactericidal antibody from rabbit antisera. The *H. influenzae* strain to be used for absorption was inoculated on chocolate agar plates with an internal diameter of 8.7 cm in such a way as to produce confluent growth after incubation for 18 hr at 37°C. The growth on four such plates was suspended in 1 ml of saline. To 0.5 ml of the type-b antiserum to be absorbed, 0.5 ml of fresh normal rabbit serum was added as a source of complement. The mixture was divided into two: 0.5 ml of the organism suspension was added to one-half and 0.5 ml of saline was added to the other. Both parts were shaken, incubated at 37°C for 6 hr and left at 4°C overnight. They were then centrifuged at 5000 r.p.m. for 5 min. to remove most of the organisms in suspension and 0.5 ml of each supernatant was pipetted off and inactivated at 56°C for 30 min.

Detection of agglutinins against non-capsulated strains. The antigen was a suspension in saline of organisms of the appropriate strain from an 18-hr culture at 37°C on chocolate agar. The opacity of the suspension was adjusted to 250 against distilled water in the MRC grey wedge spectrometer with filter no. 2. The suspension was used immediately. A drop of test serum was mixed on a slide with a drop of antigen suspension and, in a positive reaction, agglutination visible to the unaided eye occurred within a minute.

Titration of human sera for their content of agglutinins against non-capsulated strains. Doubling dilutions of the sera were made in saline to 1 in 128. The antibody in each of these dilutions was detected as described above, except that the incubation period at room temperature was increased to 10 min. The highest dilution causing agglutination was recorded.

RESULTS

The age-distribution of the agglutinins in human sera against non-capsulated strains 1 and 2 is shown in table I. These distributions resemble that of the bactericidal antibody against *H. influenzae* type b (Mpairwe, 1971).

TABLE II

The bactericidal effect of rabbit sera on Haemophilus influenzae type b before and after immunisation with type b

Rabbit no.	<i>H. influenzae</i> type-b colony counts in									
	pre-immunisation sera					post-immunisation sera				
	undiluted	at dilutions of			undiluted	at dilutions of				
		10 ⁻¹	10 ⁻²	10 ⁻³		10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵
1	186	91	87	62	189	234	73	0	0	0
2	233	92	26	26	0	0	0	0	...	...
3	327	118	125	75	0	0	0	0	...	...
4	147	68	56	40	0	0	0	0	...	...
5	7	23	31	66	16	0	0	0	...	...

... = Test not carried out at this dilution.

The agglutinin titre against non-capsulated strain 1 was determined in the sera of ten mothers who had just been delivered and in the sera of their 11 babies (including one set of twins). The titres varied from 8 to 64, but the titre in a given mother's serum was in each instance equal to that in her baby's serum.

TABLE III

Bactericidal effect of the sera of one rabbit on Haemophilus influenzae type b before and after immunisation with non-capsulated strain 2

Expt no.	<i>H. influenzae</i> type-b colony counts in neat serum	
	before immunisation	after immunisation
1	53	4
2	53	1
3	49	2
4	32	2
5	68	4

Bactericidal antibodies against *H. influenzae* type b developed in all rabbits immunised with *H. influenzae* type b (table II) with a prozone phenomenon in antisera from rabbits 1 and 5. With one exception (table III), all rabbits

immunised with non-capsulated strains failed to develop bactericidal antibodies against *H. influenzae* type b.

The bactericidal antibody against *H. influenzae* type b in antisera from three rabbits was removed by absorption with 11 non-capsulated strains. The results obtained using rabbit no. 2 antiserum and non-capsulated strain 2 are shown in table IV.

The titre of type-b capsular antibody in absorbed and unabsorbed portions of serum from rabbit no. 2 was in each instance 128 indicating that absorption did not remove the capsular antibody.

TABLE IV
Bactericidal effect of one rabbit antiserum on Haemophilus influenzae type b before and after absorption with non-capsulated strain 2

Expt no.	<i>H. influenzae</i> type-b colony counts in neat serum	
	before absorption	after absorption
1	0	Numerous
2	0	193
3	0	159
4	4	99
5	0	86
6	0	194
7	0	103
8	0	141

DISCUSSION

Capsulated strains form only about 5 per cent. of all *H. influenzae* isolated from the upper respiratory passages of people in ordinary open communities. In spite of this, virtually all cases of acute primary *H. influenzae* infections are caused by capsulated strains (Turk and May, 1967). This suggests that virulence of the organism is capsule-dependent. In support of this, capsulated strains are more virulent than non-capsulated strains for experimental animals (Pittman, 1931; Leidy *et al.*, 1960), and less susceptible to the killing action of normal rabbit (Wright and Ward, 1932) and human blood (Fothergill and Wright, 1933).

The fact that of the six known capsular types, type b is responsible for virtually all acute primary infections due to *H. influenzae* (Turk and May) suggests that the apparent virulence-potentiating qualities of the capsule are, in the case of man, specifically limited to the type-b capsule. For such reasons it has been suggested that the bactericidal antibody, which is believed to be protective, is produced against the type-b capsular antigen (Turk and May).

This conclusion must, however, be accepted with caution. In the case of *Bacillus anthracis*, virulence for the rabbit, the sheep and the guinea-pig is also associated with the presence of a capsule and yet the protective antibodies are not those produced against the capsular antigen (Raffel, 1953). Moreover, the

capsular antigen of *H. influenzae* type b may not be the only virulence factor. In mice (Leidy *et al.*) capsulated strains are more virulent than non-capsulated strains, but the degree of virulence of capsulated strains is determined not by the type-specificity of the capsule but by the somatic constitution of the organism. In other words, a somatic factor also plays a part in virulence. In man the virulence of *H. influenzae* type b might also depend, in part, on a somatic factor.

An apparently type-specific somatic antigen was demonstrated in *H. influenzae* type f (May, 1965) and there is reason to believe that *H. influenzae* type b also possesses its own kind of type-specific somatic antigen (MacPherson, 1948). It is, therefore, possible that the reason why *H. influenzae* type b features so prominently in acute primary *H. influenzae* infections is not because the type-b capsular substance has special virulence qualities not possessed by other capsular substances, but because it is always associated with a type-specific somatic virulence factor.

The view that the bactericidal antibody is produced against a somatic antigen is supported by a substantial body of evidence. The age-distributions of children with the type-b capsular antibody and of those with the bactericidal antibody have not, to the best of my knowledge, been reported to be similar. Indeed, in one study (Mpairwe, 1971) they were found to be dissimilar. The only *H. influenzae* antibodies that have previously been reported to have an age-distribution similar to that of the bactericidal antibody were those produced against a somatic antigen (Tunevall, 1953). In the present studies agglutinins in human sera against non-capsulated strains have been shown to have an age-distribution similar to that of bactericidal antibody. Their transplacental passage from maternal to foetal circulation is strongly suggested by the finding that the titres in mothers at delivery and in their babies at birth were the same.

Absorption studies indicated that the type-b bactericidal antibody in rabbit antisera could be absorbed by non-capsulated strains without removing capsular antibody. Dingle, Fothergill and Chandler (1938) immunised 12 guinea-pigs with *H. influenzae* type b, and the resulting antisera, which were practically devoid of anti-capsular antibodies, were nevertheless bactericidal to *H. influenzae* type b. In the horse immunised by Pittman (1933) it was observed that the titre of the "anti-infectious" factor which developed (and which was presumably bactericidal antibody) continued to rise several months after the capsular antibody in that same horse had attained its maximum titre.

Dubos (1942) produced immunity to serotypes b and d (which could be passively transferred) in rabbits that he immunised with an extract of *H. influenzae* type b that was evidently free from capsular material, since antisera to the extract did not react with capsular antigen. If this immunity was mediated through bactericidal antibody, it must have been different from capsular antibody.

Observations on the communicability of *H. influenzae* type b in orphanage children amongst whom the carriage rate of this serotype is sometimes high (Turk, 1963; Mpairwe, 1970) would seem to suggest that in ordinary open communities *H. influenzae* type b is very rarely communicated from one

household to another (Mpairwe, 1970). Hence very few children would be expected to have come into contact with this serotype by the age of 5 yr. Yet by that age most subjects have bactericidal antibody (Fothergill and Wright; Mpairwe, 1971) and there thus seems to be a discrepancy between the circulation of *H. influenzae* type b and the development of bactericidal antibody to it.

H. influenzae type-b infections have not been reported to occur in epidemic form; it is now tempting to explain this on the basis of widespread immunity to this serotype due to contact with the much more abundant non-capsulated strains.

In the present studies, rabbits immunised with non-capsulated strains that could absorb type-b bactericidins failed on the whole to produce type-b bactericidal antibodies. This poses a problem, but does not rule out the possibility that in man under natural conditions these strains could stimulate the production of the bactericidal antibody.

SUMMARY

It has been suggested that the bactericidal antibody associated with immunity to acute primary *Haemophilus influenzae* infections is that produced against the capsular antigen of *H. influenzae* type b. In a previous study, however, the bactericidal and the type-b capsular antibodies were found to have different age-distributions. In the present studies the age-distributions of children with bactericidal antibody and of those with agglutinins against non-capsulated strains were found to be similar. Furthermore, it was possible to absorb type-b bactericidins from rabbit antisera with non-capsulated strains without removing the type-b capsular antibody. These findings, together with other evidence in the literature, suggest that the antigen responsible for the production of the bactericidal antibody is somatic and is possessed by non-capsulated strains, although attempts to produce type-b bactericidins in rabbits immunised with these non-capsulated strains were unsuccessful.

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