Human Serum Bactericidal Activity against *Haemophilus influenzae* Type b

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We examined bactericidal and opsonizing activity of pooled adult 'immune' serum against *Haemophilus influenzae* type b with and without the addition of phagocytes. Four type b strains from cerebrospinal fluid (CSF) and three such strains from the nasopharynx (NP) of healthy children were examined. Duplicate reaction mixtures contained organisms in exponential (E) or stationary phase (S) of growth, serum, a complement source (human agammaglobulinaemic serum), and culture medium (bactericidal assay); separate assays contained the above components and polymorphonuclear leucocytes (opsonization system). A decrease in bacterial density of $\geq 1 \log_{10}$ unit was considered significant. All four S-CSF strains, three of four E-CSF strains and one of three S-NP strains were sensitive to the bactericidal activity of pooled serum. The other E-CSF strain, two S-NP strains and all three E-NP strains were resistant to the bactericidal activity of pooled serum. Two of three E-NP strains were opsonized by pooled serum; the other strains resistant to the bactericidal activity of pooled serum were also resistant to opsonization. Bactericidal and opsonizing activity of serum from an immunized adult was greater than or equal to that of pooled serum against each strain. Assuming normal adults are immune to invasive *H. influenzae* type b infection, an experimental test reflecting this immunity is the bactericidal activity against CSF isolates tested in stationary phase. We conclude that protection against invasive disease due to *H. influenzae* type b appears more complex than the presence of bactericidal and opsonizing activity in serum.

INTRODUCTION

The virulence of certain Gram-negative bacteria is attributed to the composition of the cell wall (Roantree, 1976). Conversely, 'protective antibodies' directed towards cell wall components have bactericidal and opsonic activity (Muschel, 1968). Correlations have thus been sought between the absence of serum bactericidal or opsonic activity, and host susceptibility. For example, lethal infections in adult mice could be produced by strains of *Escherichia coli* which were resistant to the bactericidal and opsonic activity of serum (Howard & Glynn, 1971). In other studies, both serum-sensitive and serum-resistant *E. coli* persisted for 48 h in the blood of normal adult rabbits after intravenous injection (Roantree & Pappas, 1960). Thus the presence of serum bactericidal activity *in vitro* did not correlate with susceptibility to infection, as assessed by bacterial persistence in the bloodstream.

Fothergill & Wright (1933) reported an age-dependent relationship between the absence of bactericidal activity and the attack rate of *Haemophilus influenzae* meningitis. They used a single cerebrospinal fluid (CSF) type b isolate to demonstrate bactericidal activity in fresh, defibrinated blood. The selection of a single strain was based on the assumption that all type b

**Abbreviations**: CSF, cerebrospinal fluid; E, exponential phase; HS, immune serum; NP, nasopharynx; PBS, phosphate-buffered saline; PMN, polymorphonuclear leucocytes; PRP, purified type b capsular polysaccharide; PS, pooled human serum; REF, resistance-enhancing factor; S, stationary phase; sBHI, supplemented brain heart infusion.
H. influenzae strains were homogeneous with respect to their immunogenicity and susceptibility to bactericidal activity of blood (Rivers, 1922). The presence of serum bactericidal activity became prima facie evidence of 'immunity' to invasive infection by H. influenzae type b. Thirty years later, strains resistant to the bactericidal activity of normal adult serum were found (Norden, 1972); opsonization of these strains was not investigated.

Bactericidal assays using a single strain of H. influenzae have shown that 35% of adults had no detectable titre (Feigin et al., 1971), or that all adult sera had detectable activity (Anderson et al., 1971). The basis for the opposite conclusions may arise from the use of different test strains or organisms in different phases of growth (exponential or stationary). One portion of the discrepancy between the original observation by Fothergill & Wright (1933) and more recent data may have resulted from the choice of blood or serum for the bactericidal assay: certain investigators used whole blood (Fothergill & Wright, 1933), while others used serum (Feigin et al., 1971; Anderson et al., 1971; Norden, 1970). Thus a portion of the bactericidal activity could be due to phagocytosis and subsequent intracellular killing. In addition there is a discrepancy between the presence of immunoglobulins which bind to the bacterial cell surface and the bactericidal titre of the same sera (Norden, 1970; Gump et al., 1971).

We examined the bactericidal and opsonizing activity of pooled normal adult serum and serum derived from an adult immunized with purified type b capsule against nine strains of H. influenzae type b. These strains were isolated from the nasopharynx (NP) of healthy children or the CSF of children with meningitis. We investigated the effect of growth phase of the organisms on their resistance phenotype in the bactericidal and opsonization assays. Our data suggest that bactericidal activity is an inadequate indicator of susceptibility.

**METHODS**

**Bacteria.** The H. influenzae isolates studied, their source, antibiotic susceptibility and biotype are given in Table 1. All organisms had an absolute requirement for NAD + and haemin; they were typed by slide agglutination with four anti type b antisera obtained from Difco Associates, Detroit, Mich., USA, Dr J. Robbins, Federal Bureau of Biologics (Burro 132), Hyland Laboratories, Research Triangle Park, N.C., USA, and polyvalent capsular antigens from Difco Associates; the agglutination results were identical for all four sera.

The 'DC' strains (provided by Dr David Scheifele, Vancouver, Canada) were isolated from nasopharyngeal cultures of children two to five years of age between April and October, 1979, at the time of one-day hospital admission for minor elective surgery. The 'C' strains were isolated from the CSF of children hospitalized with acute meningitis at the Children's Orthopedic Hospital and Medical Center, Seattle, Wash., USA between August 1978, and December 1979. These strains were selected because their phenotypic characteristics are representative of CSF isolates. The 'DC' strains and 'C' strains were suspended in skim milk from the first or second subplating of the primary culture and stored at −70°C. E1, a stably capsulated reference strain, has been previously described (Anderson et al., 1971). S2 is a mutant of E1 which contains <1/1000 the quantity of type b capsule as E1 (Anderson et al., 1971). Biotyping was performed by the method of Edberg (Edberg et al., 1980). Antibiotic susceptibility testing was performed as previously described using an inoculum of 10^3 c.f.u. (Wong et al., 1982).

**Culture techniques.** The methods for storage of H. influenzae and growth in supplemented brain-heart infusion (sBHI) have been previously described (Roberts & Smith, 1980). Mid-exponential phase (E) organisms were harvested at an OD_600 of 0.2. Stationary phase (S) organisms were grown in broth for 16–20 h at 37°C. Tubes containing the organisms were placed in crushed ice and used within 3.5 h of harvesting. In each assay a suspension of 32 × 10^6–3.4 × 10^7 organisms ml^-1 was prepared by adding a 0.2 ml aliquot of organisms in sBHI broth to 9.8 ml Medium 199 (Grand Island Biological Co., Grand Island, N.Y., USA) at 4°C.

**Sera.** Antibodies to purified type b capsular polysaccharide (PRP) were kindly assayed by Dr J. Kuo (see Kuo et al., 1981) of Lederle Laboratories, Pearl River, N.Y., USA. Pooled human serum (PS) was a mixture of heat-inactivated (56°C for 45 min) sera from five healthy adult hospital employees not involved in Haemophilus research. The anti-PRP content of PS was 3.88 µg ml^-1. This serum was stored at −20°C prior to use. Immune serum (HS) was obtained from one of the authors (A.L.S.) seven years after intradermal immunization with 100 µg PRP, and after working with type b strains for nine years. The anti-PRP content of this serum was 5–10 µg ml^-1; this serum was heat-inactivated (as above) and stored at −70°C until use. Serum from a 29-year-old male with X-linked agammaglobulinemia, obtained just prior to a gammaglobulin injection, was used as a source of complement. The anti-PRP content of this serum was 0.24 µg ml^-1; immunoglobulin concentrations of this serum were IgA < 10, IgM < 10, IgG 160 mg (100 ml)^-1. The CH_50 activity on a sample before and after the assays was greater than 100 units ml^-1. One unit of CH_50 is defined as that amount of serum necessary to produce lysis of 50% of a suspension of IgM-coated sheep erythrocytes under standard conditions. All assays were...
performed within 6-5 h of thawing from -70 °C to 4 °C. Weanling rat serum (Charles River Breeding, Wilmington, Mass., USA; CD/COBS Sprague-Dawley) was collected from the severed neck vessels after decapitation; the animals had been maintained in a pathogen-free environment. The blood from each litter was pooled, and the resulting serum harvested, filtered through a 0.22 μm Millipore filter, and stored at -70 °C. Prior to use the serum was pooled with that of other litters and heated at 56 °C for 45 min.

Phagocyte preparation. Polymorphonuclear leucocytes (PMN) were recovered by separation from heparinized (10 units ml⁻¹) blood on Ficoll-Hypaque gradients, suspended in M199, and counted as described (Jacobs et al., 1982). Viability, determined by trypan blue dye exclusion, was >97%; the percentage of PMN, determined by staining for myeloperoxidase, was ≥96%. PMN were resuspended in M199 to produce a final concentration in the reaction mixture of 1.2 x 10⁶ PMN ml⁻¹ or 4.0 x 10⁵ PMN ml⁻¹, giving bacteria:PMN ratios of 3:1 and 10:1, respectively.

Bactericidal and opsonic assays. M199 (0.2 ml, bactericidal assays) or PMN (0.2 ml, opsonic assays), 0.1 ml HS or PS, and 0.1 ml of complement source were combined in a 12 x 75 mm polystyrene tube (Falcon) at 4 °C. Then, 0.2 ml bacteria (1 x 10⁶ organisms) was added. The mixture was vortexed, and a sample was immediately removed, diluted and cultured; the mixture was then incubated at 37 °C in a shaking water bath (120 cycles min⁻¹). After 30 min another sample was removed, diluted and cultured. In the opsonic assay, 0.01 ml was initially diluted into 0.99 ml sterile water for 10 min; this resulted in lysis of all PMN as determined by phase-contrast microscopy. In preliminary experiments each strain survived for more than 10 min in water without detectable reduction in viability. Serial 10-fold dilutions were performed in phosphate-buffered saline (0.15 M-sodium chloride; PBS) with 0.1% gelatin. Quantitative cultures were performed in duplicate by spreading 0.01 ml of sample on sBHI agar. Cultures were incubated at 37 °C in 5% (v/v) CO₂ for approximately 18 h and the bacterial colonies counted. The fraction surviving was calculated as the ratio of the bacterial density at the end of the assay to that present at the beginning. Most strains were tested two or more times in stationary and exponential growth phases. The average fraction surviving was the mean of at least two tests performed on separate days. Serum bactericidal activity was considered present if the fraction surviving was ≤0.1.

HS or PS with heat-inactivated complement source, HS or PS alone, complement source without HS or PS, or M199 was mixed with bacteria as controls: none produced a significant reduction in bacterial density. A 0.01 ml aliquot of HS, PS, and complement source was cultured on a sBHI plate after each assay. PS was contaminated once; repeating the experiment with fresh PS did not change the results.

Assay for inducing bacterial resistance to serum bactericidal activity. The assay to detect phenotypic resistance was performed as reported with minimal modifications (Shaw et al., 1976). Briefly, organisms grown in sBHI to mid-exponential phase were diluted 1:50 in weanling rat serum, PS, or dialysate of PS (Anderson et al., 1980) and incubated at 37 °C for 30 min in a shaking water bath (120 cycles min⁻¹). The strains were centrifuged from that medium, resuspended in M199 and used in the bactericidal or opsonization assays.

Immunofluorescent determination of antibody or complement binding to organisms. Fluorescein-conjugated goat antisera to human IgM, IgG, and complement (βC/βA fraction) was obtained from Kent Laboratories, Redmond, Wash., USA). IgG and IgM binding to bacteria was detected by incubation of 0.2 ml of 1:10 dilution of broth-grown organisms in M199 with 0.1 ml HS or PS for 30 min at 37 °C. After centrifugation at 10000 g for 2 min at room temperature, organisms were washed three times with 0.5 ml cold PBS by vortexing and centri-

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Table 1. Phenotypic characteristics of H. influenzae used in the bactericidal and opsonization assays

<table>
<thead>
<tr>
<th>Organism</th>
<th>Type</th>
<th>Biotype</th>
<th>Origin</th>
</tr>
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<tbody>
<tr>
<td>DC15</td>
<td>b</td>
<td>IV</td>
<td>NP</td>
</tr>
<tr>
<td>DC33</td>
<td>b</td>
<td>I</td>
<td>NP</td>
</tr>
<tr>
<td>DC37</td>
<td>b</td>
<td>II</td>
<td>NP</td>
</tr>
<tr>
<td>C562</td>
<td>u*</td>
<td>I</td>
<td>CSF</td>
</tr>
<tr>
<td>C616</td>
<td>b</td>
<td>I</td>
<td>CSF</td>
</tr>
<tr>
<td>C654</td>
<td>b</td>
<td>I</td>
<td>CSF</td>
</tr>
<tr>
<td>C668</td>
<td>b</td>
<td>I</td>
<td>CSF</td>
</tr>
<tr>
<td>E₁</td>
<td>b</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>S₂</td>
<td>†</td>
<td>I</td>
<td></td>
</tr>
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* Type b on the primary culture; untypable after the first subplating.
† Strain S₂ is a spontaneous derivative of the reference strain E₁ which produces ≤1/1000 capsular polysaccharide as the parent [10].
fugation at 10000 g. Then, 0.25 ml diluted fluorescein-conjugated antisera (1:150 anti-IgG or 1:100 anti-IgM) was added; the mixture was incubated at 37 °C for 30 min. The suspension was then layered on 0.5 ml foetal calf serum and centrifuged at 10000 g for 2 min; the supernatant was discarded, and the pellet was resuspended in 0.05 ml PBS. Binding of C3 to organisms was similarly detected except that after incubation with HS or PS and subsequent washing with PBS, the bacterial pellet was suspended in 0.25 ml of a 1:3 dilution of agammaglobulinaemic serum in M199 and incubated at 37 °C for 5 min. The mixture was then centrifuged, washed, and incubated with diluted fluorescein-conjugated antisera directed against human C3 at room temperature for 30 min and processed as described for anti-IgG and anti-IgM. A 0.01 ml sample was placed on a glass slide, sealed, and read in a coded fashion by two observers using a Zeiss fluorescence microscope with epit-illumination. Organisms appearing as sharp fluorescent rings with dark centres were scored as positive. When results were equivocal, the assays were repeated.

Electron microscopy. The cells were fixed in 2.5% (v/v) glutaraldehyde in sodium cacodylate buffer (pH 7.2) for 4 h, post-fixed for 30 min in 2% (w/v) OsO₄ in o-collidine buffer (pH 7.4), centrifuged at 10000 g for 2 min at room temperature, dehydrated in ethanol and infiltrated with a mixture of 50% (v/v) propylene oxide and Epon 812. Thin sections were stained with uranyl acetate for 1 h and Millonig's lead acetate for one minute. Sections were examined and photographed in a Zeiss EM-9 electron microscope.

Animal studies. Sprague-Dawley rats (5-d-old were inoculated intranasally with 10⁹ organisms; blood cultures were obtained 48 h later as described by Roberts & Smith (1980).

RESULTS

Bactericidal and opsonic assays

Figure 1 shows the survival curve of a representative strain during incubation in the bactericidal and opsonic assays with HS and PS for 1.5 h. No additional killing was detected after 30 min incubation.

Table 2 shows the results of bactericidal activity of HS and PS against each strain. All strains were sensitive to the bactericidal activity of HS except DC15-S (stationary phase) and DC15-E (exponential phase). Killing in HS was not clearly enhanced by PMN. The relative contributions of anti-PRP and anti-somatic antibodies to bactericidal activity were not investigated. In experiments in which PMN (three organisms per PMN) were added to the incubation tubes containing PS, only strains DC33-E and DC37-E showed a reproducibly significant decline in viability (survival ratios 0.77 declining to <0.01 and 0.63 declining to 0.025, respectively).
**Table 2. Human serum bactericidal activities against H. influenzae strains**

<table>
<thead>
<tr>
<th>Strains tested with:</th>
<th>Immune serum (HS)†</th>
<th>Pooled sera (PS)‡</th>
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<tr>
<td>Survival ratio*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;0.01</td>
<td>C562-S,E C616-S,E</td>
<td>C668-S,E C562-S,E</td>
</tr>
<tr>
<td>DC33-S DC37-S E₁,S</td>
<td>DC33-S E₁,S S₂,S,E</td>
<td></td>
</tr>
<tr>
<td>S₂-S,E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;0.01 &lt;0.10</td>
<td>DC33-E DC37-E</td>
<td>C616-E E₁,E</td>
</tr>
<tr>
<td>&gt;0.10 &lt;0.70</td>
<td></td>
<td>DC37-S,E</td>
</tr>
<tr>
<td>≥0.70</td>
<td>DC15-S,E</td>
<td>C668-E DC15-S,E</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DC33-E</td>
</tr>
</tbody>
</table>

* A total of $10^6$ organisms were incubated for 30 min in diluted serum and complement source (see Methods). The fraction surviving was calculated as the ratio of the c.p.m. at the end of the assay to those present at the beginning.

† Anti-PRP content 3.88 µg ml⁻¹.
‡ Anti-PRP content 5.10 µg ml⁻¹.

Strain DC33-E also showed this effect when the organism numbers were increased to 10 organisms per PMN giving survival ratios of 0.63 declining to 0.04.

Only one CSF isolate (C668-E) was resistant to the bactericidal activity of PS. In contrast to the results with CSF isolates, the only NP isolate sensitive to the bactericidal activity of PS was DC33-S. Two additional exponential phase NP strains, DC33-E and DC37-E were opsonized by PS, since the addition of PMN enhanced killing. To distinguish adherence of organisms to the surface of PMN from true phagocytosis, PMN from the opsonic assay were visualized by electron microscopy; the enhanced killing with the addition of PMN was associated with phagocytosis and intracellular degradation of organisms (Fig. 2).

**Effect of preincubation in decomplemented serum**

Pre-incubation in infant rat serum makes the organism resistant to non-capsular bactericidal activity, which is thought to be a reflection of the in vivo phenotype (Shaw et al., 1976). This effect has been attributed to a 'resistance-enhancing factor' (REF) in infant rat serum. We investigated the effects of preincubation in heat-inactivated PS and weanling rat serum on the susceptibility of a very serum-sensitive strain (C562-E), and two moderately serum-sensitive strains (C616-E and E₁,E) to the bactericidal and opsonic activity of HS and PS (Table 2). Pre-incubation in weanling rat serum, but not PS, increased the resistance of the moderately sensitive strains but not the very sensitive strain to PS; each strain remained susceptible to the bactericidal activity of HS. To eliminate the possibility that pre-assay exposure to antibodies and enzymes in PS might mask the effects of REF, strain E₁ was tested after incubation in PS dialysate; there was no detectable enhancement of resistance to the bactericidal activity of PS or HS. PS dialysate was used in dilutions of 1:2 and 1:100; neither decreased the sensitivity of these strains to bactericidal activity of PS.

**Immunofluorescence**

Antibody binding is assumed to be a prerequisite for bactericidal activity against H. influenzae. We investigated the class of immunoglobulin binding to intact H. influenzae by an immunofluorescence assay. IgG was present on the surface of each strain harvested from each growth phase after incubation in HS or PS. Incubation in PBS, HS, or PS, followed by incubation in complement source resulted in binding of C3 to each strain harvested from each growth phase. After incubation in HS, IgM was present on one of nine strains in exponential phase and three of nine strains in stationary phase; after incubation in PS, IgM was present on seven of nine strains in stationary phase and none in exponential phase ($P = 0.001$, Fisher's exact test). IgM bound to three strains with incubation in either HS or PS; all were in stationary phase and were sensitive to the bactericidal activity of PS.
Fig. 2. Electron micrograph of a PMN after incubation with *H. influenzae* in the opsonization assay (strain DC33; pooled serum). Phagocytosis (small arrow) and intracellular degradation (large arrow) was associated with bacterial killing. The bar marker represents 1 μm.

**Animal studies**

Of 21 5-d-old rats, 15 showed bacteraemia 48 h after intranasal inoculation of DC15. This rate of bacteraemia is similar to the rate of bacteraemia after intranasal inoculation of strain E₁ (Roberts & Smith, 1980).

**DISCUSSION**

Serum bactericidal activity has been used to screen selected populations for immunity to *H. influenzae* type b. Several assumptions are implied: all type b *H. influenzae* are homogeneous and can be represented by a single reference strain; phagocytosis is relatively unimportant for *in vivo* bacterial clearance; and the phase of growth is unimportant in the assay. We sought to examine these assumptions.

Of our isolates, one NP isolate (DC15), was resistant to the bactericidal activity of serum from an immunized adult and to pooled human serum. Strains DC 15-S, DC15-E, DC33-E, DC37-S, DC37-E (all NP isolates) were resistant to the bactericidal activity of pooled normal adult serum. Only one of the four CSF isolates, C668, was resistant, and in exponential phase only. Thus, systemic humoral protection against invasive disease may be directed against a sub-population of *H. influenzae* that has a propensity to invade. However, the *in vivo* relationship between the source of the isolate and invasiveness is unclear; DC15, an NP isolate, was as virulent in 5-d-old rats as E₁, a CSF isolate.

One potential variable in bactericidal assays is growth phase of the test bacteria, where assayed. Stationary phase *E. coli* were less susceptible to the bactericidal effect of normal human serum in comparison to exponential phase organisms (Davis & Wedgwood, 1965). Our results indicate that the opposite is true for *H. influenzae*. Stationary phase *H. influenzae* were as sensitive or more sensitive than bacteria in exponential phase by both humoral and phagocytic killing. An exception is strain DC37 which had greater resistance to phagocytosis in pooled
serum during stationary phase than during exponential phase. The enhanced resistance to humoral and phagocytic killing during exponential growth may be due to an increased ability to repair antibody–complement mediated damage. Alternatively, our observation of the increased binding of IgM on stationary phase organisms suggests that antigens may be exposed on stationary phase organisms which are not exposed during exponential growth. The presence of an increased number of minor outer membrane proteins in stationary phase has been demonstrated (Loeb & Smith, 1980). The growth phase of organisms in studies of serum bactericidal activity of *H. influenzae* has not been consistent. Some investigators (Fothergill & Wright, 1933; Norden, 1970) used stationary phase organisms (18 h cultures) while others used mid-exponential phase bacteria (Anderson *et al.*, 1971).

Another variable in bactericidal assays of *H. influenzae* is the 'resistant' phenotype of the organisms. A dialysable heat-stable factor in weanling rat serum allowed broth-grown organisms to acquire resistance to serum non-capsular bactericidal activity (Shaw *et al.*, 1976). This factor is also present in dialysates of human serum (Anderson *et al.*, 1980). We confirmed the presence of this factor in weanling rat serum using two serum-sensitive strains (C616 and E1); however, weanling rat serum did not detectably enhance the resistance of an exquisitely serum-sensitive strain (C562) to human serum bactericidal activity. Further, we could not detect an increase in the resistance of the remaining strains after preincubation in heat-inactivated pooled human serum. After preincubation in rat serum, pooled human serum, or dialysate of human serum, none of the organisms tested showed enhanced resistance to the bactericidal activity of serum from an immunized adult. This suggests that the phenotypic change induced by preincubation, which increases resistance to human serum bactericidal activity is strain-dependent. This phenotypic change is undetectable when organisms are tested against serum which contains bactericidal activity greater than pooled serum from normal adults.

'Natural' human antibodies of the IgG class reactive with somatic antigens of Gram-negative bacteria were detected better by immunofluorescence than by bactericidal assays (Cohen & Norins, 1968). However, natural bactericidal and opsonizing antisomatic antibodies against *E. coli* and *Salmonella typhosa* are principally of the IgM class (Gitlin *et al.*, 1963). We detected the presence, by immunofluorescence, of IgG on each *H. influenzae* strain whether harvested in exponential or stationary growth phase, after incubation with either HS or PS. IgM antibodies were more frequently detected on stationary phase organisms (*P* = 0.001) than on exponential phase organisms after incubation with PS. Since *H. influenzae* release capsular material during transition from exponential phase to stationary phase, these IgM antibodies may be directed against newly exposed somatic antigens (Anderson *et al.*, 1976). However, there was no relationship between IgM binding and serum bactericidal activity. There was also no difference in the binding of antibody by CSF or NP isolates. The greater bactericidal activity of the sera of one of us (A.L.S.) is undoubtedly due to repeated immunization: 100 µg PRP was injected seven years prior to harvesting of the serum. In addition he was exposed to aerosols of strain E1, and has manipulated litre quantities of stationary phase cultures of at least 100 other type b strains. These situations afford ample opportunity for an immune response to *H. influenzae*.

Intracellular survival of *H. influenzae* type b isolate was demonstrated in CSF-derived leucocytes, minimizing the importance of phagocytosis in bacterial killing (Fothergill *et al.*, 1937). Clearance of *H. influenzae* type b from the blood of rats suggested that reticuloendothelial phagocytosis could account for the elimination of the majority of the inoculated bacteria (Weller *et al.*, 1978). In our study, all strains killed in the bactericidal assay were also killed with the addition of phagocytes. The addition of phagocytes increased the killing of two of six strains which were resistant to the bactericidal activity of pooled serum; however, the only serum-resistant CSF isolate, C668-E, was also resistant to phagocytosis. Our in *vitro* data suggest that antibody and complement may be adequate for killing most strains by a bacteriolytic mechanism, but that phagocytosis is necessary for killing certain isolates. Alternatively, a serum concentration greater than 1:6 (that used in our assay) may kill other *H. influenzae* isolates by bacteriolysis.

Strain dependent susceptibility of *H. influenzae* type b to the bactericidal activity of normal adult human sera has been noted by others. In one study, only 2 of 16 mid-exponential phase
strains were killed by serum from six adults (a 50% reduction in number of viable organisms) (Norden, 1972). In another study, 11 of 20 adult sera differed more than twofold in their bactericidal titre against two mid-exponential phase type b strains (Anderson et al., 1971). Both of these studies used CSF isolates. One of their strains (b-Eagan) is identical to our reference strain E; except for high level streptomycin resistance, selected in a single step. We found greater variability in the susceptibility of different strains to bacteriolysis with mid-exponential phase organisms in comparison to those harvested from stationary phase.

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