Variable Susceptibility of *Hemophilus influenzae*, Type B Strains to Serum Bactericidal Activity[1] (36076)

CARL W. NORDEN[2]
(Introduced by H. A. Feldman)
Department of Medicine, University of Rochester, School of Medicine and Dentistry, Rochester, New York 14620

Resistance to systemic infection with *Hemophilus influenzae*, type B (HIB) has been associated with serum bactericidal activity for the organism (1). While performing tests for such antibodies to estimate their prevalence, we observed different strains of HIB to vary in their susceptibility to the bactericidal action of the same serum. The present study reports the results of experiments to determine the degree of heterogeneity of HIB in bactericidal assays.

Methods. Sera were diluted serially in Dulbecco's phosphate buffered saline (DPBS) in U-shaped microtiter plates (Microbiological Associates). To 0.05 ml of serum (undiluted or diluted) in each well was added 0.025 ml of human complement (undiluted serum) from a donor with hypogammaglobulinemia. This complement contained 120 units of activity (normal 80–160) and had no bactericidal activity against all HIB strains tested.

All HIB strains had been isolated from the cerebrospinal fluids of children with meningitis and had been typed by agglutination with specific antiserum. Multiple samples of a broth culture grown from a single colony of each organism were frozen at —70°. For each test, a thawed culture was inoculated into Levinthal's broth and incubated for 18 hr; a fresh broth subculture was incubated for 4 hr in a 37° water bath. This was diluted 1:1000 in DPBS and 0.025 ml (about 7×10^3 colony forming units) was added to each well.

Immediately after the addition of bacteria, and again after 1 hr of incubation at 37°, the contents of each well were streaked onto chocolate agar plates with a standard milk loop calibrated to deliver 0.001 ml. The plates were incubated overnight at 37° in 5% carbon dioxide. A colony count reduction of 50% or greater was required for bactericidal activity to be recorded as positive; the coefficient of variation for colony counts from a single well was 15%. All sera were tested in duplicate and their titers were reproducible within a single twofold serum dilution. Each set of tests included complement and bacterial controls.

Absorption of sera with bacteria. The bacteria in 50 ml of a 4 hr broth culture were killed by the addition of 0.5% formalin, washed three times in DPBS, and then suspended in 0.6 ml of DPBS. To each milliliter of serum was added 0.2 ml of killed bacteria; after mixing at 37° for 30 min, the bacteria were removed by centrifugation. This procedure was repeated twice more prior to testing for bactericidal activity.

Absorption of sera with PRP. A solution (5 μg/ml) of polyribose phosphate (PRP),[3] prepared from the capsular material of *H. influenzae*, type B, was added to human type O red blood cells (RBC) and shaken for 0.5 hr at 37°. After centrifugation it was washed five times in PBS and then concentrated to a 50% suspension of RBC. Equal volumes of sensitized RBC and serum were shaken together for 45 min and then centrifuged. The RBC were removed and the process was repeated a second time prior to testing for bactericidal activity.

[1] This investigation was supported by PHS/GRSG Research Grant No. FR 05403 and by a Center for Disease Control Grant CC 00536.

[2] Present address: Montefiore Hospital, 3459 Fifth Avenue, Pittsburgh, Pa. 15213.

[3] Kindly provided by Drs. David Smith and Porter Anderson, Children's Hospital, Boston, Massachusetts.

TABLE I. Susceptibility of *H. influenzae*, Type B Strains to Human Serum Bactericidal Activity.

HIB strains	Effect of serum samples	
	A, B, C	D, E, F
1–14	Killed[a]	Not killed
15, 16	Killed[a]	Killed[a]

[a] Titers of 1:2 to 1:8.

Preparation of antisera. New Zealand white rabbits (2–3 kg) were injected intravenously with gradually increasing doses of a formalin killed 4 hr broth culture of HIB. After 10 such injections on alternate days, the rabbits were rested for 5 days, bled, and their sera were stored at —70°.

Results. Bactericidal susceptibility of different strains. Sixteen HIB strains were tested with six sera obtained from healthy adults aged 21–39 years (Table I). Three sera (A–C) killed 14 strains in dilutions of 1:2 to 1:8. These same 14 strains were not killed by either undiluted or diluted sera from donors D, E and F. In contrast strains 15 and 16 were killed by all six sera at dilutions of 1:2 to 1:8.

TABLE II. Effects of Absorption with Either Whole Bacteria or PRP on Human Serum Bactericidal (HIB) Activity.

Serum	Absorbed with	HIB activity vs strain	
		1	15
A	0[a]	+[b]	+
	Strain 1	—	—
	Strain 15	—	—
	PRP[c]	—	—
B	0	+	+
	Strain 1	—	—
	Strain 15	—	—
	PRP	+	+
C	0	+	+
	Strain 1	—	+
	Strain 15	—	—
	PRP	+	+

[a] Not absorbed.

[b] + = killed; — = not killed.

[c] Polyribose phosphate prepared by Smith and Anderson from capsular material of *H. influenzae*, type B.

Absorption of human sera with whole bacteria and with PRP. In several experiments in which sera A, B, and C were absorbed with either strain 1 (killed only by these three sera), strain 15 (killed by all six sera) or PRP sensitized erythrocytes, it was found that the bactericidal actions of sera B and C against both strains were unaffected by absorption with PRP, although PRP completely removed bactericidal activity from serum A (Table II). Absorption of sera A and B with either strain 1 or 15 completely removed bactericidal activity against either strain. The bactericidal activity of serum C was consistently removed against strain 1 by absorption with strain 1 or 15 and against strain 15 by absorption with strain 15. The bactericidal titer of serum C against strain 15 was incompletely reduced from 1:4 or 1:8 to 1:2 by absorption with strain 1, but never to 0 as in the other absorption experiments.

TABLE III. Effect of Absorption with Either Whole Bacteria or PRP on Rabbit Antisera Bactericidal (HIB) Activity.

Serum	Absorbed with	HIB activity vs strain	
		1	15
Anti-HIB 1	0[a]	2048[b]	2048
	Strain 1	<4	<4
	Strain 15	<4	<4
	PRP[c]	64	1024
Anti-HIB 15	0	8192	4096
	Strain 1	<4	32
	Strain 15	<4	<4
	PRP	64	256

[a] Not absorbed.

[b] Reciprocal of bactericidal titer.

[c] Polyribose phosphate prepared by Smith and Anderson from capsular material of *H. influenzae*, type B.

Absorption of rabbit antisera with either whole bacteria or PRP. Sera from unimmunized rabbits consistently failed to kill strain 1, but regularly killed strain 15 in titers of 1:8 to 1:32. The results of absorbing antisera from rabbits immunized with strain 1 or 15 are shown in Table III. The bactericidal activity of antiserum 1 against either strain was completely absorbed by both strains 1 and

15. In contrast, antisera 15 was completely absorbed by its homologous strain, but absorption with strain 1 only incompletely reduced its activity against strain 15. Absorption of both sera with PRP sensitized erythrocytes markedly reduced the bactericidal titer against strain 1, but less effectively reduced the titer against strain 15.

Discussion. It has been shown that 2 of 16 HIB strains differed significantly from the other 14 in their susceptibilities to the bactericidal activities of human and rabbit sera. All strains had ample capsular material as identified by agglutination reactions. Additionally, it was found that bactericidal activity of human sera reacted differently to absorption with either whole bacteria or capsular material.

These data suggest that there may be other antigenic characteristics among HIB strains, perhaps similar to those reported for group C meningococci (2). The present study suggests that one (A) of the three human sera used in the absorption experiments had its major bactericidal activity to the capsular polysaccharide since it was removed by treatment with PRP. In contrast, sera B and C were unaffected by such treatment suggesting that their bactericidal activities are directed at antigens other than the polysaccharides. What other antigenic site(s) might be the target(s) of bactericidal antibodies in sera B and C is not identifiable from the present data. Mpairwe (3) has suggested that bactericidal antibody to *H. influenzae,* type B may be directed at somatic rather than capsular antigens. Hopefully, further absorption studies may resolve these questions.

What is clear from these studies is that further testing of serum bactericidal activity must consider these variations in the susceptibilities of different HIB strains. Were strain 15 to be used in a prevalence survey, then essentially 100% of individuals tested would be expected to have bactericidal antibodies; quite different results would have obtained with strains 1 through 14. Such comparative surveys can yield valid results only if defined HIB strains are employed.

Summary. Sixteen strains of *Hemophilus influenzae,* type B were used for bactericidal assays with sera from six healthy adults. Two strains were killed by all six human sera and by normal rabbit sera while the other 14 strains were killed only by three human sera. Absorption experiments suggested a diversity of antibodies among the human sera, since treatment with capsular material removed bactericidal activity from one, but left it unaffected in two others. These findings are important for the evaluation of surveys of the prevalence of *H. influenzae,* type B bactericidal antibodies.

I am grateful to Phyllis Meyers and Linda Witinski for their excellent technical assistance and to Dr. Harry A. Feldman for advice and critical review of the manuscript.

1. Fothergill, L., and Wright, J., J. Immunol. **24**, 273 (1933).

2. Gold, R., and Wyle, F., Infec. Immunity **1**, 479 (1970).

3. Mpairwe, Y., J. Med. Microbiol. **4**, 85 (1971).

Received July 29, 1971. P.S.E.B.M., 1972, Vol. 139.