

# Human Serum Activities against *Hemophilus influenzae*, Type b

PORTER ANDERSON, RICHARD B. JOHNSTON, JR., and DAVID H. SMITH

*From the Divisions of Infectious Diseases and Immunology, Children's Hospital Medical Center, and the Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115*

**ABSTRACT** Humoral immunity to *Hemophilus influenzae*, type b was studied in normal human adults by means of assays for serum bactericidal and opsonizing activities against the organism and for passive hemagglutinating activity using erythrocytes sensitized with polyribophosphate, the type-specific capsular antigen. Hemagglutinating activity was detectable in about 60% of the 114 sera tested. Serum bactericidal and opsonizing activities were found in all sera tested; the levels in some sera, however, were quite low. The antibacterial activities were due not only to antibodies directed against the polyribophosphate capsule but also to antibodies that appear to be directed against somatic antigens. Type b strains differed in their susceptibility to the antisomatic antibodies of particular sera but were uniformly sensitive to anticapsular antibody.

## INTRODUCTION

*Hemophilus influenzae* may be isolated with or without an amorphous polysaccharide capsule. All encapsulated strains belong to one of six serologic types (1), whose distinguishing polysaccharides have been chemically characterized (2-4). Type b strains, whose capsule has been identified as polyribophosphate (PRP),<sup>1</sup> are responsible for almost all the life-threatening childhood

infection caused by this species (5). Meningitis, the most common of these, occurs predominantly in children  $\frac{1}{2}$ -3 yr of age. Fothergill and Wright observed that in vitro bactericidal activity against a type b strain was seldom detectable in the blood of children in this age range but was generally present in neonates, older children, and adults, who very rarely have this disease (6). Ward and Wright found that the type b bactericidal activity of fresh blood in vitro was due mainly to antibody and complement with only a slight activity being attributable to antibody-dependent phagocytosis (7). Fothergill and colleagues thus suggested that the critical factor in resistance is bactericidal antibody—acquired first transplacentally, then actively, with an intervening period of susceptibility. A role for antibody-enhanced phagocytosis in resistance, however, was suggested from the observation that serotherapy of meningitis apparently increased the extent of phagocytosis by leukocytes in the cerebrospinal fluid (8). Thus, although opsonization of *H. influenzae* has not been systematically studied, the mechanism of specific immunity has come to be viewed as some combination of the complement-dependent bacteriolytic and the opsonic activities of antibody (8, 9).

Wright and Ward observed that serum bactericidal activity against their type b strain was inhibited by its culture filtrate but not by the filtrate of an unencapsulated derivative of this strain, and they suggested that bactericidal antibody was directed against the capsule ("precipitinogen"). Antiserum to the unencapsulated derivative agglutinated the derivative and its encapsulated parent to an equal extent but was bactericidal only to the former (10). Moreover, blood of children in the susceptible age range readily killed the derivative while failing to kill the parent strain (6). These results suggested that anticapsular antibodies are the key to specific humoral immunity and that antibodies to somatic antigens are unimportant for host resistance. Such a conclusion was supported by Alexander's observations that

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Dr. Johnston's present address is Department of Pediatrics, University of Alabama Medical Center, Birmingham, Ala. Dr. Smith is the recipient of Career Development Award AI-20376 of the National Institute of Allergy and Infectious Disease.

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<sup>1</sup> Abbreviations used in this paper: AGS, agammaglobulinemic serum; BC, bactericidal; PBS, phosphate-buffered saline; PHA, passive hemagglutination; PRP, polyribophosphate.

TABLE I  
The Effect of PRP Immunization on Serum Bactericidal Activity against Several Strains of *H. influenzae*

Strain	Isolation			Reciprocal bactericidal titer		
				Before	After	After (+PRP)*
b-Aquila	Alabama	1970	MEE	2	160	2
b-Madigan	Boston	1970	CSF	<2	160	<2
b-Malerba	Boston	1970	CSF	<2	160	<2
b-DeJesus	Cape Cod	1970	CSF	2	160	2
b-Weckbacher	Boston	1969	CSF	<2	160	<2
b-Eagan‡	Boston	1968	CSF	<2	160	<2
b-Rabinowitz	New York	Pre-1945	CSF	8	160	8
b-62S	Boston	1936	CSF	16	160	16
b-Madigan-u	(mutant of b-Madigan)			16	16	16
b-Rabinowitz-u	(mutant of b-Rabinowitz)			8	8	8
b-62S-u	(mutant of b-62S)			64	64	64
U (untypable)-1	New York	1954	CSF	32	32	32
a-Cascia	New York	1946	NA	8	8	NT
c-Ruggiero	New York	NA	NA	32	32	NT

MEE, middle ear exudate; CSF, cerebrospinal fluid; NA, not available; NT, not tested.

\* Serum preincubated with PRP.

‡ Source of PRP for immunization.

the efficacy of rabbit anti-*H. influenzae* b serum in serotherapy depended upon its content of anti-PRP antibody (11, 12). Tunevall, on the other hand, obtained a somatic antigen preparation common to untypable strains and all encapsulated types and found that the age incidence of complement-fixing antibodies to this preparation was very similar to that of the type b bactericidal activity observed by Fothergill and Wright (13). The contribution of such antibodies to resistance against type b infection, however, has not been evaluated, and the view has prevailed that immunity is attributable to anti-PRP antibody (8, 9).

The present study was intended to refine the methodology for serum bactericidal activity and make it an assay for bactericidal antibody per se (the Fothergill-Wright method may stress a complement component), to examine the relation of the bactericidal and opsonizing activities of serum and the role of anticapsular and anti-somatic antibodies in these activities, and to survey the distribution of the activities in the adult (immune) population.

## METHODS

*H. influenzae* strains. The strains employed and the clinical source, date, and place of their isolation are listed in Table I. Strains b-Eagan, b-Weckbacher, b-Madigan, and b-Malerba were kindly donated by Mr. K. Beckett; strain b-Aquila, by Dr. V. Howie; strain b-DeJesus, by Dr. M. Pendleton; strains b-Rabinowitz and untypable-Ramirez (henceforth designated U-1) by Miss Grace Leidy; and

strains b-62S, a-Cascia, and c-Ruggiero by the Massachusetts Institute of Biological Laboratories. The typing of the strains was confirmed by slide-agglutination at room temperature, and they were preserved by lyophilization in skim milk.

Strains b-62S-u, b-Madigan-u, and b-Rabinowitz-u are unencapsulated mutants that arose as nonmucoid sectors of colonies of their respective parent strains. The mutants produce about 1/1000 the level of PRP of the parent strains as determined by hemagglutination-inhibition assays of culture supernatants or sonic extracts of cells. By this technique, which is sensitive to as little as 1/10,000 the level of PRP characteristic of type b strains, the untypable strain U-1 produces no detectable PRP.

Isolates of *H. influenzae* b vary greatly in the rate at which unencapsulated mutants occur in culture in vitro (14). The encapsulation of strain b-Eagan is genetically very stable, and therefore it has been employed as an assay standard.

*Culture methods.* Bacteria were grown at 37°C on BHI-DB medium (brain-heart infusion plus 1/1000 volume each of supplements D and B added after autoclaving.) Supplement D is  $\beta$ -diphospho-pyridine nucleotide (grade III, Sigma Chemical Co., St. Louis, Mo.) dissolved in distilled water at 1 mg/ml and sterilized with a 0.45  $\mu$  Millipore membrane filter; supplement B was made by adding 1 volume of defibrinated horse blood to 2 volumes of distilled water, clarifying by 30 min of centrifugation at 20,000 *g* at 4°C, and filter sterilizing. These supplements were stored at -20°C and retained activity for at least 6 months. The medium was solidified with Difco agar, 2% for plates and 1% for dilution trays. Phosphate-buffered saline (PBS), employed as a diluent, contained 8.1 g NaCl, 0.157 g NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, and 1.3 g Na<sub>2</sub>HPO<sub>4</sub>·H<sub>2</sub>O/liter (pH 7.3 at 25°C).

To prepare bacteria for the bactericidal or opsonization assay, a rehydrated lyophile culture was streaked on agar and incubated overnight. A colony was suspended in PBS, typed, and inoculated to an initial optical density (OD) of about 0.01 (Lumetron colorimeter, 490 nm filter [Photovolt Corp., New York]), into 10 ml of liquid medium in a 125 ml Erlenmeyer flask. The culture was incubated with vigorous shaking until the OD increased to about 0.2 and was chilled 15 min in an ice water bath; its OD was then read to determine the appropriate dilution. The viable count of the chilled culture was approximately  $4.0 \times 10^8$ /ml per OD unit and remained constant for up to 8 hr; the culture, however, was always used within 2 hr.

**Sera.** Sera for the bactericidal or passive hemagglutination assays were stored at  $-20^\circ\text{C}$  and thawed-refrozen several times if necessary. (The titers were not changed thereby.) Sera for the opsonization assay served as their own complement source and therefore were stored at  $-70^\circ\text{C}$  in small portions thawed only once. The same handling was given the serum used as the complement source for the bactericidal assay. This was obtained from an X-linked agammaglobulinemic volunteer bled just before his prophylactic gamma globulin injections; each batch was tested to establish the absence of antibody activity. The human anti-PRP serum (also referred to as the "postimmunization serum") was obtained from an adult volunteer after immunization with 55  $\mu\text{g}$  of purified PRP. The immunization and properties of the antigen preparation are described in the accompanying paper (15). Rabbit anti-*H. influenzae* typing sera were obtained from the Massachusetts Institute of Biological Laboratories.

**Passive hemagglutination assay.** A modification of the technique of Keogh, North, and Warburton (16) was employed. Unfixed human Rh<sup>-</sup> type O erythrocytes were washed three times in PBS and stored (up to 4 wk) in Alsever's solution at  $4^\circ\text{C}$ . For antigen sensitization the cells were suspended at 1.2% (v/v) in PBS containing 0.25  $\mu\text{g}$  PRP/ml, incubated 1 hr at  $37^\circ\text{C}$ , washed three times to remove unabsorbed antigen, and suspended in PBS at 0.6%. (The optimal PRP concentration was 0.125–0.5  $\mu\text{g}/\text{ml}$ ; higher concentrations slightly depressed titers. The binding of PRP to the cell surface was stable—sensitized cells could be stored at  $4^\circ\text{C}$  for at least 4 days without loss of sensitivity.) Serial twofold dilutions (25  $\mu\text{l}$ ) of test serum in PBS containing 0.25% bovine serum albumin were prepared in U-bottom microtiter trays (type TS-MRC-96; Linbro Chemical Co., New Haven, Conn.) with the use of microdroppers and microdiluters (Cooke Engineering Co., Alexandria, Va.), and 25- $\mu\text{l}$  drops of cell suspension were added. As a control for nonspecific agglutination, each serum was diluted in duplicate with one series receiving sensitized and the other unsensitized cells. The trays were sealed, thoroughly shaken, and incubated 3 hr at  $37^\circ\text{C}$ . Agglutination was read by tipping the tray to a vertical position and observing for several minutes: a pile of unaggregated cells flowed smoothly downward in a stream, whereas aggregated cells adhered to the bottom or fell together as a clump. Cells from different type O, Rh<sup>-</sup> donors gave similar but not identical results; all assays reported below were thus done with cells from the same donor. In repeated assays the reciprocal titer of the standard human antiserum was always within one twofold dilution of 320. When cells fixed with glutaraldehyde (17) were substituted for unfixed cells, comparable titers were obtained with most sera. Many sera, however, were found to nonspecifically agglutinate the fixed

cells (but not the unfixed cells obtained from the same donor).

Hemagglutination-inhibition assays for the detection of PRP were performed by adding 25- $\mu\text{l}$  drops of test solution or control solutions with known concentrations of purified PRP to 25- $\mu\text{l}$  serial dilutions of rabbit anti-*H. influenzae* b serum. These mixtures were incubated 30 min at  $37^\circ\text{C}$ , 25- $\mu\text{l}$  drops of PRP-sensitized cells were added, and the assay was completed as described above.

**Serum bactericidal assay.** Serial twofold dilutions (25  $\mu\text{l}$ ) of test serum were made in 1 ml microtiter trays (type 96 SC, Linbro Chemical Co.) held in an ice water bath; the diluent was PBS containing 5% bovine serum albumin, 0.5 mM  $\text{MgSO}_4$ , and 0.15 mM  $\text{CaCl}_2$ . To each well was added a 25  $\mu\text{l}$  drop of a mixture consisting of 11/20 diluent, 1/4 agammaglobulinemic serum, and 1/5 PBS suspension containing approximately 400 bacteria. The trays were closed, shaken, and incubated 1 hr at  $37^\circ\text{C}$ ; 0.8 ml of agar (at  $45^\circ\text{C}$ ) was then added with thorough mixing. After 18 hr of further incubation at  $37^\circ\text{C}$ , individual colonies were readily visible in the agar, and the end point was taken to be the highest dilution producing a reduction in colony count readily detectable by eye (about 50%).

**Higher sensitivity bactericidal assay.** Sera with undetectable or equivocal activity at 1/2 dilution in the regular bactericidal assay were tested as follows. The chilled culture was centrifuged (10 min at 10,000  $g$  at  $4^\circ\text{C}$ ), and the cell pellet was resuspended in PBS before dilution. Test sera were heated 30 min at  $56^\circ\text{C}$  to remove internal complement activity. A mixture of 90  $\mu\text{l}$  serum and 10  $\mu\text{l}$  of PBS containing about 400 bacteria was incubated in a small, corked test tube for 1½ hr at  $28\text{--}30^\circ\text{C}$ , and then chilled to  $0^\circ\text{C}$ . 15  $\mu\text{l}$  of agammaglobulinemic serum was added, and after mixing, a 30  $\mu\text{l}$  sample was spread on agar to establish a 0 min count. The tubes were then incubated at  $37^\circ\text{C}$ , and samples were spread at 30 and 60 min. The coefficient of variation for colony counts was 12%. This technique of sampling was also used in calibration of the dilution assay and determining the kinetics of bactericidal activity.

**Opsonization assay.** A previously described spectrophotometric method (18) was calibrated for use with *H. influenzae*. The chilled bacteria were washed once in PBS and suspended in veronal buffer, pH 7.35, ionic strength 0.065 (19) to a concentration of  $1.5 \times 10^8$  viable bacteria/ml. The final reaction mixtures consisted of 0.4 ml of 0.2% nitro blue tetrazolium,  $2.5 \times 10^8$  peripheral blood phagocytes suspended in approximately 0.1 ml buffer, 0.1 ml of bacterial suspension, and the specified volume of fresh serum—with volumes being equalized with buffer. All components except the phagocytes were assembled at  $4^\circ\text{C}$  and incubated at  $37^\circ\text{C}$  for 10 min. Phagocytes were added, and the mixture was incubated at  $37^\circ\text{C}$  for 30 min. The reaction was stopped with HCl, and the phagocytized dye was extracted and quantitated as described (18). Results of duplicate or triplicate assays for all test sera were averaged and expressed as  $\Delta$  OD at 515 nm, a value obtained by subtracting the OD achieved by the phagocytosis of bacteria without serum (usually about 0.060) from that achieved in the presence of the test serum.

**Inhibition of antibody activity by PRP.** Sera were incubated 5 min at  $37^\circ\text{C}$  and then 1 hr at  $0^\circ\text{C}$  with 1/100 volume of a 10 mg/ml solution of PRP. This procedure provided an excess of antigen without detectable anticomplementary effect.

**Absorption of sera with the untypable strain U-1.** The bacteria were grown to saturation in liquid medium, killed

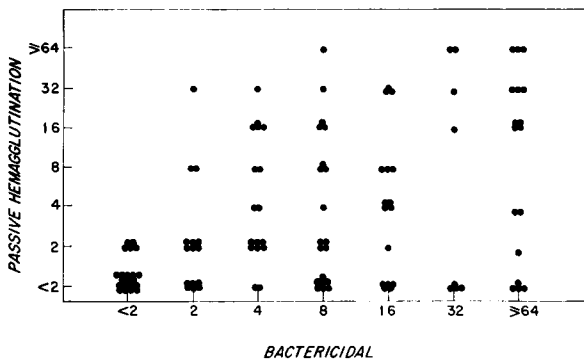


FIGURE 1 Correlation of passive hemagglutination and bactericidal activity against *H. influenzae* b, strain Eagan in normal adult sera.

and fixed by the addition of formaldehyde (0.4% final) for 15 min at 37°C, centrifuged, and washed twice in PBS. Serum, with complement inactivated, was mixed with the washed bacteria (about  $5 \times 10^{10}$  per ml of serum), incubated 1 hr at 37°C, and separated from the bacteria by centrifugation; its sterility was checked on BHI-BD agar.

## RESULTS

### Properties of the bactericidal (BC) assay

**Kinetics.** In the absence of an antibody source, the viable count increased about 60% by 1 hr. With bactericidal antibody present, samples taken before incubation at 37°C had the input number of viable cells; upon incubation the count fell exponentially. With an antibody concentration about twofold the detectable minimum, the count dropped to 17% by 10 min, 3% by 20 min, and 1% by 45 min at 37°C.

**Antibody concentration and end point.** With anticapsular antibody the full range of activity was generally achieved over a fourfold range of serum concentration: dilutions giving  $\leq 2\%$  survival were followed by one giving 20–80% followed by dilutions giving the control number. With antibody directed against non-capsular antigens the concentration-activity range was broader, about eightfold, and the dilution giving survival nearest 50% was taken as the end point.

**Bacterial concentration and end point.** An identical end point was obtained when the standard anti-PRP human serum was tested using  $4 \times 10^2$ ,  $4 \times 10^3$ , or  $4 \times 10^4$  bacteria. Thus the limiting factor in this range appears to be antibody concentration rather than the antibody/bacterium ratio.

**Complement requirement.** Agammaglobulinemic serum (AGS) was tested in twofold dilutions with and without a source of antibody (a slight excess of heat-inactivated standard antiserum). With type b strains, killing in the presence of antibody was detectable up to a dilution of 1/16 AGS; the AGS requirement for untypable strains was much lower. When substituted for AGS in

the assay against b strains, serum specifically deficient in C3 (about 15% of normal; see reference 20) was inactive below a dilution of 1/2. Thus type b bactericidal activity requires at least the first four components of complement.

### Anti-*H. influenzae* b activities of normal adult sera

Bactericidal activity against strain b-Eagan was surveyed in the sera of 114 normal adults; each serum was also titered in the PRP-specific PHA assay. The correlation of these two activities is shown in Fig. 1. 21 sera were negative in BC, 44 negative in passive hemagglutination (PHA), and 16 negative in both assays at a dilution of 1/2. A moderate positive correlation between the two was found; the relationship, however, was not reciprocal, i.e. 20 sera with BC titers of 8 or greater were negative in PHA, whereas BC activity was always found if the PHA titer was 4 or greater.

Finding that about one-fifth of the adult sera lacked bactericidal activity against type b was contrary to the expectation from the Fothergill-Wright study; these sera were therefore tested in an assay made more sensitive by increasing the serum concentration and by washing the bacteria to remove potentially antibacterial materials. Under these conditions all 21 sera were able to kill strain b-Eagan.

Fresh sera from normal adults were tested for their ability to promote the phagocytosis of viable cells of strain b-Eagan. Results for the sera of four representative normal adults and one child with congenital agammaglobulinemia are illustrated in Fig. 2. The extent of phagocytosis increased with the serum concentration until a maximum was achieved with 0.15–0.2 ml. Enhancement of phagocytosis (opsonization) was least by the agammaglobulinemic serum. Among the four normal sera opsonization was greatest by that serum

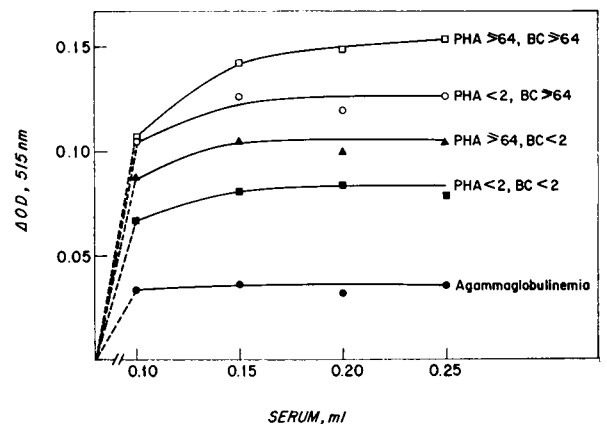


FIGURE 2 The opsonization of *H. influenzae* b, strain Eagan by normal and agammaglobulinemic sera.

TABLE II  
*The Effect of Adsorption with the Untypable Strain U-1 on Serum Bactericidal Activity against Strains b-Rabinowitz and b-62S*

Strain	Serum	Reciprocal bactericidal titer	
		Unabsorbed	Absorbed
b-Rabinowitz	Preimmunization	8	<2
	Postimmunization	160	160
b-62S	Preimmunization	16	<2
	Postimmunization	160	160
U-1	Preimmunization	32	<2
	Postimmunization	32	<2

with a high titer in both the BC and PHA assays and lowest by the serum in which both titers were <2. Intermediate opsonization values were found for the sera with a high titer in either the BC or PHA assay; opsonization correlated somewhat better with BC than with PHA activity, but it could not be exclusively related to either. This correlation was found to hold for the additional 20 normal sera tested in the opsonization assay.

#### The antigen specificity of serum bactericidal activity to *H. influenzae* b

Whether PRP-directed antibody can be bactericidal was examined directly by comparing titers of an individual before and after immunization with purified PRP. (In the PRP-specific PHA assay the reciprocal titers of the pre- and postimmunization sera were <2 and 320, respectively.) Table I shows that the preimmunization serum had generally low and variable activity against the eight tested b strains. Immunization with PRP elicited identical high titers to all eight; it did not, however, change the activity against a type a and a type c strain, a nontypable strain, and three PRP-deficient mutants of b strains. An identical specificity was demonstrated by preincubation of the anti-PRP serum with PRP: activity against b strains was reduced to preimmunization levels but that against other strains was not affected (Table I, last column).

Moderate activities against strains b-Rabinowitz and b-62S, however, were present before immunization and were not removed from the postimmunization serum by PRP. A direct indication that these activities were due to antibodies to non-PRP antigen(s) was provided by employing the untypable strain U-1, which makes no detectable PRP. Adsorption with U-1 reduced the activity against b-Rabinowitz and b-62S from the preimmunization serum but did not reduce the titers of the postimmunization serum (Table II).

The role of antibodies to PRP and non-PRP antigens in the type b bactericidal activity of normal sera was examined by preincubation with PRP (Table III). Bactericidal titers against strain b-Eagan were reduced fourfold or more by PRP in only 3 of 20 randomly selected sera. (These three had relatively high titers in the PRP-specific PHA assay.) Titers against b-Rabinowitz were not reduced, even in the sera of high PHA activity. The concentration of PRP employed was about 100-fold that necessary to inhibit completely the PHA activity of any of the tested sera. Thus the predominant bactericidal antibodies appeared to be non-PRP directed. Moreover, b-Eagan and b-Rabinowitz differed in susceptibility to these antibodies: titers against the two strains differed fourfold or more in about two-thirds of the sera.

14 of these 20 sera were absorbed with U-1 and then tested with or without PRP incubation against b-Rabinowitz. Three general patterns were discerned and are illustrated for three representative sera in Table IV: (a) U-1 reduced the activity, and the residual activity was inhibited by PRP—found in 8 of 14 sera; (b) U-1 completely removed the activity—found in 3 of 14; (c) U-1 partially removed the activity, and the residual activity was not inhibited by PRP—found in 3 of 14. Thus the bactericidal activity of normal sera may be

TABLE III  
*Effect of Preincubation with PRP on the Bactericidal Activity of Normal Sera*

Serum and its PHA titer	Bactericidal titer against strain			
	b-Eagan		b-Rabinowitz	
	-PRP	+PRP	-PRP	+PRP
1 (32)	4	<2	8	4
2 (16)	4	<2	8	8
3 (8)	8	2	64	64
4 (8)	16	16	64	64
5 (4)	<2	<2	32	32
6 (4)	64	64	32	32
7 (4)	2	<2	16	16
8 (4)	4	4	64	64
9 (2)	8	8	32	32
10 (2)	<2	<2	8	8
11 (2)	8	4	16	8
12 (2)	4	4	8	8
13 (<2)	4	4	32	16
14 (<2)	2	2	2	<2
15 (<2)	64	64	16	16
16 (<2)	4	4	32	32
17 (<2)	8	8	8	8
18 (<2)	16	16	16	16
19 (<2)	<2	<2	8	8
20 (<2)	8	8	16	16

TABLE IV

The Effect of Absorption with Strain U-1 upon the Inhibition by PRP of Bactericidal Activity of Normal Sera against Strain b-Rabinowitz

Serum* and its PHA titer	Reciprocal bactericidal titer			
	Unabsorbed		Absorbed on U-1	
	-PRP	+PRP	-PRP	+PRP
3 (8)	64	64	8	<2
16 (<2)	32	32	<2	<2
9 (2)	32	32	8	8

\* Serum number refers to Table III.

directed against PRP, non-PRP antigens, or both. Sera of type (a) had relatively high PHA activity, while types (b) and (c) did not. Thus if non-PRP antibodies are removed, a fairly good correlation between PHA and PRP-inhibitable bactericidal activities is seen. The partial removal by U-1 of bactericidal activity against b-Rabinowitz from type (c) sera suggests that the relationship between non-PRP antigens of these two strains, although close, is not identical.

#### The antigen specificity of opsonization

Table V compares the effect of absorption with PRP on the opsonization activity of the anti-PRP serum and a normal serum representative of those in which bactericidal activity was not PRP inhibitable. The activity of the former was reduced nearly to its preimmunization value, while the latter was barely affected. It thus appears that opsonization of type b strains, like bactericidal activity, may stem from antibodies both to PRP and non-PRP antigens.

### DISCUSSION

With a particular technique and test strain of *H. influenzae*, type b, Fothergill and Wright in 1932 found bactericidal activity in all of 29 tested subjects over the age of 10 yr (6). Norden, Callerame, and Baum in a very recent survey, employed a slightly different method and

TABLE V

The Effect of Preincubation with PRP on the Opsonization of Strain b-Eagan

Serum	Opsonization, OD at 515 nm	
	-PRP	+PRP
Anti-PRP, 0.2 ml (BC titers: -PRP = 160, +PRP = <2)	0.253	0.117
Normal, 0.2 ml (BC titers: -PRP = 16, +PRP = 16)	0.116	0.108

different test organisms, and found no detectable bactericidal activity in the serum of 8 of 29 normal adults (21). The present study found considerable variation among 114 normal adult sera in activity against strain b-Eagan (Fig. 1); all, however, were found to have at least some activity when a sufficiently sensitive technique was applied. In a normal serum, the titer against strain b-Eagan often differed considerably from that against strain b-Rabinowitz (Table III). Thus, the outcome of a population survey to ascertain the presence or absence of activity will depend not only upon the population studied but also upon the sensitivity of the assay technique and the type b strain employed.

The issue of what proportion of the normal population have what levels of bactericidal antibody to *H. influenzae* b is germane only if the hypothesis of Fothergill and colleagues is true—that such antibody is a critical determinant of host resistance. As evidence on this question, surveys of the activity of normal populations against an arbitrary test strain are very indirect. The ideal direct approach would be testing a significant number of preinfection sera of patients against their own infecting strain. This approach was used by Goldschneider, Gotschlich, and Artenstein to identify a protective role for antibodies against meningococcal infection. They collected sera prospectively from a large number of military recruits entering a high risk environment and found that the titers of bactericidal antibody correlated inversely to the subsequent incidence of systemic infection (22). Prospective studies are in progress for *H. influenzae* (23), but the absence of identified high risk situations and the impediments to obtaining sera from normal infants will make it difficult to assemble the number of specimens needed to attain statistical significance. Testing early acute sera of infected patients is a practical alternative, although the possibility of an insidious onset of infection may make the interpretation of the results less certain. Such data are being accumulated by this laboratory and will be reported in a subsequent communication. The success of serotherapy, while demonstrating that large doses of antiserum could clear an established infection, does not prove that serum antibody is a necessary factor in preventing invasion by *H. influenzae* b. Evidence for such a role is found, however, in the clinical experience with patients with X-linked agammaglobulinemia. These patients have a high incidence of infections caused by *H. influenzae* b (and other pyogenic bacteria), which are largely prevented by prophylactic gamma globulin (24).

Although the hypothetically protective antibodies are detectable by serum bactericidal activity in vitro, they might well protect the host by some other mechanism. Alexander observed that passive immunization with rabbit anti-*H. influenzae* b serum markedly increased the

numbers of bacteria found in phagocytes in cerebrospinal fluid smears. She proposed that the capsule of *H. influenzae* b, like that of many Gram-positive pathogens, is antiphagocytic and that opsonization by anti-PRP antibody might play a role in passive immunity (8). To explore whether this mechanism may have a role in active immunity, the opsonizing as well as bactericidal activities of human sera were studied. All of the 24 normal adult sera tested promoted the phagocytosis of strain b-Eagan to an extent much greater than the sera from three children with X-linked agammaglobulinemia. The opsonization activities of these sera correlated fairly well with their bactericidal titers against the same organism. The two systems, however, are not readily comparable in quantitative terms. In assays with some of the sera, the concentration of bactericidal antibody (and complement) was adequate to have killed the bacteria in the absence of phagocytes; with other sera, however, phagocytosis was promoted by concentrations of antibody insufficient for serum bactericidal activity. Antigenic experience indeed increases the ability of the serum to enhance phagocytosis of *H. influenzae* b in vitro, but the relative importance to human immunity of this mechanism and of the serum bacteriolytic reaction will be difficult to establish.

Any conclusion about the PRP specificity of type b bactericidal activity depends, of course, upon the antigenic purity of the material used for immunization or inhibition experiments. The preparation employed here appeared pure by the chemical and immunochemical criteria applied (15). Immunization induced high bactericidal titers against all eight b strains tested but not against the untypable, a and c strains, nor the PRP-deficient mutants of three of the b strains (Table I). The insensitivity of these mutants to the postimmunization serum is evidence against the possibility that the type b bactericidal antibody is actually induced by another type-specific antigen contaminating the PRP preparation. Were this the case, the contaminating antigen would, by coincidence, have to be lost from b strains in the same mutational event that produces the PRP deficiency. Thus, the increase in bactericidal titers by immunization and the inhibition of activity from some normal sera by the addition of PRP confirm the inference of Wright and Ward (10) that anticapsular antibody can sensitize type b strains to the bactericidal action of complement.

A substantial fraction of normal adult sera, however, had moderate type b bactericidal activity in the absence of detectable (PRP-specific) PHA activity (Fig. 1). Moreover, preincubation with PRP under conditions eliminating PHA activity did not significantly reduce bactericidal activity in most of the 20 normal sera tested (Table III). On the basis of these data alone it

could be hypothesized that bactericidal activity not inhibited by PRP was due to low affinity anti-PRP antibodies not active in passive hemagglutination. In 11 of 14 tested sera, however, activity against strain b-Rabinowitz was reduced by absorption on the PRP-negative strain U-1. It thus appears that antigens of b strains other than PRP (and common to untypable strains) are also accessible to the bactericidal action of antibody and complement and that the corresponding antibodies are quite common in the normal adult population. The opsonization of b strains, like serum bactericidal activity can apparently be performed by antibodies both to PRP and non-PRP antigens. Antigens common to untypable and all encapsulated types of *H. influenzae* have indeed been isolated (13, 25, 26), but their chemical identities and morphologic relationships are not well characterized.

Antigens of *H. influenzae* other than the type-specific polysaccharides are conventionally called somatic. The use of this term for the antigen(s) revealed in the present study should imply no particular structural arrangement. Mutation to PRP deficiency in two of the three b strains increased sensitivity to non-PRP-directed bactericidal antibody (compare b-Madigan with b-Madigan-u, etc.—Table I). This result is consistent with the possibility of masking of the somatic antigens by PRP, but a more specific model is not warranted by the present data. Rabbit hyperimmune antiserum to intact cells of strain b-Eagan when reacted in gel diffusion with a sonic extract of b-Eagan gave only one faint precipitin line in addition to the major line formed with PRP. Conceivably, the finding of Alexander, Heidelberg, and Leidy that PRP absorption removed the protective power of rabbit anti-*H. influenzae* b antisera (12) stemmed from the inactivity of somatic antigens in the immunizing preparations.

Strains b-Eagan and b-Rabinowitz, although equally sensitive to the bactericidal action of anti-PRP antibody (Table I), differed in susceptibility to the non-PRP-inhibited antibodies in normal sera (Table III). It would thus appear that the two strains differ in their content or arrangement of somatic antigens. Chandler, Fothergill, and Dingle observed that the unencapsulated mutants derived spontaneously from different type b strains were antigenically heterogeneous when examined by agglutination (14). Conceivably some of the antigens responsible for such diversity are accessible to bactericidal antibody even while the PRP encapsulation is intact. Indeed, such a relationship has been demonstrated for the meningococcus: serum bactericidal activity to group C strains stems not only from antibodies to the group-specific polysaccharide capsule but also from antibodies to a number of other antigens heterogeneously distributed within the groups (27, 28). One could speculate that bactericidal activity to a

particular strain of *H. influenzae* b might arise from contact with an antigenically related untypable or non-b typable strain; the resulting activity, however, might not apply to a b strain of different antigenic structure. In contrast, the eight tested b strains were uniformly sensitive to the antibodies elicited by PRP (Table 1). Thus if serum bactericidal activity is critical for resistance to infection, anti-PRP antibody would presumably protect against any b strain.

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