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Research Article

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The Paradox of *Hemophilus influenzae* Type B Bacteremia in the Presence of Serum Bactericidal Activity

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ABSTRACT We investigated the role of serum bactericidal activity in *Hemophilus influenzae* type b infections in infants with meningitis and in a rat model. In infected infants, 13/22 admission sera had bactericidal activity against the infecting strain, and bacteremia was as frequent in those with bactericidal activity (54%) as in those without (56%). The coexistence of bactericidal activity and bacteremia was reproduced and studied in experimentally infected weanling rats. Serum from such rats kills in vitro 95% of conventionally broth-grown bacteria within 10 min, but does not kill organisms obtained from the infected animals. Thus bactericidal activity as conventionally determined for *H. influenzae* b may have no relevance in vivo. Incubation of broth-grown bacteria in normal rat serum for 30 min at 37°C produces a resistance like that of in vivo organisms. This phenotypic conversion depends on factors that are of molecular weight less than 1,000, stable to 100°C, but destroyed by ashing.

When injected intravenously into nonimmune animals, broth-grown bacteria are quickly cleared, while serum-preincubated bacteria are not. The latter, however, are cleared when injected into bacteremic rats (half-life 30 min). Bacteremia in the rats may persist despite this capacity for clearance because bacteria are entering the blood from extravascular fluids, which contain greater than 90% of the total bacterial burden.

INTRODUCTION

In 1933, Fothergill and Wright studied the bactericidal (BC)¹ activity of whole blood from individuals of vari-

ous ages against *Hemophilus influenzae* type b (H.i.b). They demonstrated the lowest activity in infants from 2 mo to 3 yr of age, the period when children were most likely to develop H.i.b meningitis (1). From these data and supplemental studies showing that antibody and complement could account for the cidal action of blood to H.i.b in vitro, they inferred that susceptibility to H.i.b might be due to a deficiency of BC antibody. Since then, BC assays against H.i.b have been used to study population immunity (2-5), immune response to infections (6, 7), and to vaccines (8, 9), with the belief that BC activity "inhibits bacteremia and dissemination" (10). There is no direct evidence, however, for the physiologic significance of serum BC activity against H.i.b. Assessing host immunity by BC assays has revealed inconsistencies: the incidence of infection is low in adults and newborns but in some surveys a sizeable proportion of these age groups lack detectable BC activity (3, 5). Feigin et al. found BC activity in the serum of some patients very early in the course of H.i.b infection, inferring that preexisting BC activity may not protect the host from invasion (3). However, the titers were determined against a single survey strain; since strains differ in susceptibility to BC antibodies, the BC activities observed may not have been active against the infecting strains.

The present study examines the significance of BC activity. Sera obtained on the day of hospital admission from 22 patients with H.i.b meningitis were assayed against the respective infecting strains. In some of the patients bacteremia was found to coexist with homologous BC activity. This observation prompted the examination of the phenomenon in an animal model (11). In weanling rats infected with H.i.b, bacteremia coexisted several days with an elevated BC titer. One possible cause of this apparent paradox is that H.i.b from the animals' blood was less susceptible to killing in the BC assay than the same strain grown in conventional me-

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¹Abbreviations used in this paper: BC, bactericidal; CSF, cerebrospinal fluid; H.i.b, *Hemophilus influenzae*, type b; PBS, phosphate-buffered saline; PRP, polyribophosphate.

dium. The basis for this variation in phenotype was explored. Additional understanding of the paradox was gained through study of the clearance of bacteria from the blood and seeding of the bacteria into the blood from extravascular foci.

METHODS

Patients. The study population consisted of 22 infants and children with culture-proven H.i.b. meningitis from whom sera were available from the same admission blood samples cultured to determine bacteremia. The ages were 2–124 mo with a median of 16 mo. The patients had had febrile illness 1–14 days before admission; 8 had been treated with antibiotics. All received ampicillin therapy and all recovered. Convalescent sera were obtained from 19 of the 22 children, 14–30 days after the admission.

Media. Brain-heart infusion broth or agar (BioQuest Div., Becton, Dickinson & Co., Cockeysville, Md.) was supplemented as previously described (4). Phosphate-buffered saline (PBS) was previously described (4); when used as a bacterial diluent it was supplemented with 0.1% protein (bovine serum albumin in PBS-A or gelatin in PBS-G) and used at 0–4°C.

H.i.b. strains. All cerebrospinal fluid (CSF) isolates were received from the clinical bacteriology laboratory as the first subculture after primary isolation. These were streaked on agar and incubated overnight at 37°C. Typing was confirmed by slide agglutination, and the remainder of the second subculture was suspended in sterile skim milk and frozen at –70°C. A fresh (third) subculture was obtained by streaking the milk suspension on agar and incubating overnight at 37°C. The strains used in animal experiments were a meningeal isolate (E), and a mutant of E (E-1) selected in a single step for resistance to 500 µg streptomycin/ml (11).

BC activity of human sera against infecting strains. The titers were determined against bacteria from exponential broth cultures inoculated from the third subculture plate as described (4). The complement source was serum of calves denied colostrum (Bacterial Products Div., Bureau of Biologics, U. S. Food and Drug Administration, Washington, D. C.). Penicillinase (BioQuest Div.) was added at 1,000 U/ml to destroy ampicillin. As a control for nonimmunologic bactericidal effects, each serum was tested without complement and excluded from the study if positive.

Preparation of bacteria for experiments with rats. Broth-grown bacteria were used in exponential phase. In vivo-grown bacteria were obtained by inoculating 10⁸ broth-grown organisms intraperitoneally into a weanling rat, decapitating the rat 24 h later, and rinsing the peritoneal cavity with 1 ml of PBS-G. Broth- or in vivo-grown bacteria were immediately chilled to and maintained at 0°C, then diluted with PBS-A or PBS-G and used 10–60 min after chilling. “Washed” bacteria were prepared by centrifugation at 9,000 g for 2½ min at 4°C in an Eppendorf model 3200 centrifuge (Brinkman Instruments, Inc., Westbury, N. Y.), decanting the supernate, and resuspending in PBS-A or PBS-G. Bacteria were “serum-incubated” by incubating washed broth-grown bacteria in normal rat serum at densities up to 2 × 10⁶ bacteria/ml at 37°C for 30 min. (Bacterial suspensions were incubated in closed sterile 1-ml Eppendorf centrifuge tubes unless otherwise noted).

Procedures with rats. Outbred pathogen-free COBS/CB rats originally obtained from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.) were bred as previ-

ously described (11). Pups were weaned and inoculated at 21 days of age. Bacterial suspensions for inoculation were chilled, washed, diluted, and injected (0.1 ml) with a no. 25 gauge needle into a tail vein or into the peritoneum. An exception was the study of intravenous clearance in bacteremic animals, when the serum-incubated bacteria were maintained in the serum at 37°C for inoculation.

Blood for culture was obtained by lacerating the tail artery. Quantitative cultures of suspected foci of infection were performed after rapid exsanguination by cardiac puncture without anesthesia. Organ homogenates were cultured as described previously (11) with the exception that before homogenization the organs were washed with chilled PBS-G. Recovery of bacteria from body fluids was achieved as follows: peritoneal fluid, the peritoneal cavity was rinsed with 10 ml PBS-G; intraocular fluid, the contents of the globe were expelled and mixed vigorously with 1 ml of PBS-G; joints, fluid was aspirated from all erythematous joints and mixed with 1 ml PBS-G; CSF, the cranial vault (together with dura and arachnoid) was dissected off and the meningeal surfaces rinsed with 1 ml PBS-G. The total number of bacteria in each suspension was calculated by multiplying the total volume times the density of bacteria cultured from an aliquot. The animal's blood volume was assumed to be 10% of the body weight. The density of CSF infection was determined by plating a sample of the fluid film covering the meninges (before rinsing).

Rat serum preparations. Serial samples for BC assay were obtained by collecting tail artery blood in plain microcapillary tubes. Other samples were collected by rapid exsanguination by cardiac puncture without anesthesia. To assure complement preservation, all bloods were chilled immediately after clotting and centrifuged at 4°C; the serum was stored in 2-ml aliquots at –70°C. Complement activity was destroyed, where indicated, by incubation at 56°C for 30 min.

Pooled normal rat serum was derived from 20 normal weanling rats; its complement activity was destroyed before use. An exception was that the 100 ml of serum analyzed by ultrafiltration was obtained from 15 normal adult rats anesthetized with ethyl ether and used with complement preserved. “Convalescent serum” was a pool from 15 rats bled 20 days after intraperitoneal inoculation with 10⁸ H.i.b. “Reference serum” was a pool collected from 20 weanling rats 5 days after intraperitoneal inoculation with 10⁸ H.i.b.; bacteremia of greater than 5 × 10³ per milliliter was verified in these rats 10 h before bleeding. For single use standards, reference serum was thawed and 0.050 ml samples frozen with a dry ice bath in sterile 1-ml Eppendorf tubes, which were then stored at –70°C.

Serum was absorbed with bacteria by incubation for 1 h at 0°C with washed, broth-grown organisms at 10⁷–10¹⁰ per ml; the bacteria were removed by five centrifugations and sterility verified by culture. Serum was adsorbed with the capsular polysaccharide of H.i.b. (PRP; polyribophosphate) by mixing 0.050 ml of serum with 0.005 ml of a 1 mg/ml solution of PRP in water and incubating for 30 min at 37°C; because previous studies indicated no interference with the bactericidal activity of complement by this procedure (4), no effort was made to separate antigen-antibody complexes. The unabsorbed sera were incubated equivalently with water. Purified anti-PRP antibody prepared by column immunoabsorption of serum from a rabbit hyperimmunized with H.i.b. was provided by Dr. R. O'Reilly.

Normal rat serum was ultrafiltered (Model 202, Amicon Corp., Scientific Systems Div., Lexington, Mass.) at 4°C under nitrogen at 50 pounds per square inch with an Ami-

con UMO5 filter until 76% of the original volume had been filtered. The retentate was washed by twice restoring it to its original volume with PBS and repeating the ultrafiltration. 5 ml of filtrate was ashed and then reconstituted with sterile water. The pH of the filtrate and the reconstituted ash were each adjusted to 7.4 with 0.2 N HCl.

BC activity in rats. Dilution titers were determined as for human sera (4), except the end point was read by visual estimation of the highest dilution reducing the control number by 90%. Where noted, broth-grown bacteria were replaced by a comparable suspension of washed *in vivo*-grown bacteria.

Bacterial survival in reference serum or in freshly-drawn rat blood (anticoagulated with 1 U heparin per milliliter) was assayed as follows: 1 part of the bacterial suspension (10^3 – 10^8 bacteria/ml) was added to 10 parts of fluid and incubated in a stationary water bath at 37°C. Initially, and at intervals thereafter, the suspension was mixed and samples removed for determination of viable counts. Results were expressed as percentage of the initial viable count.

Kinetics of acquisition of bacterial resistance to BC activity. Broth-grown bacteria were washed and diluted 1:20 in PBS-A, and 0.010 ml was added to 0.10 ml broth or 0.10 ml normal rat serum. Each suspension was incubated at 37°C for 30 min, chilled on ice for 6 min, mixed with 0.90 ml PBS-A, washed, and resuspended in 0.1 ml PBS-A. A sample of 0.020 ml of the broth-incubated suspension was added to 0.20 ml of normal rat serum, and this mixture was divided and incubated at 0°C or 37°C. At intervals, samples were taken for viable counts and for assay in reference serum. Conversely, 0.020 ml of the normal serum-incubated bacteria were added to 0.20 ml broth; this suspension was divided, incubated at 0°C or 37°C, and assayed as described.

Assay of enhancement of resistance to BC activity. To 0.10 ml of each test sample was added 0.010 ml of PBS that contained 1% bovine serum albumin, 10 mM CaCl_2 , and 5 mM MgCl_2 . Then 0.010 ml of a 1:10 dilution of washed broth-grown bacteria in PBS-A was added, and the tube was mixed, incubated at 37°C for 30 min, and chilled to 0°C for 6 min. A sample was then assayed for bacterial survival in reference serum. Serial twofold dilutions of complement-inactivated normal rat serum in PBS were used as standards; PBS was used as a control. A standard curve was constructed by plotting bacterial survival vs. serum dilution. For each test sample, the concentration of normal serum producing the same bacterial survival was quoted as that sample's specific activity. The filtration coefficient, k , for the resistance-enhancing substance was calculated from the formula $k = \ln(cf)/\ln(vf)$ where vf = the fraction of the original volume present in the filtrate, and cf = the fraction of the substance which appears in the filtrate; derivation of this formula assumes that the concentration of the substance in the fluid being filtered at any instant is a constant fraction k of that substance's concentration in the retentate.

Measurement of PRP and anti-PRP antibody. Serum antibodies to PRP were titrated by radioantigen binding (12). The content of soluble PRP in the serum-incubated or broth-incubated *H.i.b* cultures (approx. 10^6 /ml) was estimated by adding formaldehyde to 0.4%, holding the suspension for 5 days at room temperature, centrifuging, and assaying the supernate for PRP by radioantigen-binding inhibition (12).

Statistical methods. The statistical significance of data was tested by *t* test and chi-square test with Yates' correction.

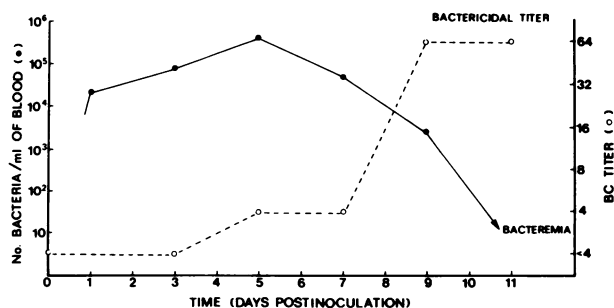


FIGURE 1 The time course of *H. influenzae* bacteremia and serum bactericidal titers in a weanling rat. Data is representative of a nonimmune rat after intravenous injection of 10^6 organisms on day zero. The arrow indicates that organisms were not present at the limit of detection (10^2 /ml).

RESULTS

Antibody activity and bacteremia in humans with *H.i.b* meningitis. Data on the 22 patients studied are given in Table I. On admission, bacteremia was demonstrated in 12 of the 22 patients. BC activity against the strain recovered from the CSF was demonstrable in the sera of 13 patients but not in the remaining 9, even in serum dilutions as low as 1:1.2. The prevalence of bacteremia was similar in patients with BC activity (54%) and those without (56%) ($P > 0.9$). The highest admission BC titer observed was 1:8, in a bacteremic patient.

Bacteremia was somewhat less frequent with short preceding illness, with prior antibiotic therapy, and when anti-PRP antibody was demonstrable, but these differences were not statistically significant. Antibiotic pretreatment was more common in the nonbacteremic patients (50%) than in the bacteremic ones (25%) ($P \approx 0.6$). Antibody activity to the capsular polysaccharide PRP, determined by radioantigen binding, generally was undetectable or very low in the admission sera, but was found more frequently in the nonbacteremic patients (55%) than in the bacteremic ones (14%) ($P \approx 0.3$). Excluding the atypical patient with prolonged treated illness (no. 8), the duration of preceding illness was generally longer in the bacteremic patients (mean = 3.8 days) than in the nonbacteremic patients (mean = 2.0 days) ($P \approx 0.15$).

Coexistence of BC activity and bacteremia in weanling rats. Bacteremia (greater than 100 organisms per milliliter) persisted in five weanling rats for at least 7 days after intravenous inoculation of 10^6 organisms. Each animal developed a BC titer of at least 1:4 by the 5th day postinoculation; thus each had a period of at least 2 days during which BC titers of at least 1:4 coexisted with bacteremia (Fig. 1). When the inoculum size and route of administration was 10^6 bacteria given intraperitoneally, BC titers reached $\geq 1:16$ by day 5 (five of five animals) and bacteremia persisted at least 2 days

TABLE I
Antibody Activity and Bacteremia in Children with H.i.b. Meningitis

Case	Preadmission		Data on admission blood sample			
	Days ill	Anti-biotics*	BC titer†	Anti-PRP titer ng/ml	H.i.b. cultured	Convalescent BC titer‡
1	4	—	8	ND§	+	64
2	1	—	2	ND	+	2
3	7	Am, Cf	1.2	<7.0	+	ND
4	2	—	1.2	ND	+	ND
5	7	—	1.2	<7.0	+	4
6	2	—	1.2	60	+	2
7	3	P	1.2	ND	+	1.2
8	14	Am	1.2	34	—	2
9	3	—	1.2	59	—	64
10	4	Am, P	1.2	<7.0	—	2
11	1	—	1.2	34	—	8
12	3	Am	1.2	ND	—	1.2
13	1	—	1.2	<7.0	—	4
14	1	—	<1.2	<7.0	+	2
15	1	—	<1.2	<7.0	+	1.2
16	7	—	<1.2	<7.0	+	2
17	7	Am, P	<1.2	<7.0	+	<1.2
18	3	—	<1.2	ND	+	<1.2
19	3	Am	<1.2	18	—	8
20	1	Am	<1.2	<7.0	—	1.2
21	1	—	<1.2	12	—	>64
22	1	—	<1.2	<7.0	—	ND

* Am, ampicillin; Cf, cephalothin; P, penicillin.

† Against patient's CSF isolate.

§ ND, not determined.

thereafter. The latter method and the 5th postinoculation day were chosen for further study of the coexistence of bacteremia and BC activity; animals so prepared are hereafter called "bacteremic" rats.

A difference between broth-grown and in vivo bacteria. Strain E-1 bacteria grown in broth were added to blood obtained from bacteremic rats (i.e., with endogenous strain E) and the survival of both bacterial populations was examined during incubation at 37°C. The endogenous bacteria survived and grew (Fig. 2). In contrast, there was a two-log decrease in the viable count of the broth-grown bacteria. To rule out possible strain-dependent effects, the experiment was repeated with reversal of strains, and the results were similar. After a 30-min incubation the mean survival (four experiments) was 130% for endogenous bacteria and 0.8% for broth-grown bacteria. The possibility that the endogenous bacteria survive in vivo because they are intracellular was excluded by recovery of greater than 95% of the circulating bacteria from cell-free plasma.

Bacterial survival was then studied during in vitro incubation in serum from bacteremic rats (hereafter called "reference serum"); bacteria harvested from the peritoneal cavity ("in vivo-grown" bacteria) were used in place of the endogenous bacteria, which had been removed by centrifugation. As in the studies with whole blood, the in vivo-grown bacteria survived (mean of 120% in four experiments), while the broth-grown bacteria were killed within 30 min (mean survival 2%). Thus, in vivo-grown and broth-grown bacteria differ in their susceptibility, and reference serum can be a reagent for differentiating the two. The designation "resistant" was applied to bacterial populations, such as those grown in vivo, which survived in reference serum. The designation "sensitive" was applied to populations, such as those grown in broth, of which greater than 95% were killed by reference serum. The possibility that the viable count of sensitive bacteria decreased in reference serum because of agglutination (13) was ruled out: the percentage survival was con-

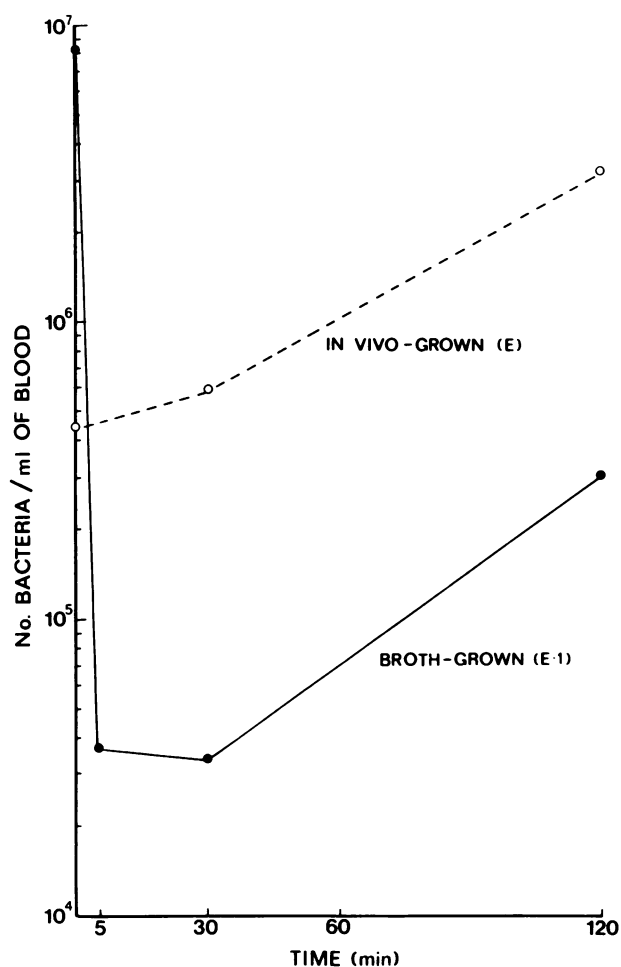


FIGURE 2 Differing survival of phenotypic variants of *H. influenzae* in blood from bacteremic rats. A weanling rat was inoculated intraperitoneally with 10^8 *H. influenzae* (strain E, open circles). 5 days later the infected blood was obtained by cardiac puncture and broth-grown bacteria (strain E-1, closed circles) were added; the mixture was then incubated at 37°C. Viable counts of strains E and E-1 were differentiated by plating on media with and without streptomycin at 500 μ g/ml.

stant (1.9–2.6%) over a wide range of bacterial concentrations (10^5 – 10^8 /ml), and no clumping was visible on microscopic examination of gram-stained smears of the incubation mixtures. Control studies showed that both antibody and complement were required for the killing of sensitive bacteria: the titer of reference serum was abolished by absorption with the homologous strain, and activity was eliminated by heating but restored with an antibody-free fresh serum. The susceptibility of sensitive (in vitro-grown) and resistant (in vivo-grown) bacteria to various antiserum preparations was then compared. Normal serum (Table II, no. 1) had no activity against either. Antibody directed against the capsular antigen PRP (no. 6) had equal activity against

TABLE II
The Activity of Different Antisera Preparations against "Resistant"* and "Sensitive"† Bacteria

Source	Preincubation with§ PRP	vs. Resistant* bacteria	vs. Sensitive† bacteria
1 Normal animals	—	<4	<4
2 Bacteremic animals¶	—	<4	32
3 Bacteremic animals	+	<4	32
4 Convalescent animals**	—	8	32
5 Convalescent animals	+	8	32
6 Normal animals + anti-PRP‡	—	128	128
7 Normal animals + anti-PRP	+	<4	<4

* Bacteria harvested from the peritoneum of infected rat.

† Bacteria grown in broth in vitro.

§ Sera incubated with PRP before BC assay designated by +, controls indicated by —.

|| Normal 21-day-old rats.

¶ 26-day-old rats, 5 days after infection with H.i.b.

** 41-day-old rats, 20 days after infection with H.i.b.

‡ Same sera as no. 1, supplemented with 1/100 volume of purified rabbit anti-PRP.

both bacterial populations. Bacteremic animals (reference serum, no. 2) had acquired BC activity only against sensitive bacteria, while convalescent animals (20 days after infection, no. 4), had acquired some BC activity against the resistant organisms as well.

Preincubation with PRP did not alter the BC activity in bacteremic or convalescent sera (nos. 3, 5), and anti-PRP antibody as determined by radioantigen binding was undetectable or very low (not tabulated). Thus, antigens other than PRP must account for the susceptibility of both populations of bacteria to these rat sera.

Generation of resistant bacteria in vitro. It was observed that sensitive bacteria became resistant during incubation in normal rat serum. Conversely, resistant bacteria became sensitive during incubation in broth. The kinetics of acquisition and loss of resistance are shown in Fig. 3. Sensitive bacteria were incubated in normal rat serum at 37°C, and samples were transferred into reference serum to determine bacterial survival. Increased resistance was apparent at 6 min and complete by 36 min. In a similar manner, samples were assayed periodically during the incubation of resistant bacteria in broth. Some loss of resistance was observed at 20 min, but loss was not complete until 4 h. Generation time was about 30 min in both media. Thus, bacteria growing in normal rat serum became resistant in approximately one generation time, while bacteria growing in broth lost resistance over several generations.

To explore whether metabolic processes were required for these changes in bacterial phenotypes, the incubations outlined above were also conducted at 0°C. There was very little acquisition of resistance (1% survival rising to 4% survival) during incubation in serum at 0°C for 60 min, and there was no loss of resis-

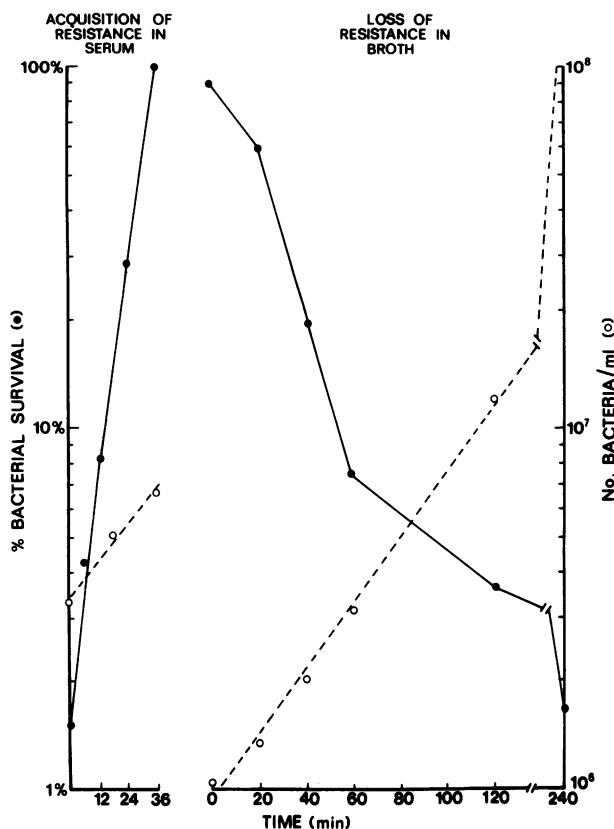


FIGURE 3 The rate at which *H. influenzae* acquires and loses resistance to serum bactericidal activity. The resistance to a bactericidal activity is expressed as the percentage of bacteria that survived a 30-min incubation in "reference serum". Left, resistance and viable counts during the incubation of broth-grown organisms in serum. Right, similar assays during incubation of serum-grown organisms in broth. The points represent the means of two experiments.

tance during incubation in broth at 0°C for 60 min. The resistance acquired by incubation in serum at 37°C was not reduced by three cycles of centrifugation and vigorous resuspension in serum-free medium at 0–4°C. Also, the sensitive and resistant bacterial preparations were compared for content of soluble PRP; both contained 0.1 ng/10⁸ cells as estimated by radioantigen binding inhibition (12).

Attempts were made to characterize the factors in rat serum responsible for the enhanced resistance. It was found that the appropriate diluent in which to assay serum fractions for enhancement of resistance was buffered saline (PBS) containing calcium and magnesium ions and a dilute protein supplement (albumin 0.1%). Broth was not used as a diluent, since it was found to inhibit the acquisition of resistance when mixed with serum. After preliminary studies showed the resistance-enhancing factor(s) to be dialysable, the molecular size was estimated by ultrafiltration through a membrane with a nominal molecular weight cut-off of 500 daltons

(Table III). Recovery of the original activity was 100%. The filtrate, which had 76% of the original volume, contained 60% of the total activity. The filtration coefficient calculated from these data is 0.6, which indicates that the filter is relatively unable to retain the substance(s) in rat serum responsible for enhancing resistance. Moreover, when the retentate was diluted and refiltered as described, its total activity was reduced to less than 10% of the starting material. The activity of the ultrafiltrate was unchanged by heating at 100°C for 30 min but was destroyed by ashing.

In vivo studies. The behavior in vivo of the two different phenotypes of *H.i.b* was first studied in nonimmune animals. Suspensions of sensitive and resistant bacteria were inoculated into weanling rats, and clearance was followed with quantitative blood cultures. Approximately 70% of the sensitive bacteria were cleared in 10 min (Fig. 4). In contrast, there was no detectable clearance of the resistant bacteria. Thus, the phenotypic difference observed in vitro correlated with a difference in the rate of clearance in vivo.

Resistant bacteria were then used to study intravascular clearance in bacteremic rats (i.e., after 5 days of infection with strain E). The animals were injected intravenously with serum-treated strain E-1 and the counts of both the endogenous and the injected bacteria examined. The counts of endogenous organisms remained constant (Fig. 5). In contrast, the number of injected organisms decreased by 95% over 2 h. After slightly accelerated clearance in the first 10 min, the decrease in viable counts of injected organisms was exponential, with a half-life of 25–30 min. Reversal of the strains, i.e., endogenous E-1 and injected E, gave qualitatively similar results; however, the small numbers of Str^s bacteria could not be quantitated among the large numbers of Str^r.

The constancy of the number of endogenous bacteria could be accounted for in either of two ways. It is pos-

TABLE III
The Effect of Ultrafiltration* on the Ability of Normal Rat Serum to Enhance Bacterial Resistance

Solution	Volume†	Specific‡ activity	Total activity
Filtrate	0.76	0.8	0.6
Retentate	0.24	1.6	0.4
Total	1.00		1.0

* Ultrafiltration through Amicon UM05 membrane.

† Volume of solution/total volume.

‡ The activity of the solution or dilutions thereof in making bacteria resistant to the bactericidal activity of serum from bacteremic rats (compared to standard of 1.0 for serum before filtration).

|| Computed as volume × specific activity.

sible that endogenous bacteria eluded clearance because they differed phenotypically in some unknown way from the injected bacteria. Alternatively, both bacterial populations may have been cleared similarly, but the expected fall in viable count of endogenous bacteria was being masked by the continuous entrance of new organisms into the blood from extravascular foci. Evidence for the latter possibility was found by culturing body fluids and tissue homogenates from bacteremic rats. In all eight animals studied, large numbers of bacteria were recovered from body fluids other than blood. In all instances, the bacteria in blood represented less than 7% of the total bacterial burden. Infected extravascular sites included CSF, peritoneal fluid, intraocular fluid, and joint fluid (Table IV). Meningitis was probably present in

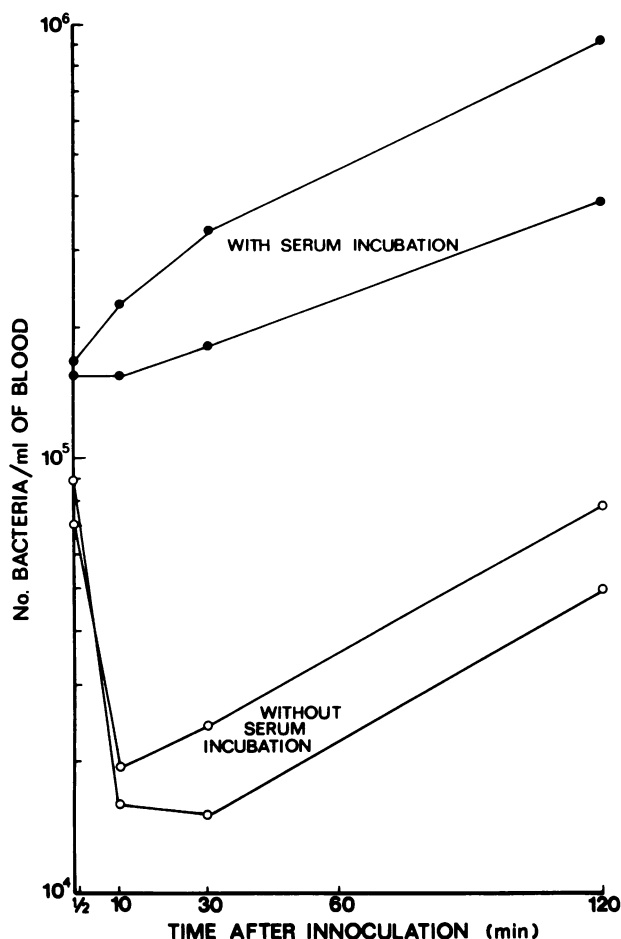


FIGURE 4 The influence of incubation in serum on subsequent intravenous clearance of *H. influenzae* in normal rats. Normal weanling rats were inoculated intravenously with "sensitive" (open circles) or "resistant" (closed circles) phenotypes of *H. influenzae*. Both strains were grown in broth, but the resistant preparation was incubated in normal rat serum at 37°C for 30 min before injection. Each curve depicts a single animal.

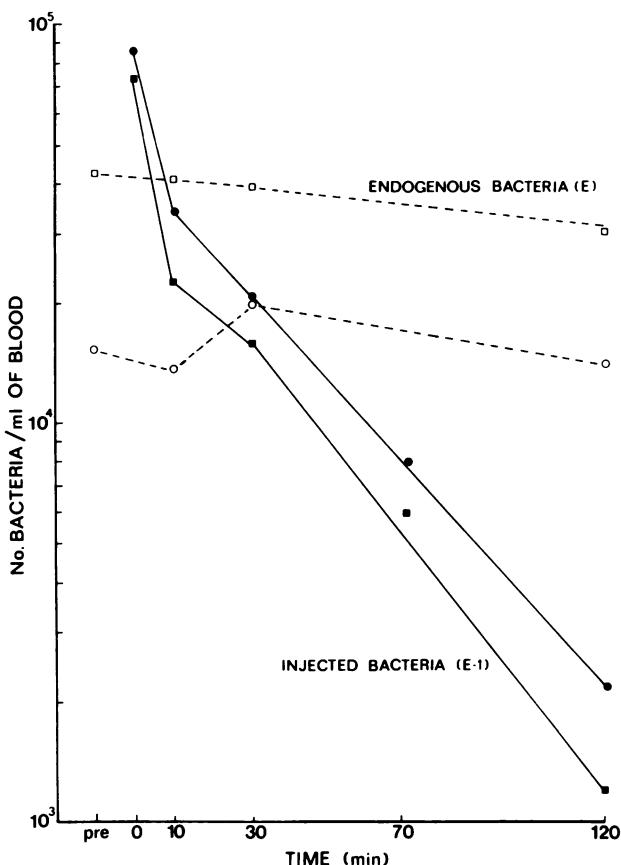


FIGURE 5 The clearance of serum-incubated bacteria in bacteremic rats. A pair of weanling rats was inoculated intraperitoneally with 10^8 *H. influenzae*, strain E. 5 days later serum-incubated strain E-1 was injected intravenously, and the viable count in the blood of the endogenous and the injected bacteria determined.

five of the eight rats which had CSF bacterial densities ranging between 2 and 250 times that in the blood. In two animals the CSF was sterile. In the remaining animal the number of bacteria in the CSF was half that of blood, which might be a reflection of meningitis, or might be an experimental artifact caused by blood contamination.

In liver, spleen, kidney, lung, brain, and muscle, the number of bacteria per gram of homogenized organ was always less than one-half, and usually less than one-fifth of the number in 1 ml of blood. It is probable that this recovery of bacteria reflected the content of bacteremic blood in uninfected organs; however, these data do not rule out the possibility of low density tissue infection.

DISCUSSION

Serum BC activity to H.i.b had been found in acute sera of patients with systemic diseases caused by this species, but the activity was measured against a single

TABLE IV
Bacterial Densities in Body Fluids in Eight Weanling
Rats 5 Days after Intraperitoneal Inoculation
of 10^3 *H. Influenzae*

Fluid	No. of animals with infected fluid	Percent of total bacterial burden*
Blood	8	1.5 (0.2-6)
CSF	6	0.11 (0-0.26)
Peritoneal	6	37 (0-99)
Intraocular	5	60 (0-99)
Joint	1	1.1 (0-9.3)

* Number of bacteria in fluid ($\times 100$)/total cultured from the animal, averaged for the eight animals (range among individual animals).

strain (3). Since isolates of H.i.b may differ in susceptibility to a particular serum, our study was performed with the patients' infecting strains. The data of Table I indicate that bacteremia can occur in the presence of homologous BC activity. This observation implies that such specimens were taken just as antibody was being made or that serum BC activity may not deter bacteremia.

By studying an experimental infection of weanling rats, we found that H.i.b bacteremia and BC activity can coexist several days (Fig. 1). Two phenomena contribute to this apparent paradox: first, the elevated BC titer can be an in vitro artifact due to a difference in bacterial phenotype, and second, the entrance of bacteria into the blood from densely infected extravascular fluids can sustain the bacteremia despite continuous clearance of bacteria from the blood.

It is well documented that organisms grown in vitro may be phenotypically different from those in vivo (14). Increased resistance of in vivo-grown organisms to phagocytosis has been reported for *Brucella abortus* (15). Increased resistance to serum BC activity has been reported for *Neisseria gonorrhoeae* (16) and decreased resistance for *Pasteurella multocida* (17). The present study demonstrates that H.i.b grown in rats or in rat serum are more resistant to host defenses than when grown in broth. When compared in BC assays, the former survive in sera which kill 95% of the latter. When compared in vivo, in intravenous clearance studies, the former survive in normal animals while greater than 70% of the latter are cleared. Since broth-grown organisms are used in the conventional assays of serum BC antibody, the activity observed in vitro may be an artifact which does not pertain to bactericidal activity in vivo. Incubations in broth and in rat serum were identical in respect to a number of variables known to change the susceptibility of a given strain to serum BC activity: growth phase (18), pH (19), temperature

(18), or added drugs (20). The factor(s) in rat serum causing the phenotypic change is not yet identified. The low activity of the washed retentate from serum ultrafiltration, and the filtration coefficient of 0.6 through a membrane with a nominal cutoff of 500 daltons suggests that the active factor(s) is of molecular weight less than 1,000. Thus antibody (21, 22) or exogenous enzymes (23) do not appear to be effecting the transformation. The destruction of activity by ashing suggests that it is not mediated by changes in the nature or concentration of inorganic ions (19, 24).

Newman et al. (25) noted that during BC assays the presence of nutrients adequate for growth made H.i.b more resistant to serum. This does not explain the difference between resistant and sensitive bacteria, since both were growing vigorously before the assay, and both had the same nutrition during the assay. (Rat serum is nutritionally complete for H.i.b). Maaløe (26) noted that the survival of *Salmonella typhimurium* was dependent upon the choice of nitrogen and carbon sources and their concentrations in the media in which bacteria were grown before the BC assay. The susceptibility of H.i.b may be similarly dependent upon the concentration of specific metabolites which vary among complete media.

The mechanism by which the serum factor(s) increases the bacterial resistance is also unknown. With *Shigella dysenteriae* the effect of small molecules on susceptibility to BC activity may be mediated by their adsorption onto the bacterial surface (27). However, with H.i.b the persistence of the enhanced resistance despite washing, and the failure of serum to enhance resistance at 0°C suggest that the change results through metabolism, not from passive adsorption. One possibility was a difference in content of PRP, for mutations reducing its synthesis increase susceptibility of H.i.b to the BC activity of non-PRP-directed antibodies (4), and mutational and phenotypic loss of PRP increases the rate of intravascular clearance in normal rats.² In the present study, however, the preparation of resistant bacteria did not contain more PRP than the sensitive. Thus, the difference in phenotypes is not attributable to differences in bacterial PRP content. However, differences in the surface distribution of PRP were not excluded.

The resistant phenotype hypothetically could resist any stage of the bactericidal sequence. Decreased antibody-binding could result from changes in the surface antigens or in their accessibility to antibody (28). Decreased complement fixation, (independent of antibody-binding) could result if the cell surface became anti-complementary, similar to K antigen function in *Es-*

² Weller, P. F., A. L. Smith, P. Anderson, and D. A. Smith. 1976. *J. Infect. Dis.* In press.

cherichia coli (29). Decreased membrane damage by complement could result from surface alterations which produced complement fixation at a greater distance from the cell membrane (30). Finally, decreased loss of viability could result if altered metabolic activity allowed better repair of complement-mediated damage (31).

If the phenotypic change were one which affected the BC sequence at or after complement fixation, it would be expected to alter bacterial susceptibility to all complement-mediated BC activity. Since the change involves increased resistance to only certain antibodies (reference serum but not anti-PRP), it is probable that the mechanism of resistance enhancement involves a change in antibody binding. One appealing hypothesis is that some substances in rat serum stimulate bacterial production of a material which overlies a critical antigen, A, on the bacterial surface. Consequently, the bactericidal antibody (anti-A) in reference serum no longer binds and kills. Growth in broth would result in dilutional loss of the surface material, concomitant exposure of antigen A, and restored BC susceptibility. This postulated behavior occurs in bacterial mutants which depend on exogenous sugars to produce complete O-side chains (32). When the requisite sugar is added to media lacking it, bacteria elongate their O-side chains and lose their reactivity with certain antibodies. Although we have been unable to demonstrate that similar blocking of antigenic determinants occurs during incubation of H.i.b in serum, all available data are consistent with this hypothesis.

Seeding of extravascular bacteria into the blood perpetuates bacteremia in rats by balancing immune clearance of bacteria. The evidence for this dynamic equilibrium can be outlined as follows: (a) Resistant H.i.b are rapidly cleared from the blood of bacteremic animals ($t_{1/2}$ of 30 min, see Fig. 5). (b) Clearance of circulating bacteria presumably occurs at the same rate. (The incubation in rat serum assured that the injected bacteria would resemble in vivo bacteria. Furthermore, if in vivo bacteria had a phenotype which escaped clearance, cessation of clearance would be expected as the injected bacteria acquired this phenotype; cessation of clearance was not evident during the 2 h of observation). (c) The failure to observe a fall in viable count of circulating organisms could be explained by rapid entrance of bacteria into the blood from extravascular foci. (d) Such extravascular foci were found in bacteremic animals, and contained the majority (> 93%) of bacteria present. (e) Rapid entrance of bacteria into the blood from such foci has been demonstrated in a similar model; in 5-day-old rats, approximately 15% of a peritoneal inoculum was seeded into the blood in 1 h (11). (f) The seeding of about 1% of the extravascular bacteria into the blood each hour would balance the calculated

clearance in the "average" rat, while 10% seeding/h would explain the most extreme case.

From this analysis of the data, the distribution of bacteria in an "average" animal after 5 days of infection would be 10^4 organisms/ml of blood (about 10^5 total intravascular bacteria) and 10^7 organisms present in extravascular fluids (such as peritoneal fluid or intraocular fluid). Of the extravascular bacteria, about 1% (10^5 organisms) migrate into the blood every hour. Concurrently the bacteria in the blood are cleared with a half-life of 30 min, resulting in a clearance of 10^5 organisms/h. The net result of this dynamic flux of bacteria is a constant bacteremia of 10^4 organisms/ml. If seeding from extravascular foci persisted, then even a 100-fold increase in the rate of bacterial clearance (corresponding to a bacterial half-life of 20 s) would still be accompanied by a bacteremia of 10^3 organisms/ml. Thus, sterilization of the blood stream depends not only on rapid clearance of bacteria from the blood, but also on eradication of extravascular foci from which seeding is occurring.

The clearance of resistant bacteria from the blood of bacteremic rats is probably not the result of destruction by circulating phagocytes, since whole blood is unable to kill the endogenous bacteria in vitro. More likely the fixed phagocytes are responsible. Since clearance of resistant bacteria did not occur in nonimmune animals, clearance probably depends on opsonization by specific antibody; however, nonspecific activation of the RE system (phagocytosis independent of antibody) or of the alternate complement pathway have not been ruled out.

It is noteworthy that the location of septic foci in this animal model is principally in fluids (peritoneal, intraocular, cerebrospinal, and joint fluids) rather than tissues. Similarly, in the common human infections with H.i.b bacteria may be recovered in high titer from infected body fluids. This predilection is poorly understood, but probably in such fluids H.i.b, like other encapsulated bacteria, is less susceptible to nonspecific phagocytosis (33).

The relation of the animal model findings to human immunity to H.i.b remain to be established. Immediate questions are whether such a phenotypic change occurs in response to factors in human blood, in relation to human antibodies, and in other or all strains of H.i.b. If the phenomenon were demonstrable, one could ponder whether it has affected the outcome of the various BC assays heretofore employed in human studies. Questions remain about the bactericidal action of antibody and complement: is the mechanism itself important in host defense against H.i.b, and is the in vitro assay a useful index of immunity?

Operation of the bacteriolytic mechanism in vivo has not been established or disproven. From previous ob-

servations that bacteria can be cultured from the blood despite BC activity of the serum against the same strain, the inference was made that "the action of serum in vivo is much less effective than testing in vitro would indicate" (34). The validity of such observations would be enhanced by ruling out phenotypic differences (as in the H.i.b-rat model) that artifactually increase susceptibility in vitro. It is of interest that blood from bacteremic rats was unable to kill in vitro the bacteria that were circulating in vivo (Fig. 2), even though the animals were able to clear comparable bacteria from circulation (Fig. 5). Thus clearance from circulation appears not to require BC activity. The rats, however, eventually developed BC activity effective against in vivo-grown bacteria (Table II, no. 4), so the data do not rule out a contribution of bacteriolysis to eventual resolution of the infection.

BC assays in vitro detect complement-fixing antibody directed against bacterial surface antigens. With *Neisseria meningitidis* such antibodies have been found in prospective studies to correlate strongly with host resistance (35); whether the antibodies act in vivo by bacteriolysis, opsonization, or by some other mechanism, the BC assay is a useful in vitro index of immunity. Since H.i.b infections occur endemically and primarily in infants, such prospective studies are difficult to achieve. Statistical surveys of normal populations have the uncertainty of strain variation, while studies on patients at presentation (such as the present) suffer the uncertainty of how long the patient has been in contact with the bacterial antigens. To these problems it may become necessary to add the complication of phenotypic discrepancies of the type here described, further increasing misgivings about the validity of conventional BC assays as in index of immunity to H.i.b.

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