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Experimental *Haemophilus influenzae* Type b Meningitis: Immunological Investigation of the Infant Rat Model

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Infant rats inoculated intraperitoneally with *Haemophilus influenzae* type b develop bacteremia and meningitis. Rats were infected at 10 to 12 days of age and studied for the development of serum anticapsular antibody and bactericidal and opsonizing activity. Seven and 11 weeks after inoculation, convalescent animals showed a higher frequency of anticapsular antibody responses than uninfected controls, but 35 to 40% of the infected group had undetectable levels of anticapsular antibody (<0.10 μg/ml). In contrast, all of the convalescent animals, but none of the controls, showed moderate titers of serum bactericidal activity; and bactericidal activity persisted after absorption of the convalescent sera with type b capsule. Bactericidal activity was detected primarily in the eluted fraction corresponding to a molecular weight of 150,000 and was present in the offspring of convalescent females. Offspring of convalescent females were protected against challenge with *H. influenzae* type b, and control offspring could also be protected by passive immunization with convalescent serum which lacked detectable anticapsular antibody. Convalescent serum samples efficiently opsonized *H. influenzae* type b, and this activity persisted after absorption of the serum with capsular antigen. These data are consistent with the hypothesis that antibody to the noncapsular surface antigens of *H. influenzae* type b play an important role in host defenses.

Haemophilus influenzae type b (Hib) is the most frequent cause of childhood bacterial meningitis in the United States (16). Previous studies conducted in both humans and other animals have shown the importance of anticapsular (AC) antibody in host defenses against this organism (1, 2, 17, 20, 25). Less information is available concerning other immunological factors, such as antibody to the other surface antigens of *Haemophilus*. The recent failure of the experimental type b polysaccharide vaccine to elicit AC antibody and protect infants under 15 months of age from invasive *Haemophilus* infection (15, 22) emphasizes the need for additional information concerning alternative immunological defenses against this organism.

Infant rats infected intraperitoneally (i.p.) or intranasally with Hib develop bacteremia and meningitis (11, 18, 27). Convalescent sera from surviving animals have been reported to be bactericidal (BC) (27). In a preliminary communication, this laboratory reported that many convalescent animals had low or undetectable levels (<0.07 μg/ml) of AC antibody (11). In contrast, in similar experiments, Myerowitz and Norden reported that uninfected rats and rats surviving neonatal Hib infection both showed age-related increases in serum AC and BC antibody (20).

In other studies of the infant rat model, Smith et al. (27) and Weller et al. (29) reported that rats showed a marked age-dependent susceptibility to *Haemophilus* infection. Older rats were found to be more resistant to infection and cleared type b encapsulated strains from the bloodstream more rapidly than younger animals. The mechanisms responsible for the increased clearance and greater resistance of older rats to *Haemophilus* infection remain undefined, but in these two studies, the age-dependent susceptibility was found to be unrelated to serum BC activity. Furthermore, in our earlier report, we noted that uninfected rats 60 days of age also lacked detectable AC antibody (11). Thus, older rats resist Hib infection despite the absence of serum AC or BC antibodies.

In the studies cited above, the possible role of serum factors which promote phagocytosis of Hib were not investigated. The present investigation was undertaken, in part, to examine the role of serum-opsonizing activity to Hib in experimental infection, and also to resolve some of the discrepancies among the published obser-

vations concerning the BC and AC antibody responses of neonatal rats to *Haemophilus* infection (11, 20, 27).

MATERIALS AND METHODS

Animals. Suckling albino rats from an outbred strain of Sprague-Dawley rats originally obtained from Charles Rivers Laboratory, Wilmington, Mass. were used. Some rats were also obtained from Sprague-Dawley Farms, Madison, Wis., and were used in the third protection experiment and as a source of convalescent sera for the fractionation experiment. Food and water were available ad libitum, and infant animals were maintained with nursing mothers. Infected and uninfected litters were housed separately to prevent cross-infection.

Bacteria. The test organism (b3a) has been described previously (10, 11). It consisted of a type b encapsulated strain obtained from the cerebrospinal fluid of a patient with *H. influenzae* meningitis. The organism was passed three times in 14-day-old rats and stored frozen at −70°C in multiple aliquots of sterile skim milk.

Radioisotope-labeled Hib were prepared by inoculation of a single colony from an overnight growth of the organism on chocolate agar into 5 ml of RPMI 1640 medium without leucine (Grand Island Biological Co., Grand Island, N.Y.) to which was added 2.5 μCi of L-[^{14}C]leucine (New England Nuclear, Boston, Mass.) and 0.05 ml of Fildes extract (Difco Laboratories, Detroit, Mich.). The culture was incubated for 8 h at 37°C on a shaker to a density of 10^9 bacteria per ml and harvested by centrifugation at 12,000 × *g* for 10 min at 4°C in a model IEC B20A centrifuge (Demon Corp., Needham Heights, Mass.). The sedimented bacteria were washed three times in cold phosphate-buffered saline (PBS) containing 0.3% bovine serum albumin (PBS-A) without added preservative (Armour Pharmaceutical, Kankakee, Ill.) and resuspended in the original volume of PBS-A. Bacterial purity and colony counts were ascertained by culturing appropriate dilutions of the bacterial suspension on chocolate agar. The radiolabeled bacteria were rapidly frozen, using liquid nitrogen, and stored at −70°C in multiple aliquots. In preliminary phagocytosis experiments using a wide range of test antisera, opsonization of freshly prepared and thawed ^{14}C-labeled Hib was similar.

Animal inoculation. The test organism was inoculated into brain heart infusion broth supplemented with 1% Fildes extract and incubated for 4 to 6 h at 37°C in a shaker to a density of 10^8 colony-forming units/ml. The organism was harvested as described by Smith et al. (27), except that PBS-A was used instead of PBS containing 0.1% gelatin.

Infant rats, 12 to 14 days old from six litters, were randomly divided and reassigned to nursing mothers. Blood was obtained from 14 of the infants by rapid exsanguination by cardiac puncture. The remaining 43 pups were divided into two groups and were inoculated i.p. either with 0.1 ml of PBS-A or PBS-A containing 10^4 resuspended bacteria. Forty-eight hours later, 0.01 ml of blood was obtained from the tail artery and cultured on chocolate agar, showing Hib bacteremia in all but one of the experimental animals and in none of the controls.

Groups of 14 to 21 animals were anesthetized with ethyl ether and bled by cardiac puncture at 3, 7, and 11 weeks after inoculation. Most animals were bled on more than one occasion, but the size of the groups decreased because of mortality associated with the procedure. Blood samples were cultured by spreading 100 μl directly on chocolate agar, and the remaining blood was allowed to clot at room temperature and immediately refrigerated for 1 h at 4°C. Serum was separated by centrifugation in the cold at 4°C and stored at −70°C in multiple vials for subsequent immunological studies.

Passive protection experiments were performed by administration of 0.1 ml of test or control sera to groups of 10 to 13 infant rats 7 days of age. In the initial experiment, the animals received the serum i.p. One day later they were challenged intranasally with 10^7 Hib in 0.01 ml of PBS-A, using techniques described by Moxon et al. (18). In subsequent experiments, we administered dilutions of serum or physiological saline subcutaneously, and 1 day later the animals were infected i.p. with 3×10^2 Hib in 0.1 ml of PBS-A. Seventy-two hours after bacterial challenge, blood was obtained by cardiac puncture, and quantitative counts of viable bacteria were obtained by spreading 0.01 and 0.1 ml, respectively, of undiluted and 10^{-1} dilutions of heparinized blood (25 U/ml) directly on chocolate agar.

Immunological methods. Antibody to the capsular antigen of Hib was measured by radioimmunoassay according to the methods of Robbins et al. (23). The ^{125}I derivative of the type b polysaccharide and samples of pooled human plasma containing 1.1 μg of AC antibody per ml (accuracy control) were kindly supplied by John Robbins and Rachel Schneerson of the Bureau of Biologics. Adult human serum (DG immunized twice with 50 μg of Hib vaccine) (4) was used as the reference standard and contained 210 μg of antibody per ml, as determined by quantitative precipitin analysis (23). This serum was diluted in gamma-globulin-free fetal calf serum (Grand Island Biological Co.), which had been assayed by electrophoresis on cellulose acetate to ensure absence of gamma globulin (13). The lowest concentration of antibody detected was 0.10 μg/ml. Serum samples were studied in duplicate, and all samples from control and experimental animals of different ages were studied simultaneously in the same assay.

BC activity was measured utilizing the homologous test organism (b3a). The source of complement consisted of serum obtained from a 24-h-old calf which had been denied colostrum (3). This complement source lacked detectable AC antibody, and opsonizing and BC activity, and was stored in multiple vials at −70°C. BC activity was measured as previously described (3, 10), except that 0.1 ml of serial twofold dilutions of sera were incubated with 0.03 ml of complement and 0.01 ml of bacteria (approximately 1,000 organisms) of a log-phase broth culture of Hib resuspended in PBS-A. The BC titer was defined as the serum dilution which resulted in a >90% decrease of bacterial growth. In some experiments, BC activity was also measured using bacteria which had been

rendered "resistant" to bacteriolysis by preincubation of the test organism for 30 min at 37°C in heat-inactivated normal rat serum, according to the methods of Shaw et al. (26).

Serum-opsonizing activity to Hib was assayed by the method of Anthony, using rabbit macrophage cells adhered to glass cover slips (5). Briefly, test or control sera were diluted 1:5 in McCoy medium with 10% gamma-globulin-free fetal calf serum and incubated in a stationary water bath at 37°C for 15 min with an equal volume of an appropriate dilution of ^{14}C-labeled Hib in PBS-A. The bacteria-serum mixture was further diluted 1:10 in McCoy medium with 10% fetal calf serum. One milliliter of this mixture was added to each Leighton tube containing a cover slip with 10^5 cells for a bacteria-macrophage ratio of 100:1. The Leighton tubes were incubated at 37°C and rocked gently on a platform (Bellco Glass, Inc., Vineland, N.J.). Cover slips were removed at 0, 30, 60, and 120 min and were washed three times with McCoy medium with 10% fetal calf serum. They were air dried and placed in a scintillation vial containing Ready-Solv HP (Beckman Instruments, Fullerton, Calif.). Counts per minute were determined in an LS-330 liquid scintillation counter (Beckman Instruments).

The number of sera tested in a single assay was limited by the number of available macrophage cells harvested from each rabbit (range, 40×10^6 to 60×10^6 cells). For interassay comparisons, we included a negative and a positive control consisting of, respectively, bacteria preincubated with media and bacteria preincubated with a convalescent serum pool obtained from previously infected 60-day-old rats. The media control was assumed to have 0% activity, and the convalescent pool was assigned a value of 100%. The percent activity for the unknown test serum was calculated according to the following formula: $[(X - M)/(P - M)] \times 100$, where X = counts per minute for the test serum, M = counts per minute for the media control, and P = counts per minute for the convalescent pool. For the results reported, the variation in counts per minute among duplicate or triplicate cover slips was <15%.

Gel filtration of rat serum. A 10-ml amount of pooled convalescent sera from 60-day-old, previously infected rats ($n = 9$) was precipitated with an equal volume of 3.2 M $(NH_4)_2SO_4$, resuspended in distilled water, and dialyzed against PBS. This serum fraction was further concentrated to 3 ml and applied to a Sephadex G-200 column eluted with a buffer composed of 0.1 M tris(hydroxymethyl)aminomethane and 0.2 M NaCl at pH 8.1. Three-milliliter fractions were collected, and protein concentrations were determined by the optical density at 280 nm. The fractions in each peak were pooled and assayed for protein content (Lowry method [14]), using bovine serum albumin as a standard. BC activity was measured after sterilizing the fraction by membrane filtration and adjusting the concentration of each pool to 10 mg of protein per ml.

Absorption of antisera. Serum samples were absorbed for AC antibody by the addition of 50 μg of purified capsular antigen (Hib vaccine) (4) to 0.5 ml of serum and incubation for 30 min at 37°C and then overnight at 4°C (24). Unabsorbed control sera were treated similarly, but received PBS. Adequacy of serum absorption of AC antibody was monitored by radioimmunoassay. Sera were absorbed with washed, viable Hib, using 1 volume of serum and 1 volume of packed bacteria. These were mixed and incubated at 37°C for 1 h and then overnight at 4°C. Sterilization was accomplished by membrane filtration.

Statistical analysis. We performed statistical comparisons with an Olivetti P652 programable calculator. The exact probability of the distribution of the AC antibody responses was determined using the program for a 2×3 contingency table. We performed other statistical comparisons by using a chi-square test and a t test for unpaired samples. Logarithmic transformations were used for comparison of bacterial counts in blood.

RESULTS

AC antibody. There was no detectable AC antibody (<0.10 μg/ml) in the mother rats ($n = 6$) or in their 12- to 14-day-old infants ($n = 14$), which were studied prior to inoculation (Table 1). In contrast, 7 weeks after inoculation (61 to 63 days of age), convalescent sera from the experimental animals showed significantly higher levels of antibody than control, uninfected littermates ($P < 0.009$). Similar increases in antibody were observed in experimental rats at 91 days of age ($P = 0.05$). However, 35 to 40% of the infected group had undetectable AC antibody.

TABLE 1. *Experimental Hib infection: AC antibody*

Rats	Age (days)	n	No. (%) of rats with AC antibody (μg/ml):		
			<0.10	0.10–0.5	>0.5
Uninfected					
Mothers	120–150	6	6 (100)	0 (0)	0 (0)
Offspring	12–14	14	14 (100)	0 (0)	0 (0)
	61–63[a]	21	17 (80.9)	4 (19.1)	0 (0)
	91–93[b]	15	12 (80)	3 (20)	0 (0)
Infected					
Offspring	61–63[a]	20	8 (40)	7 (35)	5 (25)
	91–93[b]	14	5 (35.7)	3 (21.4)	4 (28.6)

[a] $P = 0.009$ (exact probability for a 2×3 contingency table).
[b] $P = 0.050$.

BC activity. BC activity was absent in serum from the mother rats ($n = 6$), their 12- to 14-day-old suckling infants ($n = 14$), and the uninfected control rats studied at 61 to 63 days of age ($n = 21$). Two of 15 control animals studied at 91 to 93 days of age had BC activity detected in undiluted sera. In contrast, all of the convalescent serum samples from the experimental group obtained 7 and 11 weeks after infection ($n = 20$ and 14 animals, respectively) were BC and had titers ranging from 1:2 to 1:16. The difference between control and experimental animals at each age was significant at $P < 0.0001$. ($\chi^2 = 41$ and 22).

There was no significant decrease in BC activity after absorption of the convalescent rat serum with purified type b capsule. This was true even in the serum samples from four of the experimental animals with the highest levels of antibody to the capsular antigen (Table 2). Thus, BC activity persisted after serum absorption of AC antibody, and it would appear that bacteriolysis may result from serum factors other than AC antibody.

As previously reported by Shaw and co-workers (26), significant decreases in BC titer resulted when the test organism was rendered resistant to serum bacteriolysis by preincubation with normal rat serum which lacked AC or BC antibody (Table 2). A decrease in BC titer occurred with all of the convalescent sera tested, including those preabsorbed with type b capsule. However, even using the resistant test organism, low levels of BC activity were detected in convalescent serum which lacked detectable AC antibody.

Convalescent serum samples from nine previously infected animals were obtained at 60 days of age and pooled and fractionated on a Sephadex G-200 column. BC activity was found primarily in the eluted fraction corresponding to a molecular weight of 150,000 (immunoglobulin G class), and a minor peak of activity was present in the high-molecular-weight fraction. Significantly, there was complete absence of activity in the fraction corresponding to the peak containing immunoglobulin A.

In other experiments, BC activity was also present in the sera of the offspring of 120- to 150-day-old convalescent female rats who survived neonatal infection. Ten first-generation rats from three litters were tested. We detected serum BC activity in all of the infants studied up to 21 days of age, with titers ranging from 1:2 to 1:8.

Opsonizing activity. Figure 1 illustrates the results of a typical experiment measuring the uptake of ^{14}C-labeled Hib pretreated with various test sera and incubated with rabbit pulmonary macrophage cells adhered to glass cover slips.

Sera from rats 2 and 3 were obtained 7 weeks after inoculation and contained <0.10 μg of AC antibody per ml. Both convalescent sera caused progressive enhancement of the uptake of ^{14}C-labeled organisms. This increase was less than that observed with hyperimmune rabbit serum

TABLE 2. *BC activity of convalescent rat serum:[a] effect of absorption of serum with capsular antigen*

Rat no.	Unabsorbed serum		Absorbed serum	
	BC titer (reciprocal)	AC antibody (μg/ml)	BC titer (reciprocal)	AC antibody (μg/ml)
1	8 (1)[b]	0.54	8 (1)	<0.10
2	8 (2)	<0.10	8 (1)	<0.10
4	4 (1)	1.90	4 (1)	0.22
5	16 (2)	1.80	8 (2)	0.13
6	4 (1)	1.20	4 (1)	<0.10

[a] Serum obtained at 7 weeks postinoculation (age, 61 to 63 days).

[b] Titer obtained with organisms rendered resistant to bacteriolysis by preincubation in normal rat sera.

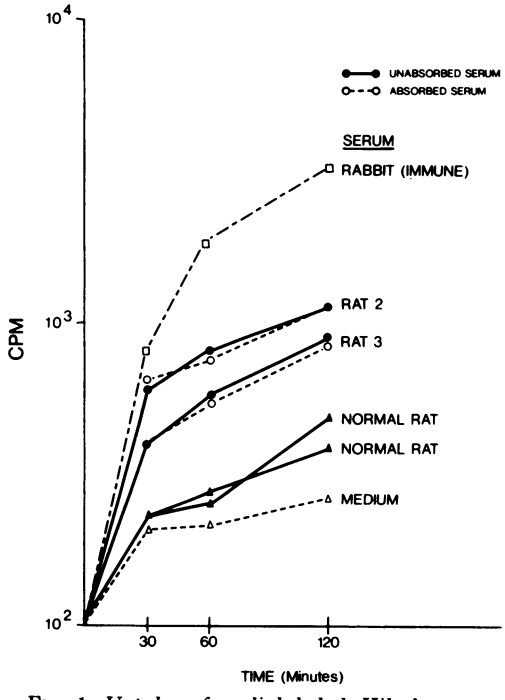

FIG. 1. *Uptake of radiolabeled Hib by macrophage cultures. Rats 2 and 3 were infected i.p. at 12 days of age. Convalescent serum samples obtained 7 weeks later contained <0.10 μg of AC antibody per ml but resulted in significant enhancement of phagocytosis. Opsonizing activity was unaffected by absorption of serum with capsular antigen.*

(AC antibody, 1,400 μg/ml) but was greater than that observed with normal rat serum obtained from uninfected control littermates. Furthermore, as indicated by the dashed lines in Fig. 1, opsonizing activity in the convalescent rat sera persisted after absorption with purified capsular antigen.

When other convalescent rat sera were selected with higher titers of AC antibody, significant decreases in opsonizing activity were obtained after absorption with capsular polysaccharide (Fig. 2). Sera from rats 4 and 6 were obtained 7 weeks after inoculation and contained 1.9 and 1.2 μg of AC antibody per ml, respectively. Absorption effectively lowered the titers of AC antibody in these sera and also resulted in decreased opsonizing activity. Although the differences between absorbed and unabsorbed sera were small, decreased opsonizing activity was a consistent finding in these and other absorbed sera with high AC titers.

To further study the effect of serum absorption on opsonization of Hib, convalescent rat serum samples were pooled and absorbed with PBS, purified capsular antigen, intact Hib, and *Neisseria gonorrhoeae*. The convalescent serum pool contained 0.37 μg of AC antibody per ml. Absorption with purified capsular antigen or intact Hib organisms lowered the AC antibody level to <0.10 μg/ml and resulted in decreased uptake of opsonized ^{14}C-labeled Hib (71.3 and 32.2%, respectively). In contrast, preabsorption of convalescent serum with PBS or *N. gonorrhoeae* caused no significant loss of opsonizing activity to Hib (102.5 or 92.6%, respectively).

Figure 3 illustrates the results from a series of experiments in which we measured serum-opsonizing activity to Hib in experimental and control rats of different ages. The data shown represent the 2-h incubation period of labeled bacteria with macrophage cells and are expressed as percent activity compared to the levels measured in a convalescent serum pool, which served as a positive control in each assay.

There was little or no opsonizing activity in serum samples from animals 12 to 14 days of age obtained prior to inoculation. In older animals, the levels of opsonizing activity increased with age in both the experimental and control groups. However, the levels found in the experimental group were significantly greater than those found in the age-matched, uninfected littermates ($P < 0.001$ for each age).

The age-related increase in serum-opsonizing activity among the normal rats is of particular interest since these animals lacked serum BC activity and had undetectable or low levels of AC antibody (Table 1). Furthermore, an increase in serum-opsonizing activity with age was not observed when heat-killed, ^{14}C-labeled group B *Streptococcus* (kindly provided by Bascom Anthony, Harbor General Hospital, Torrance,

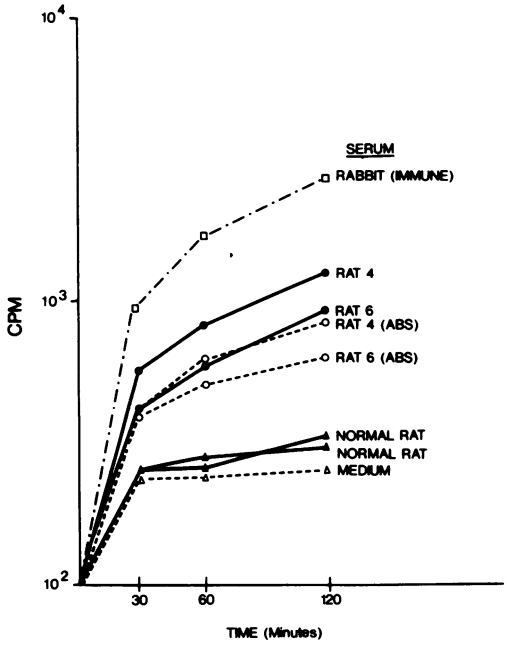

FIG. 2. *Uptake of radiolabeled Hib by macrophage cultures. Rats 4 and 6 were infected i.p. at 12 days of age. Convalescent sera obtained 7 weeks later contained 1.9 and 1.2 μg of AC antibody per ml, respectively. Considerable opsonizing activity remained after removal of AC antibody by absorption of serum with capsular antigen.*

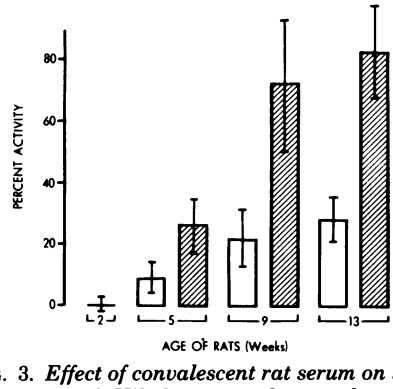

FIG. 3. *Effect of convalescent rat serum on uptake of radiolabeled Hib by macrophage culture. Rats were infected i.p. at 12 days of age and were bled before injection and at 5, 9, and 13 weeks. Convalescent animals (▨) showed higher levels of serum-opsonizing activity to Hib than uninfected littermate controls (□). The age-related increase in serum-opsonizing activity in the normal rats is also of interest since these animals lacked detectable AC and BC antibodies. Data shown are the means ± standard deviation (brackets) for groups of 15 to 20 animals.*

Calif. [5]) was used as the test organism.

Protection. Convalescent serum from previously infected animals which lacked capsular antibody, but which exhibited BC and opsonizing activity for *Haemophilus*, was next tested for protection in 8-day-old infant rats challenged by sublethal doses of the homologous organism. The results of two such experiments are shown in Table 3. Bacteremia was detected less frequently and in lower levels in the animals pretreated with the convalescent serum pool as compared with the animals which received saline or normal serum. Lower levels of bacteremia were also present in the animals pretreated with convalescent serum diluted 1:2.5, but at higher dilutions the apparent decreases in quantitative bacterial counts were not significant.

In another experiment, 12- to 14-day-old offspring of two surviving females previously infected during the neonatal period with Hib were inoculated i.p. with 10^3 colony-forming units of the homologous organism. Serum samples from the mothers and infants were BC for Hib but contained no detectable AC antibody (<0.10 μg/ml). Twenty-four hours after inoculation, bacteremia was detected in four of eight offspring of the previously infected mothers compared to 15 of 15 surviving offspring from three mothers with no previous exposure to *Haemophilus* ($\chi_c^2 = 5.93$, $P < 0.02$). The offspring of the previously infected females also had lower geometric mean bacterial counts per microliter of blood (0.216 compared to 18.6 in the control offspring; $t = 5.05$, $P < 0.001$).

DISCUSSION

The infant rat model for systemic Hib infection simulates disease in humans with respect to a number of factors including hematogenous-spread, pathological lesions induced in the central nervous system and other organs, and the marked age-related susceptibility to infection (11, 17, 18, 27). Previous studies have provided ample evidence that older rats require a higher mean lethal dose of Hib and also more rapidly clear type b encapsulated strains from the blood stream than do younger animals (27, 29).

The mechanisms responsible for increased resistance of older rats to Hib infection remain unknown. Our present data confirm earlier observations that serum BC activity is absent in most normal rats up to 91 to 93 days of age (27). Thus, the enhanced resistance of older animals to *Haemophilus* infection appears to be unrelated to the presence of serum BC activity. It also appears to be unrelated to antibody to the capsular antigen of *Haemophilus*, since nearly all of the uninfected control rats we studied at different ages lacked detectable AC antibody (Table 1). Despite the absence of BC and AC antibodies, control rats showed an age-related increase in serum-opsonizing activity to Hib

TABLE 3. *Experimental challenge of 8-day-old rats with Hib: effect of pretreatment with serum to development of bacteremia*

Expt[a]	Source of serum	No. of rats challenged	Survivors (72 h)				
			No.	No. with bacteremia (%)	Bacteria/μl of blood (geometric mean)	Significance[b]	
						P	(*t*)
1. 0.1 ml of serum i.p. followed by 10^7 Hib i.n.	Normal rat[c]	11	11	10[d] (91)	4.51		
	Convalescent rat (undiluted)[e]	13	13	4[d] (30.7)	0.0063	<0.001	(4.9)
2. 0.1 ml of serum s.c. followed by 3×10^2 Hib i.p.	Saline control	10	8	8 (100)	86.0	NS[f]	(0.11)
	Normal rat[c]	10	8	8[g] (100)	77.0		
	Convalescent rat[e]						
	Undiluted	11	8	1[g] (12.5)	0.0002	<0.0001	(11.4)
	1:2.5	10	9	8 (88.9)	1.56	<0.027	(2.48)
	1:5	10	8	8 (100)	4.31	NS	(1.46)
	1:10	10	7	7 (100)	7.09	NS	(1.22)

[a] i.n., Intranasally; s.c., subcutaneously.
[b] Data for each group are compared to bacterial counts in animals pretreated with normal serum.
[c] AC antibody, <0.10 μg/ml; BC titer, <1.
[d] $\chi^2 = 8.87$; $P < 0.005$.
[e] AC antibody, <0.10 μg/ml; BC titer, 1:4.
[f] NS, Not significant.
[g] $\chi^2 = 12.2$; $P < 0.001$.

(Fig. 3). The factors responsible for this increase remain to be determined. In addition, other potential mechanisms responsible for enhanced resistance of the older animals, such as macrophage function (6, 7), need to be explored.

Infant rats infected with Hib developed higher levels of AC antibody than uninfected control animals (Table 1), and convalescent animals also developed serum BC activity. These results are different from those of Myerowitz and Norden, who detected no differences in AC and BC antibodies among control and infected rats studied 8 weeks after inoculation (20). In their experiments, both control and infected rats had higher levels of AC antibody than our animals, and serum BC activity was present in approximately 20% of the groups.

There were a number of differences in experimental design between our study and that of Myerowitz and Norden. However, it would appear that the most important factor contributing to the higher AC antibody levels in their report relates to a recent observation made by the same investigators (20) that rat sera may nonspecifically bind some lots of radiolabeled capsular antigen, resulting in the measurement of falsely elevated AC antibody. Therefore, we utilized absorbed and unabsorbed serum samples as controls in our assay, and the results indicated that antigen-binding activity was effectively blocked by unlabeled antigen.

Myerowitz and Norden also reported that BC activity was absent from most of their previously infected rats (19), whereas it was present in 100% of the convalescent animals we studied. In this instance the different results may be explained by their use of a heterologous test Hib strain for measurement of BC activity (Myerowitz, personal communication), whereas we used the homologous strain. Further studies are needed to clarify the significance of these observations, but they support the existence of possible subtypes of Hib, capable of being defined by the BC reaction (21).

It was interesting that the BC activity in our convalescent rats was found predominantly in the serum fraction corresponding to a molecular weight of 150,000 (immunoglobulin G class) and was present in the serum of offspring of previously infected mothers. Since pregnant rats pass virtually no antibody to their infants via the placenta (28), serum BC activity in the suckling offspring was probably derived from colostrum passing into the infant's circulation via the highly permeable neonatal rat gastrointestinal tract. However, we have no direct evidence for this hypothesis, such as data from experiments utilizing cross-fostering.

A number of investigators have recently questioned the protective role of serum BC activity in immunity to Hib (8, 26). Feigin et al. detected BC activity in admission serum samples of 92% of patients with Hib meningitis and concluded that serum BC activity was not protective (8). Shaw et al. studied infected infant rats and noted that Hib bacteremia coincided for several days with an elevated BC titer (26). In these and other reports (9) serum BC activity was measured using Hib grown in broth culture. In additional experiments, Shaw et al. reported that Hib grown in vivo, or incubated in the presence of a serum factor, were more resistant to serum bacteriolysis (26). When resistant Hib were used as the test organism in the BC assay, serum activity was no longer detected in their bacteremic rats. Thus, the results of BC assays which use resistant Hib may correlate better with immunity.

When we used resistant (serum-incubated) organisms to measure serum BC activity in our animals, the titers present in the experimental group were lower, but we could still detect BC activity in convalescent serum which lacked anticapsular antibody (Table 2). Thus, even when we used more stringent conditions of testing, serum bacteriolysis in the convalescent rats appeared to depend on factors other than AC antibody.

For most bacterial pathogens, phagocytosis by leukocytes and fixed macrophages is a critical defense against invasive infection. Whereas our opsonizing data are relevant to macrophage function, it is possible that an additional set of opsonic factors may be responsible for opsonization of Hib by leukocytes. Nevertheless, our studies of convalescent sera from rats previously infected with Hib provide evidence that opsonizing activity may also result from serum factors other than AC antibody. Convalescent sera which lacked detectable AC antibody efficiently opsonized Hib. Furthermore, serum-opsonizing activity to Hib persisted after absorption with capsular antigen but was markedly lowered by absorption with intact organisms. These data are consistent with the hypothesis that serum opsonization of Hib may result from antibody directed at the noncapsular surface constituents of the organism.

There is little data available to indicate whether noncapsular antibodies are also protective. Alexander et al. studied hyperimmune rabbit serum prepared to Formalin-killed Hib and concluded that the protective factor was AC antibody, and that somatic antibodies were not protective (1). In contrast, Myerowitz and Norden reported that rabbit antisera raised against

Formalin-killed, unencapsulated *H. influenzae* were weakly protective (20). Our data indicate that convalescent rat serum, with a BC titer of 1:4 but which lacked detectable AC antibody (>0.10 μg/ml) and had a BC titer of 1:4, could passively protect infant rats against intranasal or i.p. challenge by the homologous organism (Table 3). It remains to be determined whether similar protection will occur in animals challenged by heterologous Hib.

Studies in humans indicate that young infants convalescing from systemic Hib infection may possess serum BC activity in the absence of AC antibody (2, 10, 21). Johnston et al. also studied opsonization of Hib by adult human sera and reported that significant levels of opsonizing activity remained after absorption of the sera with capsular antigen (12). These observations in humans and our data from the experimental rat model suggest that the noncapsular surface antigens of Hib might be promising components for a future immunizing agent if they should also prove to be nontoxic. Finally, our data also suggest that the infant rat model may be useful for future assessment of the immunogenicity of isolated, noncapsular, outer cell wall constituents of Hib, since the antibody responses of the animals to neonatal infection appear to be primarily directed at these antigens.

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