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Immunology of the Infant Rat Experimental Model of *Haemophilus influenzae* Type b Meningitis

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The age-related acquisition of serum anticapsular and bactericidal antibodies to *Haemophilus influenzae* type b observed in rats was similar to that of humans. The antigenic source for this "natural" immunity was not identified, since neither pharyngeal infection with *H. influenzae* b nor enteric colonization by cross-reacting bacteria was detected. Infant rats surviving *H. influenzae* b bacteremia failed to respond immunologically to the capsular polysaccharide. However, surviving rats demonstrated no impairment of immune responsiveness to this antigen after subsequent immunization with live bacteria in adulthood. In passive protection experiments, antibodies directed against the type b capsular polysaccharide represented the major protective specificity. However, a small protective effect of antibodies to noncapsular antigens also appeared to have been demonstrated.

Haemophilus influenzae type b is the commonest cause of bacterial meningitis for individuals less than 5 years old and is also an important etiological agent of penumonia, otitis media, and septic arthritis in this age group (23). Human immunity to *H. influenzae* b was first elucidated by Fothergill and Wright (7), who showed that bactericidal (BA) antibody was present at birth (via transplacental passage from the mother), declined to undetectable levels by age 6 months, reappeared by age 3 years in almost all children, and was sustained throughout adult life. The lack of BA antibodies was associated with a higher incidence of *H. influenzae* b disease, and their presence implied protection from disease. More recent investigations (1, 2, 15, 24) have confirmed the age relationship of BA antibodies to *H. influenzae* b and have shown that the bulk of bactericidal antibodies are directed against the type b capsular polysaccharide. Based on these facts, immunization with purified polysaccharide has been suggested as a potentially effective preventive measure and field trials are currently underway (J. C. Parke et al. Pediatr. Res. **10**:402, 1976; P. W. Anderson et al., Pediatr. Res. **10**:383, 1976).

Several questions about immunity to *H. influenzae* b remain to be investigated. Since childhood infection with this organism is uncommon, the almost universal age-related acquisition of protective antibodies has been termed "natural" immunity. It has been suggested that the antigenic source for these antibodies may be enteric cross-reacting bacteria which colonize the gastrointestinal tract (4). Children surviving *H. influenzae* b meningitis prior to age 2 years make little or no anticapsular (AC) antibodies despite systemic infection, whereas survivors of meningitis after age 2 respond briskly (17). Furthermore, the subsequent response to immunization with polysaccharide vaccine is blunted in children surviving *H. influenzae* b meningitis before age 2 years. "Immunological paralysis" has been suggested as the responsible mechanism for this apparent age-related specific suppression of immune responsiveness (17, 19). Several investigators have shown that one cannot account for all naturally acquired BA antibodies with AC antibodies (2, 11). The suggested protective effect of BA antibodies directed against noncapsular antigens requires investigation.

The recent development of a reproducible model of *H. influenzae* b bacteremia and meningitis by either intraperitoneal (i.p., 25) or intranasal (i.n., 10) inoculation of infant rats affords an opportunity to study some of the above questions in a controlled manner. This report outlines the results of investigations using both models designed to determine: (i) whether natural immunity and the immune response to experimental *H. influenzae* b infection in rats is similar to that observed in humans; (ii) whether immunological paralysis can be reproduced in survivors of experimental infection; and (iii) whether antibodies directed against noncapsular antigens are protective against experimental infection.

(Portions of the data in this report were pre-

sented at the 16th Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, Ill.)

MATERIALS AND METHODS

Animals. Outbred, pathogen-free, albino Sprague-Dawley rats were purchased from Hilltop Lab Animals, Scottsdale, Pa. Some rats used for the protection experiments were bred in our own animal facility. Rats were housed in a single room at the animal quarters of the Children's Hospital of Pittsburgh.

Bacteria. The strain of *H. influenzae* b ("Pekala" strain) and the techniques of storage and culture used in our laboratory have been previously described (13). For the present experiments, the bacteria used were passaged either once or twice through 5-day-old rats before storage and use. Both strains of unencapsulated *H. influenzae* (F2 and F3) were isolated from children with otitis media. Killed bacteria for immunization and absorption were prepared by centrifugation of an overnight broth culture and resuspension in 2% neutral buffered formalin. After overnight incubation at room temperature, the bacteria were washed four times in 0.5 M phosphate-buffered saline (pH 7.1).

Stool and throat cultures. Throat swabs were planted on Levinthal "antiserum-agar" medium as previously described (9), and rectal swabs were planted on Trypticase soy agar (Difco Laboratories, Detroit, Mich.) containing a similar amount of antiserum. Plates were incubated for 24 h at 37°C followed by 24 h at 4°C. Colonies surrounded by a precipitin halo were subcultured, and the presence of a cross-reacting antigen was confirmed by immunodiffusion (21).

Immunological methods. Serum AC antibodies were measured by a radiolabeled, antigen-binding assay as previously described (18). Results are expressed as micrograms of antibody protein per milliliter of serum. The minimum detectable concentration was 0.1 μg/ml. The presence or absence of serum BA activity was detected as previously described (17). Because of the small sample size, end point titrations of BA activity were not carried out. Counterimmunoelectrophoresis for soluble type b polysaccharide antigen was performed as described (8). The minimum detectable antigen was 30 ng/ml. Absorption of hyperimmune sera with either purified antigen or killed whole bacteria was carried out at 37°C for 2 h followed by 24 h at 4°C. Hyperimmune sera were raised in rabbits against two unencapsulated strains of *H. influenzae* using intravenous injections of a saline suspension of washed, formalin-killed bacteria (10^{10}/ml). The schedule in Table 1 was used.

TABLE 1. *Schedule of intravenous injections of a saline suspension of washed, formalin-killed bacteria*[a]

Week	Intravenous injection (ml) on:		
	Day 1	Day 3	Day 5
1	0.1	0.2	0.3
2	0.4	0.5	0.6
3	0.7	0.8	0.9
4			
5		Rest	
6	0.5	0.6	0.7
7	0.8	0.9	1.0
8	1.0	1.0	1.0
9		Rest	
10			Bleed

[a] Each suspension contained 10^{10} bacteria/ml.

These sera yielded a double precipitin line by counterimmunoelectrophoresis using a broth supernatant from an overnight culture of the homologous strain as antigen. Hyperimmune burro antiserum raised against the "Rab" strain of *H. influenzae* b was kindly supplied by J. B. Robbins (24).

Animal inoculations. The techniques used to produce *H. influenzae* b bacteremia and meningitis by either the i.n. or i.p. route in 5-day-old rats were those of Moxon et al. (10) and Smith et al. (25), respectively, as slightly modified by us (13). Passive protection experiments were performed by an i.p. inoculation of 0.1 ml of the serum dilution to be tested into each of a group of six 4-day-old rats. The following day, the rats were inoculated i.p. with 10^4 live *H. influenzae* b, an inoculum that produced bacteremia in all control animals. Forty-eight hours after bacterial inoculation, 1-μl samples of blood were obtained from the jugular vein by decapitation and were planted on antiserum-agar plates. Failure to recover any colonies of *H. influenzae* b was considered positive for protection. Unprotected animals usually had colony counts greater than 10^4 colony-forming units per ml, and most were greater than 10^5 colony-forming units per ml. Fifty percent end point dilutions for protection against bacteremia were calculated by the method of Reed and Muench (20). Meningitis was detected by histological examination of the brain as described (13).

Experimental design. In the first experiment, four litters (12 animals each) of newborn rats were housed in separate cages until they were 4 weeks old, at which time they were weaned and divided into cages containing four animals each from the same litter. Mothers were bled shortly after giving birth. Growing rats were weekly bled by cardiac puncture until age 5 weeks and then biweekly until age 15 weeks. All rats (mothers and pups) had throat and rectal swab cultures after each bleeding. By age 17 weeks, 34 of the original 48 pups remained. These were divided into two groups. One group (A) was inoculated intravenously with 10^9 formalin-killed *H. influenzae* b. A second group (B) received 0.1 μg (five rats), 0.3 μg (five rats), 0.7 μg (five rats), or 1.0 μg (four rats) of polysaccharide vaccine (supplied by J. B. Robbins) subcutaneously. Postimmunization blood samples were obtained at 1 and 3 weeks. Group A was then given a second intravenous inoculation with 10^9 killed *H. influenzae* b. Group B received an i.p. inoculatlon of 10^7 live *H. influenzae* b. Blood samples were obtained 2 weeks after this second immunization.

In the second experiment, a total of 83 5-day-old rats from eight litters were inoculated i.n. with 10^7 live *H. influenzae* b. Rats were bled for culture by cardiac puncture at 48 h after inoculation. Forty (48%) of the 83 rats were bacteremic, all with

greater than 10^4 colony-forming units per ml of blood. Bacteremic rats were identified and separated from nonbacteremic rats. A control group of 40 5-day-old rats from five litters were inoculated i.n. with saline. Bacteremic rats, rats inoculated with bacteria but who were nonbacteremic, and saline-control rats were housed in separate cages. The mortality in bacteremic rats was 50% (20/40) during the first 2 weeks after inoculation. However, during the first 5 weeks after inoculation, 17 (40%) of the 43 inoculated-but-nonbacteremic rats and 20 (50%) of the 40 saline-control rats also succumbed for unknown reasons. All surviving rats were bled by cardiac puncture at 5, 9, and 15 weeks of age. At age 15 weeks, rats were inoculated i.p. with 10^5 live *H. influenzae* b. Blood was obtained 2 weeks after this immunization.

The third experiment used passive protection against i.p.-induced bacteremia to determine: (i) if the protective activity of hyperimmune antiserum to *H. influenzae* b could be completely absorbed by purified type b polysaccharide; (ii) if the protective activity would be affected by absorption with unencapsulated *H. influenzae* b; and (iii) if hyperimmune serum prepared against unencapsulated strains of *H. influenzae* would have any protective activity against i.p.-induced bacteremia.

RESULTS

Natural immunity to *H. influenzae* in rats. Each of four litters of rats demonstrated an age-related acquisition of AC antibodies (Fig. 1). At 1 week of age, only 1 of 48 rats had detectable serum AC antibodies, despite the presence of maternal serum antibodies. AC antibodies were first detectable at about 4 weeks of age,

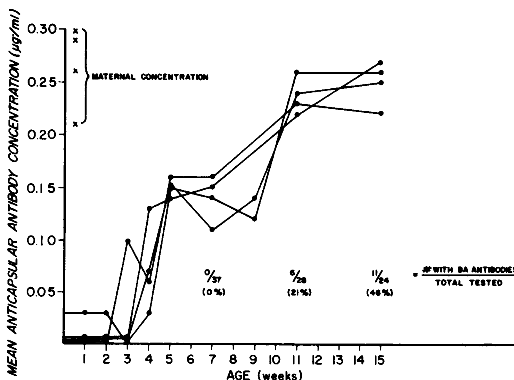

FIG. 1. *Age-related appearance of serum AC and BA antibodies during the first 15 weeks of life in four litters of rats. Each point represents the geometric mean concentration of AC antibodies for the entire litter on a particular week. Sera with no detectable AC antibodies (<0.1 μg/ml) were assigned a value of 0 for determination of the means. Thus, some points fall below the lower limit of detection by our assay. Sera were assayed for BA antibodies only during weeks 7, 11, and 15.*

and "adult" levels were achieved when rats were between 9 and 11 weeks of age. By 17 weeks of age, the geometric mean antibody concentration (± standard deviation) for the entire group was 0.25 ± 0.04 μg/ml; BA activity was not detected at 7 weeks of age (despite detectable levels of AC antibody), but 46% of the sera from 15-week-old rats had BA activity. No *H. influenzae* b or cross-reacting bacteria were isolated from the throat or rectal cultures during this period, except for one rat whose rectal culture at 9 weeks of age yielded a single colony of a cross-reacting *Bacillus* species (12).

Immunization of adult rats. Immunization of these rats at 17 weeks of age with either whole killed *H. influenzae* b (group A) or varying doses of polysaccharide vaccine (group B) failed to significantly increase the mean AC antibody concentration at 1 or 3 weeks postimmunization (Table 2). Since there was no difference in results for the different dosage groups of vaccine, the data were combined. However, the group immunized with killed bacteria (group A) did have a significant increase in the number of rats with detectable bactericidal activity ($P < 0.01$ using Fisher's exact test, Table 3). A second immunization of group A with killed *H. influenzae* b induced a mild, but not statistically significant, increase in AC antibodies. A second immunization of group B with live *H. influenzae* b induced a significant increase in AC antibodies at 2 weeks postimmunization, although the percentage of rats in this group with detectable BA activity did not change.

Immune response of survivors of experimental infection. The age-related acquisition of AC antibodies among rats surviving an i.n.-induced bacteremia when they were 5 days old, rats who had been challenged but were nonbacteremic, and rats who were saline controls was not significantly different among the groups (Fig. 2), nor was it significantly different from the unmanipulated group in the first experiment (Fig. 1). Table 4 shows the AC antibody response 2 weeks after immunization with live *H. influenzae* b. All three groups had a significant response ($P < 0.05$ using the Student's t-test for unpaired observations), and there was no significant difference in the magnitude of the response between groups. Table 5 shows that there was also no difference among the three groups in the age-related acquisition of BA activity. It also shows that after immunization with live bacteria, all three groups showed a modest increase in the percentage of rats with detectable BA activity. The individual AC antibody responses after immunization with live bacteria were quite variable in all groups (Fig. 3). Responses among the bacteremic and non-

TABLE 2. *Immune response of AC antibodies of 17-week-old adult rats to H. influenzae type b*

Adult rats in:	Immune response of AC antibodies[a]				
	Geometric mean concn (± SD) at preimmunization (μg/ml)	1st immunogen (age, 17 weeks)	Geometric mean concn (± SD) at 1 and 3 weeks postchallenge (μg/ml)	2nd immunogen (age, 20 weeks)	Geometric mean (± SD) concn of AC antibody at 2 weeks postchallenge (μg/ml)
Group A (n = 15)	0.25 ± 0.04	10^9 killed HITb i.v.	0.24 ± 0.09 0.29 ± 0.06	10^9 killed HITb i.v.	0.44 ± 0.13
Group B (n = 19)	0.25 ± 0.04	Polysaccharide vaccine s.c.	0.25 ± 0.07 0.29 ± 0.08	10^7 live HITb i.p.	1.01 ± 0.10
			($P < 0.01$)		

[a] Abbreviations: HITb, *H. influenzae* type b; i.v., intravenously; s.c., subcutaneously.

TABLE 3. *Immune response of BA antibodies of 17-week-old adult rats to H. influenzae type b*

Adult rats in:	Immune response of BA antibodies[a]				
	No. of animals with detectable BA activity at preimmunization/no. of animals in group (%)	1st immunogen (age, 17 weeks)	No. of animals with detectable BA activity at 1 and 3 weeks postchallenge/no. of animals in group (%)	2nd immunogen (age, 20 weeks)	No. of animals with detectable BA activity at 2 weeks postchallenge/no. of animals in group (%)
Group A (n = 15)	7/15 (46)	10^9 killed HITb i.v.	9/11 (82)[b] 12/12 (100)[b]	10^9 killed HITb i.v.	13/15 (87)
Group B (n = 19)	8/19 (42)	Polysaccharide vaccine s.c.	1/10 (10) 9/16 (56)	10^7 live HITb i.p.	7/12 (58)

[a] Abbreviations: HITb, *H. influenzae* type b; i.v., intravenously; s.c., subcutaneously.
[b] $P < 0.01$ when compared with preimmunization ratio.

bacteremic rats appeared to fall into three categories: high responders (>10-fold), low responders (<2-fold), and intermediate responders (2- to 10-fold). Among the control rats, almost all were either high or low responders. Only three rats in the postimmunization sample had detectable circulating polysaccharide antigen. Interestingly, two of these rats were among the highest responders (10.0 and 6.1 μg/ml, respectively).

Passive protection experiments. Hyperimmune antiserum to *H. influenzae* b had high levels of protective activity against bacteremia (Table 6). The 50% protection end point titer varied from 1:1,750 to 1:4,700 in separate experiments. All 11 rats who were protected against bacteremia were also protected against meningitis. Twenty-six of 29 rats with bacteremia also had meningitis. Rats inoculated with a 1:2,000 dilution of antiserum, which approached the 50% protection end point titer, had a serum AC antibody concentration of 0.2 μg/ml. A single absorption of the hyperimmune antiserum with formalinized *H. influenzae* b (10^{10}/ml of serum) did not completely remove AC antibodies or protective activity. Absorption of hyperimmune serum with either one of two unencapsulated *H. influenzae* strains failed to reduce either the AC antibody concentration or protective titer. Hyperimmune serum raised against either unencapsulated *H. influenzae* strain had slight protective activity when used either undiluted or in a 1:2 dilution (Table 7). This slight, but reproducible, protective activity was abolished by absorption with the homologous bacterial strain, despite the absence of a reduction in AC antibody concentration. Two lots of pooled normal adult rat serum (AC antibody concentrations of 0.20 and 0.17 μg/ml, respectively) failed to protect any animals from bacteremia, even when used undiluted.

DISCUSSION

Natural immunity to *H. influenzae* b in rats appears to be similar to that of humans. Acquisition of serum AC and BA antibodies was age related and universal in rats, despite the com-

plete absence of pharyngeal colonization by *H. influenzae* b. However, enteric colonization by cross-reacting bacteria, which might have provided an antigenic source for these antibodies, was also not observed. Previous studies have shown that the susceptibility of infant rats to bacteremia after either an i.p. or i.n. inoculation of *H. influenzae* b markedly declines during the first 3 weeks of life (10, 25). This phenomenon cannot be accounted for on the basis of the appearance of serum antibodies, since these did not reach detectable levels until the rats were 5 weeks old.

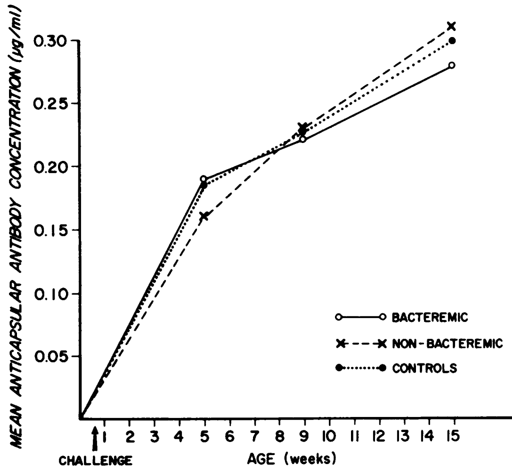

FIG. 2. *Age-related appearance of serum AC antibodies in rats challenged at 5 days of age with either live H. influenzae b or saline i.n. Twenty rats were bacteremic, and 26 rats were nonbacteremic after bacterial inoculation. There were 18 saline-control rats. Each point represents the geometric mean concentration of AC antibodies for the entire group during that week.*

Polysaccharide vaccine, which has been shown to be immunogenic in human adults and older children, appears to be either weakly or totally non-immunogenic in human infants (3, 22, 26). The vaccine failed to elicit an immune response in adult rats. The dosages used were calculated to be proportionate by weight to immunogenic doses in humans. Nevertheless, immune responses to polysaccharide antigens are dose dependent, and a wider range of doses must be studied in rats before a final conclusion regarding immunogenicity can be reached.

Infant rats surviving severe bacteremic infection failed to develop AC or BA antibodies any more rapidly than controls. Similar observations have been made in human infants surviving *H. influenzae* b meningitis (17). In contrast, Smith et al. (25) found that serum BA activity among a small number of rats surviving experimental infection was markedly increased. No data regarding AC antibodies were given in their report.

Subsequent immunization of rats who survived infantile *H. influenzae* b infection by i.p. inoculation of live bacteria induced a definite

TABLE 4. *Age-related acquisition of AC antibodies to H. influenzae type b in infected rats and controls*

Rats studied	Detectable AC antibody response (μg/ml) in rats at age:			
	5 Weeks	9 Weeks	15 Weeks	17 Weeks postchallenge
Bacteremic	0.19 (n = 20)[a]	0.22 (n = 20)	0.27 (n = 19)	1.32 (n = 19)
Nonbacteremic	0.16 (n = 26)	0.21 (n = 26)	0.28 (n = 26)	1.35 (n = 26)
Controls	0.19 (n = 18)	0.21 (n = 16)	0.30 (n = 17)	0.94 (n = 17)

[a] Geometric mean concentrations.

TABLE 5. *Age-related acquisition of BA antibodies to H. influenzae type b*

Rats studied	Detectable BA antibody response in rats at age:			
	5 Weeks	9 Weeks	15 Weeks	17 Weeks postchallenge
Bacteremic	4/20[a] (20%)	4/20 (20%)	7/16 (44%)	11/19 (58%)
Nonbacteremic	6/26 (23%)	5/26 (19%)	8/17 (47%)	14/24 (58%)
Controls	5/23 (22%)	4/19 (21%)	6/14 (43%)	8/17 (47%)

[a] Number of rats with BA antibodies per total number of rats tested.

AC antibody response which was similar to that of control animals. Thus, immunological paralysis to the capsular antigen was not observed. Immunological paralysis is a specific suppression of immune responsiveness due to antigen overload at the time of primary exposure. The phenomenon was first described in

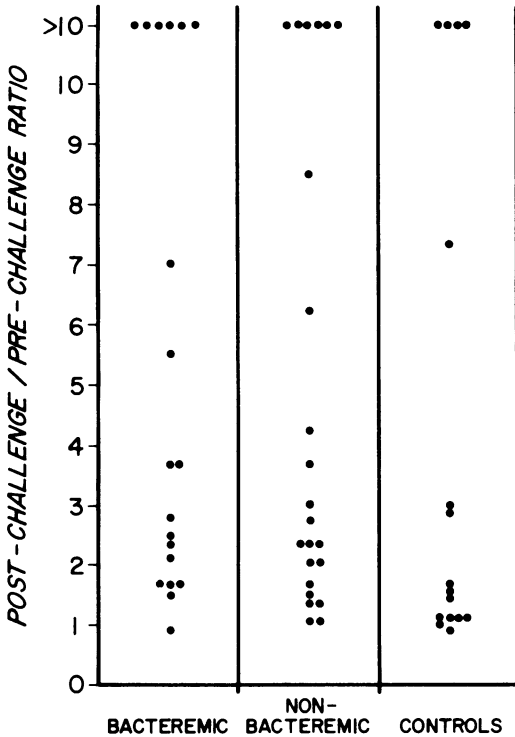

FIG. 3. *Ratio of AC antibody concentration among survivors of infant H. influenzae b bacteremia and controls before and 2 weeks after challenge with live H. influenzae b. The challenge was carried out i.p. on rats that were 15 weeks of age. Each point represents the ratio for an individual animal.*

mice with pneumococcal polysaccharide (6) and appears to be age dependent as well as dose dependent. Immunization with live bacteria was chosen because our first experiments indicated that this was the only immunogenic form of the type b polysaccharide antigen in adult rats. Since the unresponsiveness observed in human survivors of meningitis has been to polysaccharide vaccine, our inability to demonstrate specific unresponsiveness may be related to the mode of immunization rather than a true species difference. Also, we have studied survivors at only one age. It is possible that immunization at an earlier age may have demonstrated significant blunting of the immune response in survivors.

The possibility that antibodies to noncapsular antigens may also be protective is based on data from bactericidal assays. Absorption of sera from adult humans with polysaccharide antigen often cannot completely remove BA activity despite the complete removal of AC antibodies (2, 14). Several studies have shown that the presence of BA antibodies does not always correlate with AC antibodies in the same sample (2, 11, 16). Bactericidal assays in general are difficult to perform and involve many variables that are difficult to control, including the test strain of bacteria which may be particularly crucial for *H. influenzae* b (2, 14). Thus, conflicting conclusions based on bactericidal data are common (5).

A direct measurement of protective activity seemed feasible using the infant rat model. The results of passive protection experiments indicate that antibody to the capsule is the major, if not sole, protective specificity. The concentration of serum AC antibodies which protect 50% of the rats from experimental bacteremia, using the i.p. model (0.2 μg/ml), is not very different from the minimal protective concentration in humans, as predicted by calculation (0.1 μg/

TABLE 6. *Protective activity of H. influenzae type b antiserum using the infant rat model administered i.p.*[a]

Antiserum used in expt:	Absorbent	AC antibody concn (μg/ml)	Reciprocal protective titer[b]
A HI burrow serum		5,000	4,700
HI burrow serum		5,000	1,750
HI burrow serum		5,000	1,750
B HI burrow serum	Type b polysaccharide	5	2
HI burrow serum	Type b polysaccharide		4
C HI burrow serum	Polysaccharide and killed HITb	1.2	2.5
D HI burrow serum	Killed, nontypable (F2) HI serum	5,000	1,550
HI burrow serum	Killed, nontypable (F3) HI serum	5,000	2,400

[a] Abbreviations: HI, Hyperimmune; HITb, *H. influenzae* type b.
[b] 50% end point of protection against bacteremia.

TABLE 7. *Protective activity of antisera to unencapsulated H. influenzae using the infant rat model administered i.p.*

Antiserum used in expt:		Absorbent	AC antibody concn (μg/ml)	Protective effect of unencapsulated *H. influenzae* antiserum[a]			
				Undiluted	Diluted		
					1:2	1:4	1:8
A	Rabbit anti-F2		0.37	4/6		6/6	6/6
	Rabbit anti-F2		0.37	4/6	4/6	6/6	6/6
B	Rabbit anti-F2	Killed F2 bacteria	0.37	6/6	6/6	6/6	6/6
	Rabbit anti-F2	Killed F2 bacteria	0.37	6/6	6/6	6/6	6/6
C	Rabbit anti-F3		0.45	2/6	2/6	6/6	6/6
D	Rabbit anti-F3	Killed F3 bacteria	0.45	6/6	6/6	6/6	6/6

[a] Results are expressed as the number of bacteremic animals per total number of inoculated animals in each group.

ml) or measurement (0.29 μg/ml) of passively protected humans with Bruton's agammaglobulinemia (23, 26). However, a small but reproducible protective effect of antibodies to noncapsular antigens was observed with hyperimmune sera raised against unencapsulated *H. influenzae*. This effect was probably not due to AC antibodies, since absorption by the homologous strain abolished protective activity while not reducing the AC antibody content. Reduction of the i.p. bacterial inoculum used in the protection assay may increase the "sensitivity" of this assay to protective antibodies and thereby amplify the small protective effect observed with noncapsular antibodies. These noncapsular antigens should be further studied, since they might provide an alternative method of immunization against disease caused by *H. influenzae* b.

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