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Further Studies of the Role of Noncapsular Antibody In Protection Against Experimental *Haemophilus influenzae* Type b Bacteremia

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Serum antibody against polyribosylribitol phosphate, the capsular antigen of *Haemophilus influenzae* type b, confers protection against experimental *Haemophilus* infection. Antibodies against noncapsular antigens are also protective, but the antigenic specificity of the protective antibodies remains unknown. Antilipopolysaccharide antibody was prepared by immunization of rabbits with boiled *H. influenzae* type b cells. Antilipopolysaccharide antibodies present in these sera did not protect against experimental *Haemophilus* bacteremia in infant rats. Antisera were also prepared by immunization of rabbits with live *H. influenzae* type b bacteria. After absorption of anticapsular and antilipopolysaccharide antibodies, these sera contained antibody to several outer membrane proteins which were accessible on the intact bacterial surface as detected by radioimmune precipitation. These absorbed sera prevented experimental *Haemophilus* bacteremia in infant rats. Thus, antibodies against noncapsular, non-lipopolysaccharide determinants, possibly against one or more outer membrane proteins, confer protection against experimental *H. influenzae* type b disease. In contrast, antibodies against lipopolysaccharide are ineffective.

Serum antibody to polyribosylribitol phosphate (PRP), the type b capsular antigen of *Haemophilus influenzae*, confers protection against *Haemophilus* disease (1, 3, 20, 24). However, immunization with purified capsular polysaccharide fails to elicit protective levels of serum antibody in many infants and young children (20, 24), the age groups at greatest risk for *Haemophilus* meningitis (8). Recent data suggest that noncapsular antigens of *Haemophilus* are more immunogenic in young children than the type b capsule (10, 27) and are also capable of eliciting protective antibodies in experimental animals (9, 13).

Two major classes of *Haemophilus* noncapsular surface antigens against which protective antibodies might be directed are outer membrane proteins and lipopolysaccharide (LPS). Hansen et al. recently demonstrated that a monoclonal antibody to a *Haemophilus* outer membrane protein prevented experimental *Haemophilus* infection in infant rats (13). Marks et al. demonstrated that antiserum prepared against boiled cells of *Escherichia coli* J5 and containing antibody to core LPS lowered lethality in mice infected with type b *Haemophilus* (21). These results from experiments in animal models of infection suggest that either outer membrane proteins or LPS might be useful vaccine candidates. However, recent data from our laboratory suggest that antibody to LPS may not prevent disease in humans since at the time of admission to the hospital children with *Haemophilus* meningitis frequently have high levels of serum antibody to this macromolecule (27). Therefore, the present investigation was undertaken to determine whether the protection observed in the infant rat model with antibodies to noncapsular antigens (9) was a result of antibodies directed against LPS or of antibodies directed against non-LPS determinants.

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MATERIALS AND METHODS

Bacteria. *H. influenzae* type b strain Eagan (2, 3, 6), the gift of Porter Anderson, University of Rochester School of Medicine, Rochester, N.Y., was serially passaged through three infant rats and isolated from a blood culture plated on chocolate agar. *H. influenzae* type b strain S (30), a derivative of the fully encapsu-

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lated parent strain Eagan, producing approximately 1/1,000 of the amount of capsular antigen as the parent strain, was also obtained from Porter Anderson. *Salmonella typhimurium* G30 was the gift of Mary Jane Osborn, University of Connecticut Health Center, Farmington, Conn. The organisms were stored at −70°C in skim milk and recovered by inoculating a small portion of the frozen stocks onto chocolate agar and incubating overnight at 37°C in an atmosphere of 95% air–5% CO_2. Portions of 10 representative colonies from an overnight plate were transferred to 10 ml of brain heart infusion broth supplemented with hemin and NAD (27). After overnight incubation, this broth culture was diluted 1:20 in 50 ml of fresh medium. Cells were grown to mid-log phase at 37°C in 250-ml Erlenmeyer flasks, using a shaker-incubator (model no. G25; New Brunswick Scientific Co., Edison, N.J.) at 250 rpm as previously described (27), and harvested as described below.

Animals. New Zealand white rabbits were obtained from local breeders. Timed-pregnant Sprague-Dawley rats were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass., and upon arrival were housed in separate cages. To prevent cross-infection, litters of infected animals were housed on separate floors from uninfected animals. All animals received food and water ad libitum.

Preparation of antisera to *H. influenzae* type b. A logarithmic-phase culture of *H. influenzae* type b strain Eagan was harvested by centrifugation, and the bacterial cells were washed twice with phosphate-buffered saline (PBS). The pellet was resuspended in PBS at a concentration of 10^{10} bacteria per ml, boiled for 2 h, and frozen. Rabbits 75 and 81 were immunized by eight weekly intravenous injections of 0.1 ml of this suspension. Rabbits 44 and 45 were immunized intravenously with a live cell suspension containing 10^{10} organisms per ml. The 0.1- to 0.5-ml doses were administered at weekly intervals over a period of approximately 6 months. Rabbit 44 was immunized with the capsule-deficient mutant of Eagan strain (strain S) and rabbit 45 with fully encapsulated Eagan strain. Blood was collected from the rabbits before immunization and at least 2 weeks after the last injection. Because of an inadequate volume of preimmune serum for some experiments, a rabbit preimmune serum pool was employed which had been prepared from five unimmunized rabbits of the same age, obtained from the same breeder, and housed concurrently in the same facility as rabbits 44 and 45. Serum samples were stored in multiple portions at −70°C.

Absorption of antisera and preparation of affinity-purified antibodies. PRP-Sepharose was prepared by employing methods similar to those of Robbins and Schneerson (25). In brief, PRP was purified by the method of Kuo et al. (17), activated with cyanogen bromide, and coupled to ω-aminohexyl-Sepharose (Sigma Chemical Co.). Anti-PRP antibody was absorbed to the affinity matrix for 2 h at room temperature. After washing with PBS-albumin, anti-PRP antibody was eluted from the solid phase with 3.5 M $MgCl_2$ (16) and immediately diluted with 1 volume of PBS-albumin followed by dialysis against PBS at 4°C.

LPS was prepared from strain Eagan by the hot phenol method and then coupled to Sepharose as previously described (27). Sera were absorbed on this matrix, and affinity-pure anti-LPS antibody was eluted as described above for anti-PRP antibody.

Antisera were also absorbed with detergent-insoluble preparations enriched in major outer membrane proteins. Sarcosyl-insoluble outer membrane preparations were prepared as previously described (4), suspended in PBS, and harvested at 105,000 × *g* for 60 min twice to remove residual detergent. Antisera at a dilution of 1:4, previously treated to remove anti-capsular and anti-LPS antibody as described above, were incubated with an equal volume of the detergent-insoluble outer membrane preparation (1 mg of protein per ml) for 3 h at 37°C. Insoluble material was removed by centrifugation at 105,000 × *g* for 60 min and the absorption was repeated. After centrifugation, the residual antibody preparation was sterilized by filtration.

For some experiments, serum preparations were concentrated by dialysis against dry Sephadex (Pharmacia Fine Chemicals).

Serological methods. (i) Anti-PRP antibody. Serum anti-capsular antibody was measured by radioimmunoassay, using a tritium-labeled type b capsule preparation (17) kindly provided by J. S.-C. Kuo (Lederle Laboratories, Pearl River, N.Y.). Samples of human reference serum containing 40 μg of anti-PRP antibody per ml (kindly provided by Rachael Schneerson, U.S. Bureau of Biologics, Food and Drug Administration, Rockville, Md.) were used to standardize the assay. The limit of detection of the radioimmunoassay was 25 ng of anti-capsular antibody per ml.

(ii) Anti-LPS antibody. The concentrations of antibodies to LPS in hyperimmune rabbit sera were measured by quantitative precipitin analysis (14). In a preliminary assay, nonspecific precipitation of serum protein was observed at high LPS concentrations. Therefore, the immunoglobulin fraction from each serum was first partially purified by precipitation with 50% saturated ammonium sulfate. This fraction was then suspended in the original volume of saline containing 0.02% sodium azide and dialyzed. Recovery of anti-LPS antibody was quantitative as monitored by enzyme-linked immunosorbent assay (see below). Partially purified immunoglobulin was added to various amounts (0 to 100 μg) of purified LPS in a final volume of 1.0 ml. The antigen-antibody mixtures were incubated at 37°C for 1 h and then at 4°C for 3 to 7 days. The precipitates were pelleted by centrifugation, washed three times with PBS, and dissolved in PBS with 1% sodium dodecyl sulfate. Protein was determined spectrophotometrically by the method of Kalb and Bernlohr (15).

The concentrations of antibodies to LPS in absorbed preparations were quantitated by enzyme-linked immunoassay as previously described (27). Briefly, microtiter plates were coated with LPS isolated from strain Eagan (40 μg/ml). After incubation with antibody, plates were developed with goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma). This antiserum had both heavy and light chain specificities. Sera with a known amount of antibody as measured by quantitative precipitin analysis were employed to standardize the enzyme-linked immunoassay. The limit of detection was 75 ng of anti-LPS antibody per ml.

Anti-outer membrane protein antibody. Immunoglobulin G (IgG) antibodies directed against surface-exposed outer membrane proteins were identified by

the staphylococcal protein A-dependent radioimmunoprecipitation system described by Hansen and coworkers (11). In brief, cells from a fresh log-phase growth were harvested, and cell surface proteins were iodinated by the lactoperoxidase method (12). Iodinated cells were then incubated with sera and washed, and immune complexes were solubilized in detergent as described by Hansen and co-workers. In previous work (11), this technique was shown to solubilize 95% of the radioactivity associated with extrinsically labeled cells. Solubilized antigen-antibody complexes were recovered by coprecipitation with protein A-Sepharose (29). The radioiodinated proteins were dissociated from antibody and identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (19) and autoradiography. This technique visualizes a minimum spectrum of surface-exposed outer membrane protein antigens which were recognized by antibodies in our immune serum.

Experimental infection of infant rats. Passive protection experiments were performed with the infant rat model as previously described (9, 22, 28). Briefly, infant rats were removed from their mothers, randomized, and marked for identification. Portions (0.1 or 0.2 ml) of either PBS-albumin, serum, or affinity-purified antibodies in PBS-albumin were administered subcutaneously, and the infants were returned to nursing mothers. One day later, infant rats were challenged intraperitoneally with 10^2 to 10^3 CFU of *H. influenzae* type b in 0.1 ml of PBS-albumin, prepared by dilution of a logarithmic-phase broth culture. In preliminary experiments, 50% of untreated rats challenged intraperitoneally with an average of 5 CFU per rat were bacteremic at 18 h, and all control rats were bacteremic at the higher challenge doses used in the protection experiments. In protection experiments, blood was obtained from each animal by cardiac puncture 18 h after intraperitoneal challenge, and 0.1- and 0.01-ml volumes and a dilution corresponding to 0.001 ml of blood were plated on chocolate agar as previously described (9). The minimum protective quantity of antibody per rat was defined as the amount of antibody which prevented bacteremia in at least 80% of the animals challenged.

Statistical analysis. Geometric mean bacterial concentrations in blood were determined. Statistically significant differences between treatment groups were determined by the *t* test for unpaired samples.

RESULTS

Passive protection by anti-PRP and anti-LPS antibodies. Immune serum prepared against boiled bacterial cells (*E. coli* J5) contains anti-LPS antibody and previously has been shown to decrease lethality in mice challenged with type b *Haemophilus* (21). We therefore characterized the ability of antisera prepared against boiled *Haemophilus* cells to protect infant rats from *H. influenzae* type b bacteremia caused by the homologous organism. Antisera were first absorbed with PRP-Sepharose to remove low-level background anti-PRP antibody. Animals were given 0.1-ml portions of pre- or postimmune serum, challenged 1 day later with *H. influenzae* type b strain Eagan, and tested for bacteremia 18 h later. In control animals, administration of 500 ng of affinity-purified anti-PRP antibody per rat completely prevented bacteremia. In contrast, antiserum containing 3,300 ng of anti-LPS antibody failed to protect animals against bacteremia. However, compared to animals treated with preimmune serum, the level of bacteremia was decreased in the rats treated with dilutions of immune boiled cell antiserum (Table 1, experiment 1). But the lower levels of bacteremia did not appear to result from anti-LPS antibody since similar low levels of bacteremia were observed in rats treated with the same immune serum from which anti-LPS antibody had been absorbed (Table 1). In another experiment (Table 1, experiment 2) infant rats were treated with 0.2 ml of another boiled cell immune antiserum (no. 75), which contained severalfold more anti-LPS antibody than the antiserum used in the first experiment. Animals were then infected intraperitoneally with *H. influenzae* type b, and blood cultures were obtained 18 h later as described above. As with serum 81, serum 75 did not prevent bacteremia in any of the experimental animals. But, compared to control animals treated with preimmune serum, experimental animals had lower levels of bacteremia (Table 1). Once again, however, the lower levels of bacteremia were also observed in the experimental animals treated with immune serum in which 99% of the anti-LPS antibody had been absorbed. Thus, anti-LPS antibody in the boiled cell antiserum did not appear to be responsible for the decreased level of bacteremia.

Passive protection by non-PRP, non-LPS antibodies. Immune serum prepared against live cells of strain Eagan contains high concentrations of antibodies against PRP, LPS, and outer membrane proteins (data not shown). After absorption with PRP and LPS, this live cell antiserum completely prevented bacteremia in infant rats when 0.1 ml of dilutions of serum up to 1:16 were administered subcutaneously (Table 2). In other experiments, this same serum completely prevented bacteremia at dilutions up to 1:32 (data not shown). When immune serum was further absorbed with a sarcosinate-insoluble outer membrane protein preparation (4), protective activity was lost (Table 2). In other experiments, an immune serum prepared against *H. influenzae* type b strain S and absorbed with PRP and LPS also prevented bacteremia when administered in dilutions up to 1:16 (data not shown).

The preimmune serum pool used in both protection experiments did not contain detectable antibody to surface-exposed outer membrane proteins as measured by radioimmune precipitation (Fig. 1). The protective immune serum (Table 2) which lacked detectable antibody to

TABLE 1. Effect of boiled cell antiserum on *Haemophilus* bacteremia in the infant rat model

Serum (dilution)[a]	Anti-LPS (ng per rat)	No. of rats bacteremic/no. challenged	CFU/0.1 ml of blood (geometric mean)[b]
Expt 1			
Preimmune no. 81, absorbed PRP			
1:2	NT[c]	6/6	23,045
Immune no. 81, absorbed PRP			
1:2	3,300	6/6	1,896
1:4	1,650	5/5	3,086
Immune no. 81, absorbed PRP, LPS			
1:2	<100	5/6	1,079
1:4	<50	6/6	8,967
Affinity-purified anti-PRP			
500 ng per rat	NT	0/5	0
Expt 2			
Preimmune no. 75, absorbed PRP			
Undiluted	460	5/5	2,200
Immune no. 75, absorbed PRP			
undiluted	13,400	5/5	110
1:2	6,700	5/5	210
1:4	3,400	5/5	1,200
Immune no. 75, absorbed PRP and LPS			
Undiluted	<160	4/5	30
1:2	<80	5/5	430
1:4	<40	5/5	790

[a] Antiserum was prepared against boiled cells of *H. influenzae* type b strain Eagan (rabbits 75 and 81, see the text) and absorbed with PRP-Sepharose. In experiment 2, all rats received <8 ng of anti-PRP antibody. In experiment 1, anti-PRP antibody levels were not determined.

[b] Levels of bacteremia in animals treated with immune serum were lower than in rats treated with comparable dilutions of preimmune serum ($P < 0.05$). However, the lower levels of bacteremia remained unchanged when animals were treated with comparable dilutions of the same immune serum in which 97% of the anti-LPS antibody had been absorbed ($P > 0.10$).

[c] NT, Not tested.

PRP and LPS contained antibody to at least three or four outer membrane proteins. When this immune serum was further absorbed with a sarcosinate-insoluble preparation of outer membrane proteins from *H. influenzae* type b strain Eagan, most of the antibodies to surface proteins were removed (Fig. 1), and, as already noted, this absorbed antiserum was no longer protective (Table 2). Absorption of the anti-LPS antibody depleted serum with an equal quantity of a Sarkosyl-insoluble outer membrane preparation from *S. typhimurium* had no effect on its protective activity (data not shown).

Previous studies have suggested significant heterogenity in the major outer membrane proteins among different *H. influenzae* type b isolates (4, 18). These observations led us to describe a subtyping scheme for *H. influenzae* type b based on strain differences in outer membrane protein profiles on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4). The protection experiments described above utilized *H. influenzae* type b strain Eagan, a fully encapsulated and virulent prototype 1L strain. We next tested the protective activity of non-PRP, non-LPS antibody present in the antiserum prepared against Eagan, using two other *H. influenzae* type b test organisms: strain 1499, a blood isolate randomly chosen from our collection with the same outer membrane protein subtype (1L) as strain Eagan; and strain Durst, a prototype outer membrane protein subtype 2L organism originally obtained from a patient with meningitis. After intraperitoneal inoculations, all three isolates caused bacteremia in infant rats (Fig. 2). Non-PRP, non-LPS antibody prepared against strain Eagan protected against bacteremia caused by both *H. influenzae* type b strain Eagan and strain 1499 (both subtype 1L) but failed to protect against bacteremia caused by strain Durst (subtype 2L). Anti-PRP antibody (120 ng per rat) conferred protection against all three strains.

DISCUSSION

Antibodies against cell surface antigens of *H. influenzae* type b appear to confer protection against bactermia by either activating comple-

TABLE 2. Effect of anti-outer membrane protein antiserum on *Haemophilus* bacteremia in the infant rat model

Pretreatment (serum dilution)[a]	Protection expt		
	No. of rats bacteremic/ no. challenged	Bacteria per 0.1 ml of blood (geometric mean)	P^b
Preimmune absorbed PRP and LPS			
1:2	5/5	15,600	
Immune absorbed PRP and LPS			
1:8	0/5	0	$<10^{-10}$
1:16	1/5	4	$<10^{-3}$
1:32	5/5	1,200	$<10^{-3}$
Immune absorbed PRP, LPS, and outer membrane protein			
1:2	5/5	2,500	NS[c]
1:4	5/5	9,500	NS
1:8	5/5	11,500	NS

[a] Antiserum was prepared against live cells of *H. influenzae* type b strain Eagan (rabbit 45, see the text) and absorbed with PRP and LPS, both coupled to Sepharose. All rats received <10 ng of anti-PRP antibody.

[b] Levels of bacteremia in each treatment group were compared, using the unpaired *t* test, to levels of bacteremia in rats treated with preimmune serum absorbed with PRP and LPS.

[c] NS, Not significant ($P > 0.05$).

ment-mediated bactericidal activity or by opsonization (3, 7, 9, 31). Fothergill and Wright first demonstrated an inverse relationship between the presence of serum bactericidal activity and the age incidence of *H. influenzae* meningitis in children and adults (7). Subsequent studies suggested that most of the bactericidal antibodies were directed against the type b capsule (1) but investigators more recently have found that bactericidal and opsonic antibodies could also interact with noncapsular antigens (3, 9, 23). However, the protective role of bactericidal antibody to *H. influenzae* type b has been questioned by a number of workers who observed *H. influenzae* type b bacteremia in children or in laboratory animals in the presence of serum bactericidal antibody (5, 26).

Because of the controversy with regard to whether serum bactericidal activity confers protection against *H. influenzae* disease, in the present studies we employed the infant rat model to assess the protective activity of antibodies directed against noncapsular antigens of this organism. In these experiments, only antibodies to PRP and to non-LPS, non-PRP determinants conferred protection against experimental *Haemophilus* bacteremia. Surprisingly, anti-LPS antibody was not protective. Failure of anti-LPS antibodies to prevent bacteremia in infant rats may relate to previously reported observations that, after exposure to serum, *H. influenzae* type b cells develop resistance to the complement-mediated bactericidal effect of anti-LPS antibodies, but not to the bactericidal effect of anti-PRP

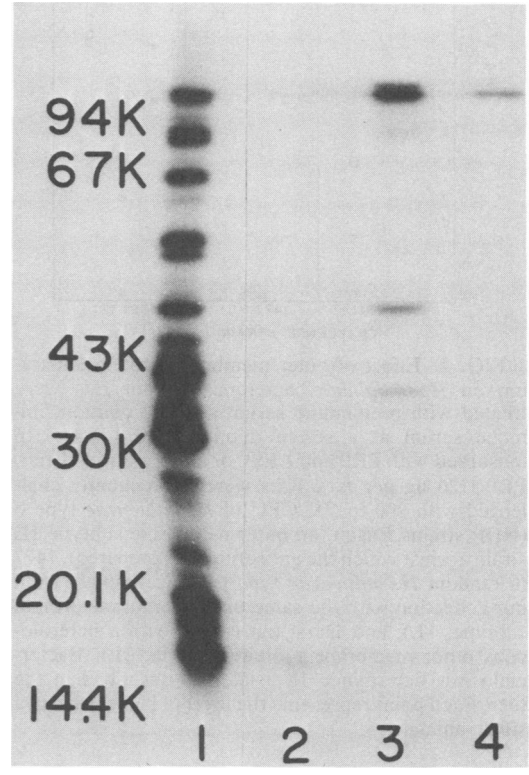

FIG. 1. Radioimmunoprecipitation of surface-exposed *H. influenzae* type b outer membrane proteins according to the method of Hansen et al. (11). Bacterial cells were iodinated (lactoperoxidase) and incubated with preimmune serum (lane 2), hyperimmune rabbit serum absorbed with PRP and LPS (lane 3), or hyperimmune serum absorbed with PRP, LPS, and an outer membrane protein preparation (lane 4). Outer membrane proteins were solubilized from the bacterial cell with detergent, and the preformed immune complexes were precipitated with protein A-Sepharose, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and visualized by autoradiography. For reference, total solubilized iodinated peptides are shown in lane 1. Preimmune serum lacked antibody to outer membrane proteins (lane 2) and was not protective. Antibodies directed at several high-molecular-weight proteins were present in immune serum (lane 3), and this serum protected infant rats against bacteremia caused by the homologous strain (Table 2). Protective activity and antibody to surface-accessible proteins (lane 4) were removed by absorption with the outer membrane protein preparation.

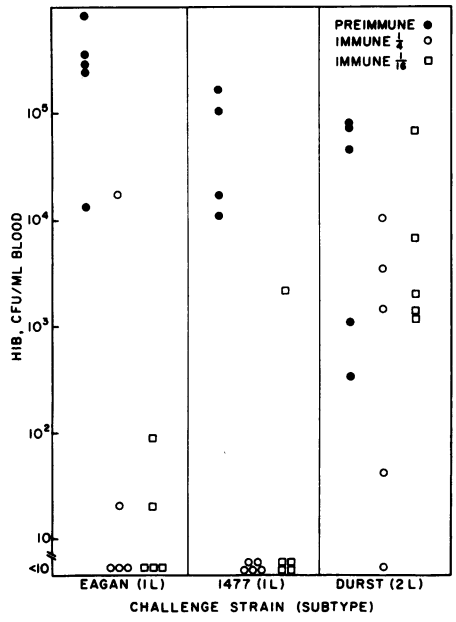

FIG. 2. Effect of outer membrane protein antiserum on *Haemophilus* bacteremia. Infant rats were treated with preimmune serum at a 1:4 dilution, immune serum at a serum dilution of 1:4 and 1:16 (absorbed with PRP and LPS), or affinity-purified anti-PRP (120 ng per rat). Rats were subsequently challenged with 200 to 500 CFU of *H. influenzae* type b (HIB) strains Eagan (an outer membrane subtype 1L strain against which the antiserum was prepared), 1477 (a random *H. influenzae* type b clinical isolate from our collection with the same outer membrane protein subtype, 1L), and Durst (an isolate with a heterologous outer membrane protein subtype, 2L). Bacteremia was determined 18 h later as described in the text. Each point represents the level of bacteremia in a single animal.

antibodies, or possibly to anti-outer membrane protein antibodies (2, 26).

Marks et al. recently reported that passive administration of immune serum prepared against boiled cells of *E. coli* J5, which contained antibody to core LPS, prevented lethality in mice challenged intraperitoneally with *H. influenzae* type b suspended in mucin (21). Bacteremia was not measured in this study. In preliminary experiments by us and others (G. Siber, personal communication), antiserum prepared against *E. coli* J5 failed to prevent or lower the level of bacteremia in infant rats challenged with *H. influenzae* type b (strain Eagan). These preliminary results are consistent with our present data, indicating that homologous anti-LPS antibody does not prevent *Haemophilus* bacteremia in the infant rat model. Taken together with our observations that many children with *H. influenzae* type b meningitis have high levels of *H. influenzae* type b anti-LPS antibodies present in serum obtained at the time of hospital admission (27), it seems likely that anti-LPS antibodies at quantities readily achievable by immunization or infection have a minor role, if any, in the prevention of *H. influenzae* type b bacteremia. However, anti-LPS antibody may prevent some of the adverse effects of endotoxin released during *H. influenzae* type b bacteremia, as suggested by the observation of decreased lethality in infected mice treated with antiserum to boiled *E. coli* J5 cells (21).

In contrast to the results obtained with anti-LPS antibody, immune serum prepared against whole cells and absorbed to remove anti-capsular and anti-LPS antibodies prevented bacteremia in infant rats challenged with *H. influenzae* type b (Table 2). This serum contained antibody directed against surface-accessible determinants of at least three outer membrane proteins. Absorption of this antiserum with a detergent-insoluble fraction enriched in outer membrane proteins removed the majority of the detectable anti-outer membrane protein antibody (Fig. 1) and removed the protective activity (Table 2). Although it is conceivable that antibodies directed against unknown determinants were responsible for this protective activity, it seems more likely that the protective antibodies were directed against one or more outer membrane proteins.

We have recently purified and characterized a 37-kilodalton major outer membrane protein of *H. influenzae* type b (R. S. Munson, Jr., S. J. Barenkamp, J. L. Shenep, D. M. Granoff, J. Clin. Invest., in press). Antisera prepared against this purified protein was protective in the infant rat model and antibody directed against this protein was present in the conventional antiserum used in the present protection experiments (Fig. 1). Also, Hansen and co-workers (13) have recently demonstrated protection with anti-protein monoclonal antibody. Their results and our data document strain differences in relation to protection by antiprotein antibody which require further investigation.

Outer membrane proteins appear to be more immunogenic in human infants than the type b capsule (10). Our data provide further evidence that antibodies against outer membrane proteins, but not LPS, protect against experimental *Haemophilus* bacteremia caused by the homologous organism. These results support further investigation of outer membrane proteins as potential *H. influenzae* type b vaccine candidates.

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