INFECTION AND IMMUNITY, Dec. 1991, p. 4576–4582
0019-9567/91/124576-07$02.00/0

Vol. 59, No. 12

Antibody to a 145-Kilodalton Outer Membrane Protein Has Bactericidal Activity and Protective Activity against Experimental Bacteremia Caused by a Brazilian Purpuric Fever Isolate of *Haemophilus influenzae* Biogroup aegyptius

LORRY G. RUBIN,* ATQIA RIZVI, AND THE BRAZILIAN PURPURIC FEVER STUDY GROUP†

Division of Infectious Diseases, Department of Pediatrics, Schneider Children's Hospital of Long Island Jewish Medical Center, Long Island Campus for Albert Einstein College of Medicine, New Hyde Park, New York 11042

Received 22 May 1991/Accepted 6 September 1991

The immunologic basis for protection against Brazilian purpuric fever, a septicemic infection associated with *Haemophilus influenzae* biogroup aegyptius bacteremia, is unknown. Passive immunization of infant rats with antiserum to whole bacterial cells of the homologous strain protects them from experimental bacteremia following bacterial challenge. In immunoblotting, antibody to a 145-kDa protein (P145) was present in protective antisera but not in nonprotective antisera. As judged by analysis of the antibodies eluted from whole bacterial cells and the agglutination of bacteria by antisera to P145, this protein is surface exposed. We prepared monospecific rat antisera to this protein by three methods: (i) immunization with whole bacterial cells and absorption with a Brazilian purpuric fever strain not expressing P145, (ii) immunization with gel-purified P145, and (iii) immunization with a P145-expressing transformant of a laboratory *H. influenzae* strain expressing this protein and absorption of the antiserum with the laboratory *H. influenzae* strain. These antisera had low antilipooligosaccharide antibody titers, were reactive only with P145, and had bactericidal activity in vitro. Following passive immunization, these antisera partially protected infant rats from bacteremia resulting from intraperitoneal challenge with bacteria. As assessed by immunoblotting, pooled adult human sera contained antibodies reactive with P145. Antibody to P145 may contribute to protection against Brazilian purpuric fever.

Brazilian purpuric fever (BPF) is a recently recognized fulminant infection affecting young children (7). Following recovery from purulent conjunctivitis, patients show acute onset of symptoms, which include fever associated with systemic toxicity and then the development of petechiae, purpura, and vascular collapse. This frequently fatal illness is associated with bacteremia caused by particular strains of *Haemophilus influenzae* biogroup aegyptius, a pathogen long associated with conjunctivitis (7) but not with bacteremia. All isolates of *H. influenzae* biogroup aegyptius from Brazilian children with BPF share certain phenotypic and genotypic features, suggesting that these isolates are clonally related (2, 19). All cases of BPF have occurred in children 3 months to 10 years of age; newborns, adolescents, and adults appear to be immune to this infection. Although antibodies against cell surface antigens play an important role in protection against many bacterial pathogens, the immunologic basis for protection against BPF strains of *H. influenzae* biogroup aegyptius is unknown.

We have recently reported the development of an infant rat model of bacteremia with BPF strains of *H. influenzae* biogroup aegyptius (15). Although this model does not simulate human infection, injection with *H. influenzae* biogroup aegyptius BPF strains caused bacteremia more frequently than did injection with *H. influenzae* biogroup aegyptius strains not associated with BPF. When infant rats were passively immunized with antiserum to whole bacterial cells, they were protected from developing bacteremia following challenge with an *H. influenzae* biogroup aegyptius BPF strain. Antiserum prepared with whole cells of *H. influenzae* biogroup aegyptius BPF isolates afforded protection, while antiserum prepared with conjunctival, *H. influenzae* biogroup aegyptius strains not associated with BPF failed to protect these animals from developing bacteremia (15). Here we report that a cell envelope protein with an apparent molecular mass of 145 kDa is recognized by protective but not by nonprotective antisera. Antisera to this protein have bactericidal activity in vitro and protective activity in an infant rat model of bacteremia.

[This work was presented in part at the 29th Interscience Conference on Antimicrobial Agents and Chemotherapy, 17 to 20 September 1989, and published in abstract form (L. G. Rubin, A. Rizva [sic], and BPF Study Group. 1989. Antibody to a 145 kd outer membrane protein (OMP) of a Brazilian purpuric fever (BPF) strain of *Haemophilus influenzae* biogroup aegyptius (Hae) is associated with protection against experimental bacteremia. Program Abstr. 29th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 348.]

* Corresponding author.

† Members include the following: Gloria Ajello, Robert J. Arko, William F. Bibb, Kristin A. Birkness, Don J. Brenner, Claire V. Broome, George M. Carlone, Robert C. Cooksey, Linda L. Gheesling, Leo Gorelkin, Lee H. Harrison, Leonard W. Mayer, Roger M. McKinney, Stephen O'Connor, Michael W. Reeves, Frances O. Sottnek, Arnold G. Steigerwalt, B. Swaminathan, Jana M. Swenson, Jay D. Wenger, and Robbin S. Weyant, Centers for Disease Control, Atlanta, Ga.; Maria Cristina de Cunto Brandileone, Kinue Irino, Ilka Maria Landgraf, Carmo Elias Andrade Melles, Maria Lucia Cecconi Tondella, Vera Simonsen Dias Vieira, Alexandre Vranjac (deceased), and Eliseu Alves Waldman, Adolfo Lutz Institute, São Paulo, Brazil; David W. Fleming, Oregon State Health Department, Portland, Oreg.; Mituka Kahu, São Paulo State Health Department, São Jose do Rio Preto, Brazil; Ligia Regina Kerr Pontes, São Paulo State Health Department, Ribeirão Preto, Brazil; and Lorry G. Rubin, Schneider Children's Hospital of Long Island Jewish Medical Center, New Hyde Park, N.Y.

MATERIALS AND METHODS

Bacterial strains, growth, and handling. *H. influenzae* biogroup aegyptius BPF strain F3037 was recovered from the blood of a child with BPF and has the "case-clone" characteristics, i.e., has several phenotypic features common to *H. influenzae* biogroup aegyptius isolates from children in Brazil with BPF (2). We found it to be the most virulent of the *H. influenzae* biogroup aegyptius BPF isolates tested in infant rats (15) and used it in these studies. It was passaged through the blood of infant rats prior to use for infant rat inoculation, adult rat immunization, lipooligosaccharide (LOS) purification, and outer membrane protein preparation. We have recently observed that this animal-passaged isolate differs from the original strain in that it is nonpiliated and has an altered LOS phenotype (16). F3031 is an *H. influenzae* biogroup aegyptius BPF case-clone isolate (2) which has a pattern of sarcosinate-insoluble proteins in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) identical to that of F3037 but which lacks a visible protein band at 145 kDa (data not shown). F3052 and NCTC 8502 are *H. influenzae* biogroup aegyptius conjunctival isolates which are not related to the case-clone (2). Eag^{sm} is a streptomycin-resistant mutant of *H. influenzae* type b Eag (1). Rd is a nontypeable laboratory *H. influenzae* strain originally derived from a type d strain (21). *Escherichia coli* ATCC 25922 was used. Bacteria were stored at −70°C, subcultured on chocolate agar plates, grown in brain heart infusion broth supplemented with hemin and diphosphopyridine nucleotide, and processed as previously described (14).

Agglutination. Bacterial cells from an overnight culture of F3037 on a chocolate agar plate were suspended in 0.01 M phosphate-buffered saline (pH 7.2) (PBS) to an optical density at 490 nm of 0.6 (Spectronic 20; Bausch & Lomb, Rochester, N.Y.). Serum (0.01 ml) was mixed with 0.05 ml of bacterial suspension on a glass slide. The slide was rotated for 5 min and examined for agglutination (visible clumping and clearing of the background). PBS mixed with the bacterial suspension was included as a negative control.

SDS-PAGE and immunoblotting. Sarcosinate-insoluble membrane protein preparations were prepared, solubilized by boiling for 5 min in specimen buffer containing SDS and 2-mercaptoethanol, and subjected to SDS-PAGE in 7 or 11% gels as previously described (4). Gels were stained with Coomassie brilliant blue or equilibrated in blotting buffer and electrophoretically transferred (2.5 h; 60 V; Trans-Blot apparatus; Bio-Rad Laboratories, Richmond, Calif.) to 0.45-μm-pore-size nitrocellulose transfer membranes (Schleicher & Schuell, Keene, N.H.) as previously described (20). Immunoblotting was performed by blocking membranes with PBS–0.1% Tween 20–0.1% gelatin for 0.5 h at room temperature. The nitrocellulose was dried and stored at 4°C until used or incubated with rat serum (or sera pooled from four human adult volunteers from the United States) in PBS–0.1% Tween 20 for 1 h at room temperature on a rotating platform. Following five washes with PBS, the nitrocellulose was incubated in peroxidase-conjugated goat anti-rat immunoglobulin G (IgG), IgM, and IgA or peroxidase-conjugated goat anti-human IgG, IgM, and IgA (Organon Teknika Corp., Cappel Research Products, Durham, N.C.) diluted in PBS–0.1% Tween 20 for 1 h at room temperature. Following five washes with PBS, peroxidase substrate (60 mg of 4-chloro-1-naphthol dissolved in 20 ml of methanol and added to 100 ml of Tris-buffered saline [pH 7.5] and 0.06 ml of 30% H_2O_2) was added and the appearance of protein bands was observed. Protein bands were directly identified with the use of colloidal gold total protein stain (Bio-Rad Chemical Division, Richmond, Calif.). To compare LOSs of strains, we prepared proteinase K digests of whole-cell lysates and subjected them to electrophoresis in SDS–urea–4 to 24% gradient polyacrylamide gels as described previously (8). Gels were stained with silver stain as described previously (8).

Animals and techniques. Pathogen-free female Sprague-Dawley albino rats with natural litters were obtained from Harlan-Sprague-Dawley, Frederick, Md. Six-day-old rats were passively immunized by subcutaneous injection on their dorsal surface with 0.1 ml of PBS containing a dilution of serum 3 h prior to intraperitoneal injection with 10^5 CFU of logarithmic-phase F3037. Blood was obtained for culturing following tail vein puncture with a lancet. To obtain serum for use as a complement source in bactericidal studies, we obtained blood from 8- to 10-day-old rats by cardiac puncture following ether anesthesia. Antisera were prepared by immunization of adult female rats by three weekly injections of whole bacteria as previously described (15) or three or four weekly to biweekly subcutaneous injections of polyacrylamide gel slices (from 7% gels loaded with ~1 mg of cell envelope protein containing an estimated 15 μg of P145 per rat per injection) emulsified and combined with Freund's adjuvant (complete Freund's adjuvant for the first injection and incomplete Freund's adjuvant for subsequent injections). Alternatively, rats were immunized in an identical fashion with a similar amount of P145 eluted from P145-containing gel slices (elution conditions: 10 mA; 4.5 h; 0.025 M Tris–0.192 M glycine–0.1% SDS; 422 Electroeluter; Bio-Rad). While anesthetized by ether, rats were bled by cardiac puncture 1 week after the last injection.

DNA preparation and transformation. High-molecular-weight DNAs from F3037 (not animal passaged) and *H. influenzae* Eag^{sm} were prepared with SDS, heat, and proteinase K as previously described (9), except that the RNase step was omitted. Nontypeable *H. influenzae* Rd was made competent with Herriot's MIV medium as described previously (9). Transformation was carried out as described previously (9). Streptomycin-resistant transformants were detected by plating serial dilutions of the transformant mixture on solidified medium supplemented with streptomycin (250 μg/ml). Transformants expressing P145 were detected by a colony blot assay with colonies of the transformant mixture plated on agar plates as described below.

Monoclonal anti-LOS antibodies. Cell culture supernatants containing monoclonal anti-*H. influenzae* LOS antibodies were provided by Eric Hansen, University of Texas Southwestern Medical Center, Dallas (12D9, 8G8, 3D9, 4C4, 6A2, 17E12, 5G8, and 11E7), and William Bibb, Centers for Disease Control, Atlanta, Ga. (B3B1B7 and A4E8E5). The monoclonal antibodies used in the colony blot assays were 12D9, 8G8, B3B1B7, A4E8E5, and 3D9 (reactive with Rd) and 4C4, 6A2, 17E12, 5G8, and 11E7 (nonreactive with Rd). All antibodies except for 17E12 were reactive with *H. influenzae* biogroup aegyptius BPF strain F3037.

Enzyme immunoassay for anti-LOS antibodies. The enzyme immunoassay for anti-LOS antibodies used LOS purified from animal-passaged F3037 (5 μg/ml) to coat the solid phase and was performed as described by Shenep et al. (17). Starting at a dilution of 1:100, serial twofold dilutions were tested; the dilution of test serum which gave an optical density reading equivalent to that of a 1:100 dilution of nonprotective, normal adult rat serum (preimmunization serum) was recorded.

Colony blot assay. Colonies were transferred to Whatman no. 42 filter paper by placing filter paper cut to the appropri-

ate size on the surface of an agar plate containing colonies or by inoculating filter paper with individual colonies by use of a wooden stick or 0.001 ml of a turbid suspension of bacteria. After being dryed, the filter paper was blocked with PBS containing 1% bovine serum albumin for 1 h at room temperature. The filter paper was incubated with antibody (rat anti-F3037 antibody absorbed with control *H. influenzae* biogroup aegyptius F3052 and NCTC 8502 diluted 1:2,000 to detect P145-expressing colonies or monoclonal anti-LOS antibody in cell culture supernatant diluted 1:5 to assess the LOS phenotype) in PBS–0.1% Tween 20 for 1 h at room temperature or 4°C overnight. After five washes with PBS, the filter paper was incubated in peroxidase-conjugated goat anti-rat or goat anti-mouse IgG, IgM, and IgA (Organon Teknika Corp.) diluted 1:1,000 in PBS–0.1% Tween 20 for 1 h at room temperature. After five washes, peroxidase substrate (as described for immunoblotting) was added and color development was observed.

Bactericidal assay. A 0.25-ml reaction mixture was prepared in 0.01 M PBS (pH 7.4) with 1 mM $CaCl_2$–0.5 mM $MgSO_4$–0.1% bovine serum albumin, 10^4 logarithmic-phase *H. influenzae* biogroup aegyptius, serial twofold dilutions of sera (preheated at 56°C for 30 min to inactivate complement activity), and a 20% final concentration of infant rat sera (complement source). The reaction mixture was maintained in an ice water bath during preparation; 0.01 ml was plated in duplicate on chocolate agar plates, and tubes were incubated at 37°C for 60 min in a gyrotary water bath. Tubes were returned to ice water, and 0.01 ml was again plated in duplicate. Plates were incubated at 37°C overnight. Percent killing was calculated as the mean number of CFU at time zero − the mean number of CFU after 60 min of incubation, divided by the mean number of CFU at time zero, multiplied by 100. Results for assays with control tubes containing bacteria and only preheated serum generally showed a net multiplication of organisms during the 60-min incubation period (100 to 200% survival). The bactericidal titer was defined as the highest dilution of serum killing ≥50% of bacteria. Each serum was tested at least twice on separate days.

Absorption and elution of sera. Elution of antibody bound to the surface of bacteria was performed by a method similar to that described by Engleberg et al. (5). Bacteria (10^{10}) were scraped from an overnight culture on a chocolate agar plate, suspended in PBS, and washed once prior to suspension in 0.5 ml of decomplemented sera–0.5 ml of PBS. After incubation for 1 h at 4°C on a rotator platform, the bacteria were pelleted by centrifugation and washed three times. The pellet was resuspended in 0.5 ml of cold 0.2 M NaCl–0.2 M glycine buffer (pH 2.5) and incubated for 15 min at 4°C on a rotator platform. Following centrifugation, the supernatant was filtered through a 0.45-μm-pore-size filter and neutralized by the addition of 1 M $NaHCO_3$. Two procedures were used for absorbing sera with bacteria to remove antibody: ~10^9 bacteria were suspended in 0.5 ml of sera diluted 1:2 in PBS and incubated at 37°C for 1 h. Following centrifugation, the sera were again incubated with 10^9 bacteria at 4°C overnight. The bacteria were removed by centrifugation and filtration through a 0.45-μm-pore-size filter. Alternatively, sera were incubated three times at 4°C with ~10^{11} bacteria for 2 h, 2 h, and overnight (for anti-F3037 absorbed with F3031 and anti-Rd and anti-Rd P145 absorbed with Rd, respectively).

RESULTS

The protective activity of antiserum to whole cells of the homologous strain was preserved when the antiserum was preabsorbed with a control *H. influenzae* biogroup aegyptius strain or a strain of *E. coli* but was partially removed by absorption with the homologous *H. influenzae* biogroup aegyptius BPF strain (Table 1). These results indicated that protection was mediated, at least in part, by antibodies to antigens unique to the case-clone *H. influenzae* biogroup aegyptius BPF strain. We analyzed the reactivity of antisera to sarcosinate-insoluble cell envelope proteins (outer membrane proteins) by immunoblotting. A limited number of proteins were recognized by the antisera (Fig. 1). A protein band located just above the 200,000-dalton marker on an 11% polyacrylamide gel had an apparent molecular mass of 145,000 daltons (P145), as estimated after electrophoresis in a 7% polyacrylamide gel (data not shown), and was recognized by protective antiserum raised against the *H. influenzae* biogroup aegyptius BPF strain but not by antiserum raised against an *H. influenzae* biogroup aegyptius control strain (Fig. 1). We identified immunogenic protein antigens exposed on the bacterial cell surface by absorbing antiserum with bacteria, washing the bacteria, eluting bacterial cell surface-bound antibodies, and analyzing the eluted antibodies in an immunoblot assay (see Materials and Methods). P145 and two or three additional polypeptides, including one with an apparent molecular mass of 46,000 (P46), were detected (Fig. 2) and were therefore surface exposed. To ensure that the eluted antibodies were not contaminated with residual antiserum, the last wash of antiserum-exposed bacteria prior to elution was also assayed; this wash showed no reaction in the immunoblot assay (data not shown). Thus, P145 is an immunogenic protein which appears to be surface exposed. In addition, protective rat antiserum contains antibody to this protein.

To study the biologic activity of antibody to P145, we prepared antisera specific for P145. Monospecific antisera to P145 were prepared in three ways. (i) Rat antiserum to whole bacterial cells was made monospecific for P145 by absorption with strain F3031 (anti-F3037AF3031). (ii) Adult rats were immunized with slices of polyacrylamide containing P145 or buffer containing P145 which had been eluted from gel slices as described in Materials and Methods (anti-P145). Three different lots were prepared and analyzed. (iii) Rats were immunized with a transformant of nonserotypeable

TABLE 1. Protection of infant rats from *H. influenzae* biogroup aegyptius BPF bacteremia by passive immunization with preabsorbed antisera[a]

Rat sera	No. of rats with bacteremia/total no. tested (%)	Geometric mean magnitude of bacteremia (CFU/ml ± SD)[b]
Normal absorbed with F3037	8/10 (80)	160 ± 70
Anti-F3037 sham absorbed	0/10	
Anti-F3037 absorbed with F3037	7/11 (64)[c]	320 ± 720
Anti-F3037 absorbed with NCTC 8502	0/10	
Anti-F3037 absorbed with *E. coli*	0/9	

[a] Rat sera were diluted 1:32 in PBS prior to absorption as described in Materials and Methods. Infant rats were passively immunized by subcutaneous injection of 0.1 ml of a 1:128 final dilution of serum 3 h prior to intraperitoneal injection of 10^5 CFU of logarithmic-phase F3037. Blood was cultured 24 h later.

[b] A value of 50 (midway between 0 and the lower limit of detection, 100 CFU/ml) was used for the calculation of the geometric mean magnitude of bacteremia.

[c] $P < 0.005$ (Fisher's exact test, two-tailed) in comparison with anti-P145 sham absorbed or absorbed with NCTC 8502 or *E. coli*.

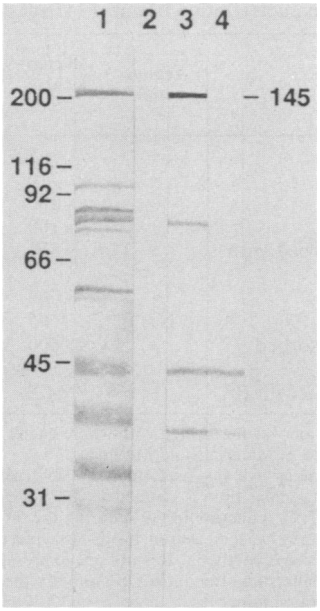

FIG. 1. Immunoblot of sarcosinate-insoluble membrane proteins from *H. influenzae* biogroup aegyptius BPF strain F3037 with rat sera following SDS-PAGE. Sera were diluted 1:100 for analysis. Lanes: 1, protein bands revealed by protein stain; 2, normal sera; 3, antisera to whole cells of F3037; 4, antisera to whole cells of control strain NCTC 8502. Molecular mass markers (in kilodaltons) are indicated on the left.

laboratory strain of *H. influenzae*, Rd, expressing P145. This transformant was prepared with chromosomal DNA from strain F3037 and was detected by a colony blot assay (see Materials and Methods). The transformation rates were approximately 10^{-6} for P145 and 10^{-4} for streptomycin resistance (with donor DNA from streptomycin-resistant *H. influenzae* Eag). The transformant (Rd/P145) and Rd had

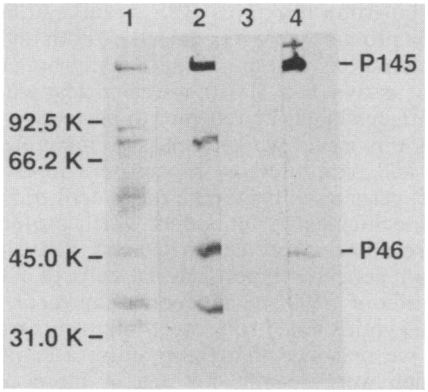

FIG. 2. Immunoblot analysis of antibodies to cell surface-exposed epitopes of cell envelope proteins of BPF strain F3037. Sarcosinate-insoluble membrane proteins were subjected to SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. Lanes: 1, protein bands stained by colloidal gold; 2, antisera to whole cells of F3037 tested at a 1:1,000 dilution; 3, normal sera eluted from F3037 and tested at a 1:40 dilution; 4, anti-F3037 sera eluted from F3037 and tested at a 1:40 dilution. Molecular mass markers (in kilodaltons) are indicated on the left. P145 and P46 (a polypeptide with an apparent molecular mass of 46 kDa) are indicated on the right.

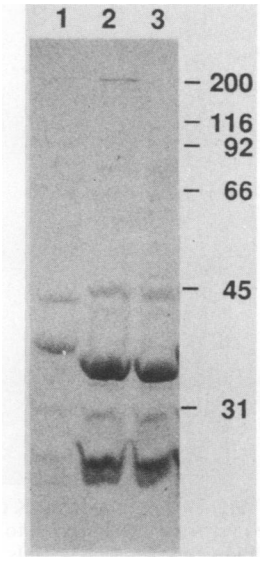

FIG. 3. Comparison of sarcosinate-insoluble membrane proteins from the DNA donor strain, F3037 (lane 1), transformant Rd/P145 (lane 2), and Rd (lane 3). Following PAGE, protein bands were revealed by staining of gels with Coomassie blue. P145 (near the 200-kDa marker) can be seen in lanes 1 and 2. Molecular mass markers (in kilodaltons) are indicated on the right.

identical electrophoretic patterns for proteins following SDS-PAGE of sarcosinate-insoluble cell envelope proteins or whole-cell lysates, except that P145 was detectable in the transformant (Fig. 3). The LOS phenotype of Rd/P145 was identical to that of Rd (and distinct from that of F3037), as judged by the electrophoretic mobilities of LOS bands visualized by silver staining following electrophoresis of proteinase K-treated whole-cell lysates of bacteria following SDS-urea-polyacrylamide gel electrophoresis (Fig. 4) and by comparison of the reactivities of these strains in a colony blot assay with a panel of 10 monoclonal antibodies (reactivity with Rd was assessed as described in Materials and Methods). Antiserum to Rd/P145, which contained antibodies reactive with several proteins, was absorbed with strain Rd to remove antibodies directed against Rd and to leave only antibodies reactive with P145. Antiserum to Rd was prepared and absorbed with strain Rd in an identical fashion for use as a control preparation.

We analyzed these antisera to determine whether they were monospecific. Figure 5 illustrates immunoblot analyses of the anti-P145 antisera prepared by the three methods. In some blots assaying antisera to gel-purified P145, faint bands corresponding to proteins with apparent molecular masses of approximately 46 kDa or a lower-molecular-mass protein were visualized. The anti-LOS antibody concentration was assessed by an enzyme immunoassay with LOS purified from F3037 to coat the solid phase. Antisera to whole cells of F3037 or purified LOS had 250- and 125-fold higher concentrations of anti-LOS antibody than did normal rat sera. In contrast, anti-P145 sera prepared with absorbed antisera to whole cells of F3037 or the P145-expressing transformant had concentrations of anti-LOS antibody similar to or lower than those in normal rat sera (Table 2). Antisera to gel-purified P145 had a two- to eightfold-higher concentration of anti-LOS antibody than did normal rat sera. Most of these anti-P145 antisera agglutinated strain F3037, and all had complement-dependent bactericidal activity against F3037

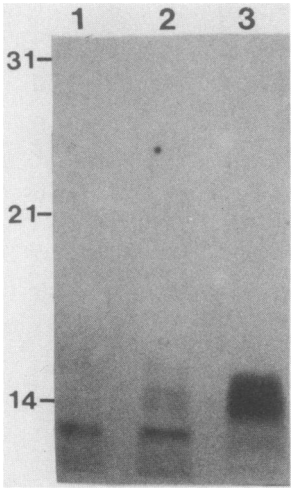

FIG. 4. LOS of *H. influenzae*. Proteinase K digests of whole-cell bacterial lysates were prepared and subjected to electrophoresis in a urea-SDS-polyacrylamide (gradient) gel. Gels were stained with silver. Lanes: 1, recipient *H. influenzae* Rd; 2, transformant Rd/P145; 3, DNA donor strain, F3037. The locations of protein molecular mass standards (in kilodaltons) are indicated on the left.

(Table 2). The bactericidal activity of the monospecific absorbed antisera was approximately fourfold lower than that of the unabsorbed antisera to whole bacteria (Table 2).

All three anti-P145 antibody preparations partially protected against bacteremia when administered passively to infant rats prior to bacterial challenge in comparison with rats passively immunized with their appropriate control serum preparations (Table 3). These results were evident in an assessment of both the incidence and the magnitude of bacteremia in comparison with immunization with an appropriate rat control serum preparation.

Pooled, adult human serum was analyzed by immunoblotting against an outer membrane protein preparation. P145 was recognized by this serum (Fig. 6).

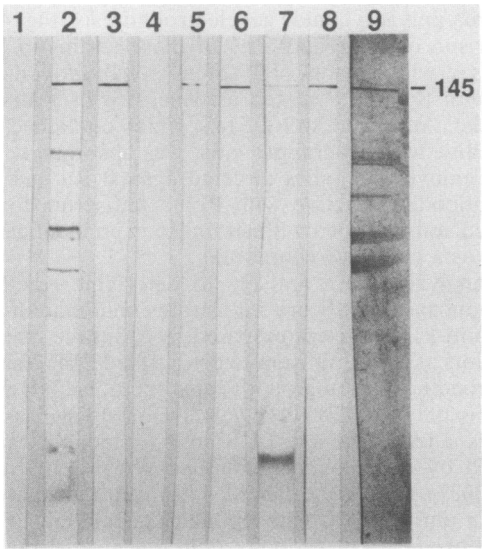

FIG. 5. Analyses of rat antisera to P145 by immunoblotting. A 1:200 dilution of antisera was reacted with sarcosinate-insoluble membrane proteins following SDS-PAGE and electrophoretic transfer to a nitrocellulose membrane. Lanes: 1, normal sera; 2, antisera to whole cells of strain F3037 (anti-F3037); 3, anti-F3037 absorbed with F3031; 4, anti-Rd absorbed with Rd; 5, anti-Rd/P145 absorbed with Rd; 6, antisera to P145 eluted from gel slices; 7 and 8, two lots of antisera to gel slices containing P145; 9, protein bands revealed by protein stain. The location of P145 is indicated on the right.

TABLE 2. Characterization of anti-P145 rat and control sera

Rat sera	Agglutination[a]	Bactericidal titer[b]	Relative anti-LOS antibody titer (no. of twofold increases)[c]
Normal	−	<5	1
Anti-P145 lot a[d]	−	10	3
Anti-P145 lot b[d]	−	100	3
Anti-P145 lot c[d]	+	100	2
Anti-F3037 absorbed with F3031	+	800	1
Anti-F3037	+	6,400	8
Anti-LOS	ND[e]	<5	7
Anti Rd/P145 absorbed with Rd	+	1,600	<1
Anti Rd absorbed with Rd	−	<5	<1

[a] Slide agglutination was performed with whole bacterial cells of F3037 and rat sera as described in Materials and Methods.

[b] The bactericidal assay was performed as described in Materials and Methods. The bactericidal titer is defined as the reciprocal of the highest dilution showing a 50% reduction in CFU during the 60-min incubation.

[c] Anti-LOS antibody was measured by an enzyme immunoassay as described in Materials and Methods. Results are expressed as the number of twofold dilutions higher than the anti-LOS antibody titer detected in normal (preimmunization) rat serum.

[d] Assays were done with three independent lots of antiserum to gel-purified P145. Lots a and b were from rats immunized with homogenized gels, and lot c was from rats immunized with P145 eluted from gels (see Materials and Methods). The results of infant rat protection assays, shown in Table 3, line 2, are the combined results of experiments with these three antisera.

[e] ND, not done.

DISCUSSION

Little is known about the role of serum antibody in the protection of children against bacteremia caused by *H. influenzae* biogroup aegyptius BPF strains. Although LOS and multiple proteins were recognized by both rat antisera to whole bacterial cells (Fig. 1) and adult human sera in immunoblot assays (Fig. 5) (12), it was unclear which, if any, of these antigens might be relevant to protective immunity. To address this issue, we used passive immunization with protective and nonprotective rat antisera in an infant rat model of experimental bacteremia as a tool to identify the antigenic specificities of antibodies which protect rats and might be relevant to protection against BPF in children. Since rat antisera to whole bacterial cells of F3037 had a protective titer of >500 and antisera to control *H. influenzae* biogroup aegyptius failed to protect infant rats from bacteremia (15), we reasoned that these antisera contained antibodies which were specific for one or more cell surface antigens and which afforded protection. Candidate antigens included cell surface-exposed proteins such as outer membrane proteins, pili, and putative surface array protein (18), LOS, and capsule. We showed that rat antisera to purified LOS did not have protective activity in this model system (11). Therefore, it is unlikely that anti-LOS antibodies contributed to the protective activity of rat antisera to whole bacterial cells. A polysaccharide capsule has not been convincingly demonstrated on these strains (3). Animal-passaged F3037 used for rat challenge and for immunization is

TABLE 3. Activity of passively administered anti-P145 sera in protection of infant rats from bacteremia[a]

Rat sera	No. of rats with bacteremia/ total no. tested (%) [P^b]	Geometric mean magnitude of bacteremia (CFU/ml ± SD)[c] [P^d]
Normal	50/63 (79)	3,630 ± 890
Anti-gel-purified P145	35/64 (55) [0.004]	210 ± 760 [$<10^{-4}$]
Anti-whole-cell F3037	0/50	
Normal absorbed with F3037	20/20 (100)	6,170 ± 390
Anti-whole-cell absorbed with F3031	13/20 (65) [0.009]	4,790 ± 1,700 [$<10^{-5}$]
Anti-whole-cell F3037	0/20	
Anti-whole-cell Rd absorbed with Rd	18/20 (90)	3,470 ± 2,040
Anti-whole-cell Rd/P145 absorbed with Rd	9/20 (45) [0.006]	280 ± 1,230 [0.01]
Anti-whole-cell F3037	0/10	

[a] Rat serum absorption was performed as described in Materials and Methods. Infant rats were passively immunized by subcutaneous injection of 0.1 ml of a 1:8 dilution of serum 3 h prior to intraperitoneal injection of 10^5 CFU of logarithmic-phase F3037. Blood was cultured 24 h later.
[b] Fisher's exact test, two-tailed, in comparison with rats immunized with control sera (indicated on the line above).
[c] A value of 50 (midway between 0 and the lower limit of detection, 100 CFU/ml) was used for the calculation of the geometric mean magnitude of bacteremia.
[d] Mann-Whitney t test, two-tailed; in comparison with rats immunized with control sera (indicated on the line above).

hemagglutination negative and appears nonpiliated in transmission electron microscopy (13). Therefore, it is unlikely that antipilus antibodies contributed to protection in these experiments. We therefore focused our attention on sarcosinate-insoluble cell envelope proteins (which are usually outer membrane proteins) because these proteins are frequently exposed on the bacterial cell surface and because antibodies to outer membrane proteins of *H. influenzae* type b or nontypeable *H. influenzae* have often been found to have bactericidal activity and/or protective activity in the infant rat model (6).

Immunoblot analyses following electrophoresis of sarcosinate-insoluble protein preparations showed that protective and nonprotective antisera recognized the same protein bands, except for a protein of approximately 145 kDa which was recognized by the protective sera but not by the nonprotective sera. Furthermore, immunoblot analyses of cell surface-bound antisera eluted from bacterial cells indicated that P145 appeared to be surface exposed. We used three independent strategies in an attempt to produce monospecific rat antisera to this protein. Since each antiserum preparation had limitations, the protection observed with each of the three procedures used to prepare monospecific antisera made it likely that antibody to P145 was indeed protective and that protection was not due to antibody specific for a contaminating antigen. The use of polyacrylamide gels to purify P145 for use as an immunogen was attractive because the migration of this high-molecular-mass protein was slow in gels and P145 was well separated from other protein bands (especially in gels with a 7% acrylamide concentration). A disadvantage of this technique is that the polypeptides injected into the animal are likely to be in a reduced and denatured form because of specimen pretreatment prior to PAGE, and cell surface-exposed antigenic determinants reactive with protective antibody may be lost. We immunized with P145 eluted from gel slices in an attempt to obtain a partially renatured protein; antisera prepared by this method agglutinated F3037 (Table 2) but had no greater bactericidal (Table 2) or protective activity than did antisera obtained by direct injection with emulsified gel slices. Some of these antisera had a modest increase in anti-LOS antibody titer which could possibly have accounted for the protective activity observed (although antisera to whole bacterial cells had a much higher anti-LOS antibody titer, and high-titer anti-LOS sera prepared against purified LOS lacked protective and bactericidal activities (Table 2) (10). We attempted to make protective antisera to whole cells of strain F3037 monospecific for P145 by absorption with strain F3031, a case-clone *H. influenzae* biogroup aegyptius BPF strain with an SDS-PAGE pattern for sarcosinate-insoluble membrane proteins identical to that of strain F3037, with the exception of P145 expression. These antisera had anti-LOS antibody titers similar to that of nonprotective, nonbactericidal normal adult rat serum. The decrease in the bactericidal titers of these antisera as compared with nonabsorbed antisera to whole cells of F3031 (from 6,400 to 800; Table 2) suggests that bactericidal activity is due to antibodies directed at other cell surface antigens in addition to P145. The use of the P145-expressing Rd transformant for immunization was attractive because P145 was separated from other antigens specific for F3037 and because the tertiary structure and cell

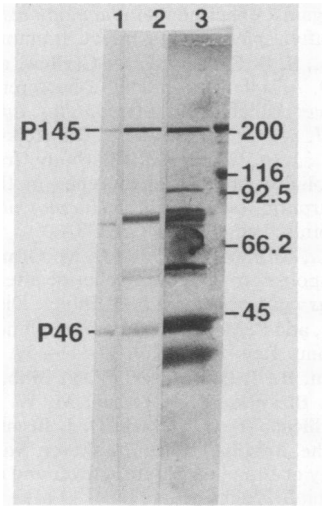

FIG. 6. Analysis of pooled adult human serum by immunoblotting. The assay was performed as described in the legend to Fig. 5 and in Materials and Methods. Lanes: 1, pooled normal human serum, 1:100; 2, rat antisera to whole cells of animal-passaged strain F3037, 1:1,000; 3, total protein bands visualized by colloidal gold stain. The locations of P145 and P46 are indicated on the left. The locations of protein molecular mass markers (in kilodaltons) are indicated on the right.

surface-exposed antigenic determinants of P145 were likely to be intact. Since high-molecular-weight DNA was used for transformation, it is possible that genes encoding other cell surface-exposed antigens in addition to P145 were simultaneously transferred. However, we were unable to detect differences in PAGE protein profiles and the LOS phenotypes of transformed Rd versus Rd. The absorption of this preparation with Rd cells lowered the anti-LOS antibody concentration to less than that of nonprotective normal rat sera so that it is extremely unlikely that the protection observed with this preparation was due to anti-LOS antibody. As noted in Fig. 2, some lots of antisera had limited reactivity with lower-molecular-mass polypeptides, including one of approximately 46 kDa. Although it is possible that the protective antibody was actually directed against this smaller polypeptide rather than against P145, the much higher antibody concentrations of anti-P145 preparations (judging by the intensity of the bands) and the inconstant presence of this antibody in various anti-P145 preparations make this possibility unlikely. It is possible that there is immunologic cross-reactivity or an association between these polypeptides or even that the 46-kDa polypeptide is a subunit or an incompletely denatured form of P145. Furthermore, attempts to prepare monospecific antiserum to gel slices containing polypeptides with apparent molecular masses of 30 to 65 kDa frequently resulted in antisera reactive with P145 (12).

The biologic function of P145 is unknown. The high apparent molecular weight and the bacterial cell surface exposure are consistent with the hypothesis that P145 is a surface array protein or protein capsule (18), although direct evidence to support this hypothesis is not available.

For a cell envelope protein to serve as a protective antigen which might be useful as a vaccine candidate, the protein must be immunogenic and must be exposed on the bacterial cell surface, where it can be recognized by antibodies. Furthermore, antibodies elicited to this cell surface component must be biologically active; i.e., they must have bactericidal and/or opsonic activity to be protective. P145 and anti-P145 antibody appear to fulfil these criteria, although the relevance of the data generated with animal sera is unknown. Our preliminary studies indicate that adult human sera contain antibodies which recognize P145 in immunoblot assays (Fig. 6). Other prerequisites for a vaccine candidate are that the cell surface antigen must be conserved among strains; P145 is not detected by PAGE of sarcosinate-insoluble membrane protein preparations of *H. influenzae* biogroup aegyptius BPF strain F3037. Because BPF occurs in young children, the antigen must be immunogenic in young children. Further studies are needed to address these issues.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Faculty Awards program of Long Island Jewish Medical Center.

We thank Eric Hansen and William Bibb for providing the monoclonal anti-LOS antibodies used in this study.

REFERENCES

1. **Anderson, P., R. B. Johnston, Jr., and D. H. Smith.** 1972. Human serum activities against *Haemophilus influenzae* type b. J. Clin. Invest. **51**:31–38.
2. **Brenner, D. J., L. W. Mayer, G. M. Carlone, L. H. Harrison, W. F. Bibb, M. C. de Cunto Brandileone, F. O. Sottnek, K. Irino, M. W. Reeves, J. M. Swenson, K. A. Birkness, R. S. Weyant, S. F. Berkley, T. C. Woods, A. G. Steigerwalt, P. A. D. Grimont, R. M. McKinney, D. W. Fleming, L. L. Gheesling, R. C. Cooksey, R. J. Arko, C. V. Broome, and the Brazilian Purpuric Fever Study Group.** 1988. Biochemical, genetic, and epidemiologic characterization of *Haemophilus influenzae* biogroup aegyptius (*Haemophilus aegyptius*) strains associated with Brazilian purpuric fever. J. Clin. Microbiol. **26**:1524–1534.
3. **Carlone, G. M., L. Gorelkin, L. L. Gheesling, A. L. Erwin, S. K. Hoiseth, M. H. Mulks, S. P. O'Connor, R. S. Weyant, J. Myrick, L. Rubin, R. S. Munford III, E. H. White, R. J. Arko, B. Swaminathan, L. M. Graves, L. W. Mayer, M. K. Robinson, S. P. Caudill, and the Brazilian Purpuric Fever Study Group.** 1989. Potential virulence-associated factors in Brazilian purpuric fever. J. Clin. Microbiol. **27**:609–614.
4. **Carlone, G. M., F. O. Sottnek, and B. D. Plikaytis.** 1985. Comparison of outer membrane protein and biochemical profiles of *Haemophilus aegyptius* and *Haemophilus influenzae* biotype III. J. Clin. Microbiol. **22**:708–713.
5. **Engleberg, N. C., E. Pearlman, D. Dixon, and B. I. Eisenstein.** 1986. Antibodies isolated by using cloned surface antigens recognize antigenically related components of Legionella pneumophila and other Legionella species. J. Immunol. **136**:1415–1417.
6. **Granoff, D., and R. S. Munson, Jr.** 1986. Prospects for prevention of *Haemophilus influenzae* type b disease by immunization. J. Infect. Dis. **153**:448–461.
7. **Harrison, L. H., G. A. da Silva, M. Pittman, D. W. Fleming, A. Vranjac, C. V. Broome, and the Brazilian Purpuric Fever Study Group.** 1989. Epidemiology and clinical spectrum of Brazilian purpuric fever. J. Clin. Microbiol. **27**:599–604.
8. **Kimura, A., and E. J. Hansen.** 1986. Antigenic and phenotypic variations of *Haemophilus influenzae* type b lipopolysaccharide and their relationship to virulence. Infect. Immun. **51**:69–79.
9. **Moxon, E. R., R. A. Deich, and C. Connelly.** 1984. Cloning of chromosomal DNA from *Haemophilus influenzae*. Its use for studying the expression of type b capsule and virulence. J. Clin. Invest. **73**:298–306.
10. **Peters, V., L. Rubin, and BPF Study Group.** Unpublished data.
11. **Peters, V. P., L. G. Rubin, and BPF Study Group.** 1989. Anti-lipoligosaccharide antibody does not protect against experimental bacteremia with a Brazilian purpuric fever isolate of *Haemophilus influenzae* biogroup aegyptius. Pediatr. Res. **25**:187A.
12. **Rubin, L.** Unpublished observations.
13. **Rubin, L., and J. St. Geme.** Unpublished observation.
14. **Rubin, L. G.** 1988. Anticapsular antibody requirements for protection against experimental *Haemophilus influenzae* type b bacteremia after splenectomy. Infect. Immun. **56**:984–986.
15. **Rubin, L. G., E. S. Gloster, G. M. Carlone, and the BPF Study Group.** 1989. An infant rat model of bacteremia with Brazilian purpuric fever isolates of *Haemophilus influenzae* biogroup aegyptius (*H. aegyptius*). J. Infect. Dis. **160**:476–482.
16. **Rubin, L. G., J. St. Geme, and BPF Study Group.** 1991. Role of pili and lipoligosaccharide phenotypes in the virulence of a Brazilian purpuric fever strain of *Haemophilus influenzae* biogroup aegyptius. Pediatr. Res. **29**:138A.
17. **Shenep, J. L., R. S. Munson, and D. M. Granoff.** 1982. Human antibody responses to lipopolysaccharide after meningitis due to *Haemophilus influenzae* type b. J. Infect. Dis. **145**:181–190.
18. **Sleytr, U. B., and P. Messner.** 1983. Crystalline surface layers on bacteria. Annu. Rev. Microbiol. **37**:311–339.
19. **Swaminathan, B., L. W. Mayer, W. F. Bibb, G. W. Ajello, K. Irino, K. A. Birkness, C. F. Garon, M. W. Reeves, M. C. de Cunto Brandileone, F. O. Sottnek, D. J. Brenner, A. G. Steigerwalt, and the Brazilian Purpuric Fever Study Group.** 1989. Microbiology of Brazilian purpuric fever and diagnostic tests. J. Clin. Microbiol. **27**:605–608.
20. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
21. **Zwahlen, A., J. A. Winkelstein, and E. R. Moxon.** 1983. Participation of complement in host defense against capsule-deficient *Haemophilus influenzae*. Infect. Immun. **42**:708–715.