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Biologic Activities of Antibody to a Peptidoglycan-Associated Lipoprotein of *Haemophilus influenzae* against Multiple Clinical Isolates of *H. influenzae* Type b

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A peptidoglycan-associated lipoprotein of about 15 kilodaltons was purified from the outer membranes of *Haemophilus influenzae* by using nondenaturing detergents. To assess its vaccine potential, rabbit antiserum to the purified protein was obtained. The antiserum was specific for the peptidoglycan-associated lipoprotein in whole cell lysates of *H. influenzae* and was bactericidal for *H. influenzae* types a, b, d, e, and f and for 181 of 182 *H. influenzae* type b clinical strains isolated in widely dispersed geographic areas. The antibody protected infant rats from challenge with each of five clinical *H. influenzae* type b isolates and was additive to and did not interfere with bactericidal and protective activities of antibody against the type b capsule. These data indicate that the purified peptidoglycan-associated lipoprotein is a potentially valuable vaccine candidate for *H. influenzae* type b disease and may enhance the effectiveness of preexisting anticapsular antibody.

Haemophilus influenzae organisms are a major pathogen of humans, both adults and children. *H. influenzae* type b (Hib) is a common causative agent of meningitis of young children (23), and nontypable *H. influenzae* is regularly implicated in sinusitis (8), otitis media (5), neonatal sepsis (29), and bronchitis (21).

A vaccine composed of the capsule of Hib, polyribosyl ribotol phosphate (PRP), has been licensed. This vaccine is effective in children over 18 months of age but is ineffective in younger children. Vaccines composed of saccharide-protein conjugates are being evaluated in field trials, and results suggest that the conjugates are immunogenic in infants (4). However, the conjugate vaccines as now formulated are not effective against nontypable *H. influenzae*.

These problems have led investigators to study other *H. influenzae* antigens for their vaccine potential. One of these antigens, an approximately 16,000-dalton outer membrane protein (OMP) called P6 by some investigators, has been shown to be present in every *H. influenzae* isolate examined, both typable and nontypable (20), and is reported to be antigenically conserved among strains (18). Recent reports have indicated that antibodies which react with P6 are present in human serum and that these antibodies are bactericidal against nontypable *H. influenzae* (19). Munson and Granoff (17) isolated P6 by using sodium dodecyl sulfate (SDS) and high salt. When they immunized rabbits with a P6-cell wall complex, they reported that the anti-P6 antibody was protective against the Hib strain from which the P6 was isolated.

P6 remains associated with the peptidoglycan after extraction of a protein-cell wall complex with SDS at 37°C (17) and recently has been reported to contain fatty acids (G. A. Weinberg, D. Towler, and R. S. Munson, Abstr. Annu. Meet. Amer. Soc. Microbiol. 1987, K142, p. 226). Thus the P6 OMP can be classified as a peptidoglycan-associated lipoprotein (PAL; for a review of PALs, see reference 15).

In this manuscript we describe the isolation and purification of an approximately 15-kilodalton PAL from the outer membranes of *H. influenzae* by using nondenaturing detergents. In a separate manuscript, the chemical and physical properties of this protein will be described and the protein will be characterized as a PAL (G. W. Zlotnick, V. T. Sanfilippo, J. A. Mattler, D. H. Kirkley, R. A. Boykins, and R. Seid, manuscript in preparation). To assess its vaccine potential, specific rabbit antiserum to the purified protein was prepared and used to determine (i) the distribution of bactericidal epitopes among a variety of Hib isolates from various geographic areas, (ii) the potential protective activity of anti-PAL antibody against multiple Hib clinical isolates, and (iii) the effect, if any, of anti-PAL antibody on the bactericidal and protective activities of anti-PRP antibody.

MATERIALS AND METHODS

Bacteria. Clinical isolates of Hib and nontypable *H. influenzae* were kindly provided by Porter Anderson, Rochester, N.Y., Eric Hansen, Dallas, Tex., William Albritton, Saskatoon, Saskatchewan, Canada, and Robert Daum, New Orleans, La. *H. influenzae* isolates were subcultured once by overnight incubation at 37°C on brain heart infusion agar (BHI; Difco Laboratories, Detroit, Mich.) supplemented with hemin (Sigma Chemical Co., St. Louis, Mo.) at 10 μg/ml and NAD (Sigma) at 2 μg/ml (BHI-XV). Isolates were stored without further passage at −70°C in BHI containing 20% glycerol.

Purification of PAL. PAL was purified from Hib strain Eagan grown in BHI-XV broth medium. After incubation at 37°C with aeration, cells were harvested by centrifugation and frozen at −20°C. Frozen cells were thawed at 37°C, and a 20% (wt/vol) suspension made in 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4, containing 1 mM disodium EDTA (buffer A). The cell suspension was homogenized by sonication of 100-ml samples with a Branson model 350 sonifier cell disruptor (Branson Sonic Power, Danbury, Conn.) at 60% power for 5 min on ice. Unbroken cells were removed by centrifugation at 10,000 × *g* for 5 min at 4°C. Unbroken cells were suspended in half the original volume of buffer A and sonicated as before. Remaining unbroken cells were removed by centrifugation, and the supernatants from the low-speed centrifugations were pooled. The supernatants

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were adjusted to 0.5 M NaCl and centrifuged at 300,000 × *g* for 1 h at 4°C. The cell envelope pellet obtained from this centrifugation was suspended in 10 mM HEPES, pH 7.4 (buffer B), adjusted to 1% sodium lauroyl sarcosinate (sarcosyl), and extracted at room temperature for 30 min. Sarcosyl-insoluble material was pelleted by centrifugation at 300,000 × *g* for 30 min at 4°C, and the sarcosyl extraction was repeated.

Sarcosyl-insoluble material was suspended in 50 mM Tris hydrochloride (pH 8.0)–5 mM EDTA (buffer C) and extracted twice with a final concentration of 1% (wt/vol) Zwittergent 3-16 for 30 min at room temperature followed by centrifugation as above. Zwittergent-insoluble material was extracted twice more with 1% sarcosyl for 30 min at 23°C in buffer C. The remaining insoluble material was suspended in buffer C at one-fifth the volume of the original suspension and stored at 4°C.

The insoluble material from above was pelleted by centrifugation at 300,000 × *g* for 30 min at 4°C. The pellet was suspended in buffer C at a final protein concentration of 5 to 8 mg/ml, adjusted to 1% (wt/vol) dodecyl-β-D-maltoside, and extracted for 30 min at room temperature. The insoluble material was pelleted by centrifugation at 450,000 × *g* for 30 min. PAL was solubilized by repeating the above extraction at 55°C. Insoluble cell wall material was removed by centrifugation at 250,000 × *g* in a swinging-bucket rotor for 1 h at 10°C. Solubilized PAL was stored at 4°C.

Protein determination. Protein was determined by the method of Lowry et al. (14) as modified by Peterson et al. (24).

Antisera. Antiserum to PAL was produced in New Zealand White rabbits by intramuscular injection of 10 μg of purified protein emulsified in Freund incomplete adjuvant (Difco). Blood was taken before immunization and was the source for normal rabbit serum (NRS). Animals received booster injections on days 14 and 21 after the primary immunization. Animals were bled 10 days after the last injection, and sera were tested for anti-PAL antibodies by an enzyme-linked immunosorbent assay and Western blot (immunoblot) analysis.

Antiserum to PRP was produced in rabbits by using oligosaccharides prepared by periodate oxidation of PRP linked to the nontoxic diphtheria toxin-related protein CRM_{197} by the reductive amination method of Anderson (1). Rabbits were injected and serum were obtained as described above.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was performed in a 70- by 100-mm minigel system (Bio-Rad Laboratories, Richmond, Calif.) by the method of Laemmli (13). Samples were reduced with β-mercaptoethanol in sample preparation buffer and boiled for 5 min. Gels were run at 150 V (constant voltage). Separated proteins were detected by staining with Coomassie brilliant blue G-250 (Sigma).

Lipooligosaccharide (LOS) was detected in SDS-PAGE gels by the silver staining method of Tsai and Frasch (28).

Western blot analysis. Proteins were transferred to nitrocellulose by a modification of the method of Towbin et al. (27). Proteins separated by SDS-PAGE were electrophoretically transferred at 100 V for 50 min to nitrocellulose (0.45-μm pore size; Schleicher & Schuell Co., Keene, N.H.) in buffer containing 25 mM Tris–384 mM glycine at 4°C. Protein-binding sites were blocked by incubating filters in 50 mM Tris (pH 7.5)–0.15 M NaCl containing 5% nonfat dry milk (wt/vol) (BLOTTO) (11). Rabbit polyclonal antiserum was diluted in BLOTTO and used to probe the filter by incubating at 37°C for 1 h. Filters were washed three times in BLOTTO for 10 min per wash to remove unbound antibody. Bound antibody was detected by incubating the filters for 1 h at 37°C in BLOTTO containing horseradish peroxidase conjugated to goat anti-rabbit immunoglobulin G (IgG; heavy and light chain specific; Kirkegaard and Perry, Gaithersburg, Md.). Three additional 10-min washes in BLOTTO were done, and the blots were developed by incubating at room temperature in a solution of 50 mM Tris (pH 7.0)–0.15 M NaCl containing 0.06% (wt/vol) 4-chloro-1-naphthol (Sigma) and 0.015% H_2O_2. The reaction was stopped by washing in distilled water.

Monoclonal antibody. Spleen cells from C57BL/6 mice immunized with PAL were fused to mouse myeloma cell line X63.Ag8.6543 by the method of Gefter et al. (9). Desired hybridomas were then recloned by limiting dilution (16) and screened against desired antigens by Western blot analysis. Selected hybridomas were injected into BALB/c mice for growth as ascites (7).

Bactericidal assays. Serum bactericidal assays were performed by a modification of the method of Anderson et al. (3). Bacteria were prepared by diluting overnight Hib cultures 1:15 in BHI-XV broth at 37°C with aeration. Cells were grown to an optical density at 490 nm of 1.0 (approximately 10^9 CFU/ml). Bacteria were diluted 16,000-fold in sterile phosphate-buffered saline containing 0.15mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PCM). The bacterial cell suspension was held at room temperature for no more than 45 min. Rabbit polyclonal antiserum was heated to 56°C for 20 min to remove complement activity. Twenty microliters of antiserum (diluted 1:10 in PCM) were placed in the first well of a 24-well microtiter plate (Becton Dickenson Labware, Oxnard, Calif.) held on ice. Twofold serial dilutions in PCM were done in the remaining wells. Twenty microliters of the undiluted complement source, precollostral calf serum (VIDO, Saskatoon, Saskatchewan, Canada), was added to each well when Hib cells were used. When nontypable strains were used, the complement was diluted 1:4 in PCM before use. The plate was removed from ice, and 20 μl of the bacterial suspension was added to each well. Plates were incubated at 37°C for 30 min, and 800 μl of BHI-XV containing 0.75% agar (Difco) at 45°C was added to each well. Agar was allowed to solidify at room temperature, and the plates were incubated at 37°C overnight. The bactericidal titer was reported as the reciprocal of the highest dilution of antiserum capable of killing greater than 50% (by visual inspection) of the viable cells as compared with control wells containing no antibodies.

Infant-rat studies. Passive immunization studies were done with the infant-rat meningitis model previously described by Smith et al. (25). The 100% lethal dose of Hib challenge strains was determined by sham-immunizing 4-day-old Sprague-Dawley rats intraperitoneally with 100 μl of either PCM or a 1:10 dilution of NRS. After 24 h, animals were challenged intraperitoneally with 100 μl of appropriate doses of mid-log-phase Hib cells in PCM. Actual doses given were determined by viable counts on BHI-XV agar medium. The 100% lethal dose was reported as the lowest dose of cells allowing no survival after 72 h. Protective titers of antisera were determined by intraperitoneal immunization of 4-day-old infant rats with 100 μl of various dilutions of the antisera in PCM. Animals were challenged i.p. with a 100% lethal dose 24 h after immunization. Survivors were counted at 72 h postchallenge. Protective titers were reported as the reciprocal of the highest dilution of antiserum which allowed 50% survival.

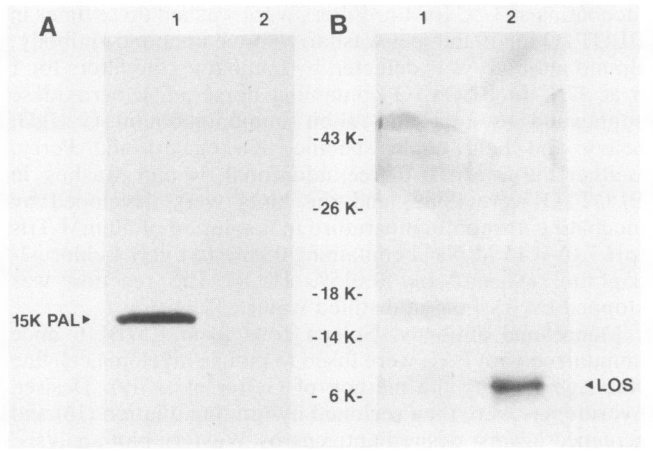

FIG. 1. SDS-PAGE analysis of purified PAL. Molecular weight standards (not shown): ovalbumin (43,000); α-chymotrypsinogen (26,000); β-lactoglobulin (18,000); lysozyme (14,000); bovine trypsin inhibitor (6,000). Lanes: 1, 10 μg of PAL; 2, 20 ng of Hib Eagan LOS. (A) Coomassie stain; (B) silver stain for LOS.

Indirect IFA studies. Surface exposure of epitopes on Hib was determined by indirect immunofluorescence assay (IFA). An IFA with air-dried cells was developed based on the procedure of Snail and Jones (26). Overnight cultures of Hib were diluted 100-fold in PCM and 100-μl samples of the diluted cells were pelleted by centrifugation at room temperature in a microcentrifuge for 2 min. Cell pellets were suspended in 100 μl of PCM. Fifteen microliters of washed cells was added to each well of a 12-well Teflon-coated slide (Cel-Line Inc., Newfield, N.J.) and air dried. After cells were washed briefly in distilled H_2O, they were air dried, and 15 μl of the appropriate antiserum diluted 1:100 in PCM was added to each well. Slides were incubated for 1 h at 37°C and washed three times in PCM. Fifteen microliters of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG or fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG, IgA, and IgM (Kirkegaard and Perry) containing 5% Evans blue (Baker Chemical Co., Phillipsburg, N.J.) were placed in each well. Slides were incubated at 37°C for 1 h and washed in PCM, followed by distilled H_2O. The slides were air dried, mounted with glycerin mounting fluid, and placed under a cover slip. Slides were examined under oil immersion at 1,000× with an Olympus fluorescent microscope (Olympus Corp., Lake Success, N.Y.). Nonimmune rabbit sera or a mouse monoclonal antibody against a viral protein were controls for nonspecific fluorescence.

The specificity of the IFA for cell surface determinants was assessed by comparing the reactivity of a monoclonal antibody with cells suspended in PCM and subsequently washed and air dried for IFA with that of cells which were air dried before incubation with monoclonal antibody. Assays with cells suspended in PCM showed that a monoclonal antibody which reacted with the 15-kilodalton PAL (15K PAL) of *H. influenzae* in the immunoblot assay did not recognize a surface-exposed determinant in *H. influenzae*. This monoclonal antibody also failed to react with air-dried cells unless they were first treated with acetone to remove lipids and expose nonsurface determinants. Thus, the IFA with air-dried cells was assessing surface determinants.

RESULTS

Purity of isolated PAL. The purity of isolated OMP was assayed by SDS-PAGE. When 10 μg of protein was analyzed, a single major band with an approximate molecular weight of 15,000 was detected (Fig. 1A). LOS contamination of the purified protein was assayed by silver staining after SDS-PAGE. The sensitivity of the assay for LOS was 2.5 ng. Purified 15K PAL contained <2.5 ng of LOS as determined by silver stain when 10 μg of protein was assayed (Fig. 1B).

Specificity of anti-PAL antiserum. Hyperimmune rabbit anti-PAL antiserum was analyzed by Western blot analysis (Fig. 2). The antiserum at a dilution of 1:500 reacted with the purified 15K PAL (Fig. 2, lane 2) and with the 15K PAL in 20 μg of an Hib cell envelope preparation (Fig. 2, lane 3). The identical pattern was obtained when a 1:50 dilution of anti-PAL was reacted with 1 μg of Hib Eagan OMPs (data not shown). Other bands reacting with the polyclonal antiserum were assumed to be multimers of the PAL since they were also reactive with monoclonal antibodies specific for the 15K PAL. No reaction was observed with purified Hib Eagan LOS (Fig. 2, lane 1).

Bactericidal activity of anti-PAL antibody on typable and nontypable *H. influenzae*. The bactericidal activity of anti-PAL antiserum against typable and nontypable *H. influenzae* was measured by an in vitro bactericidal assay (Table 1). Anti-PAL antibody was capable of killing nontypable *H. influenzae* at high dilutions of antiserum. Anti-PAL was also bactericidal in this assay system against types a, b, d, e, and f. Type c *H. influenzae* was not consistently killed by anti-PAL antibody.

Bactericidal activity of anti-PAL against clinical isolates of *H. influenzae*. Antibodies to PAL can kill a variety of heterologous *H. influenzae* clinical isolates (Table 2). The bactericidal titer of anti-PAL antiserum is shown against 12 Hib clinical isolates. Anti-PAL antiserum had bactericidal titers ranging from 40 to 320 against the clinical isolates tested. Bactericidal titers against four nontypable clinical isolates ranged from 20 to 160.

Surface reactivity of anti-PAL. The surface exposure of epitopes of the 15K PAL on a large variety of clinical Hib isolates was determined with an IFA. Assays with air-dried Hib cells and polyclonal anti-PAL as the primary antibody were done on 182 clinical Hib isolates. All 182 strains tested contained surface-exposed epitopes of the 15K PAL. The clinical isolates showed a qualitative range of reactivity with the antiserum, with some showing more reactivity than the Hib Eagan control and some showing less. No correlation

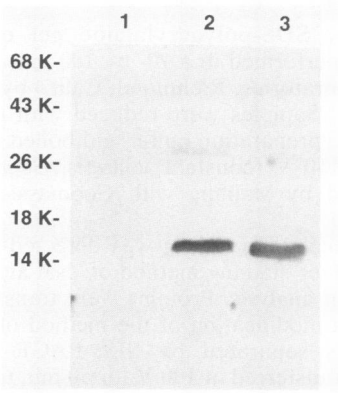

FIG. 2. Immunoblot analysis of anti-PAL antiserum diluted 1:500 in BLOTTO. Molecular weight standards (not shown): phosphorylase B (97,000); bovine serum albumin (68,000); ovalbumin (43,000); α-chymotrypsinogen (26,000); β-lactoglobulin (18,000); lysozyme (14,000). Lanes: 1, 10 ng of Hib Eagan LOS; 2, 100 ng of purified PAL; 3, 20 μg of Hib Eagan whole cell envelopes.

TABLE 1. Bactericidal activity of anti-PAL antiserum against Hib and nontypable *H. influenzae*

H. influenzae strain[a]	Bactericidal titer[b]
Typable	
Type a	40
Type b	40
Type c	±[c]
Type d	60
Type e	30
Type f	40
Nontypable	
S-2	80
HST-31	160
36	20

[a] Centers for Disease Control type strains.
[b] Reciprocal of highest dilution of antiserum killing >50% of test bacteria. Normal rabbit serum had a bactericidal titer of 1:2 or less.
[c] Approximately 50% of test bacteria survived at anti-PAL dilutions of 1:10.

was observed between reactivity of anti-PAL in the IFA and either bactericidal titer or protective activity of anti-PAL in the infant-rat model.

Bactericidal activity of anti-PAL antiserum against Hib clinical isolates from various geographic areas. The bactericidal activity of anti-PAL antiserum against a wide distribution of 182 clinical Hib strains was tested by a screening version of the bactericidal assay. In this system, bacterial strains were grown as before and then tested at two dilutions of anti-PAL antiserum (5- and 10-fold over background) for bactericidal activity. Isolates were considered capable of being killed by the rabbit antiserum if the bactericidal titer was at least fivefold greater than that obtained with NRS (Table 3). Strains used in these studies were isolated at various dates (73 from 1971 through 1974, 49 from 1976 through 1980, and 40 from 1981 through 1985) in widely separated geographic areas of North America. A single isolate from the Federal Republic of Germany was also tested. The clinical isolates from Saskatoon, Saskatchewan, and the isolate from the Gallop Indian Reservation represent isolates from Native American children. Anti-PAL had bactericidal activity of at least fivefold over background against 181 of 182 clinical Hib isolates. Anti-PAL was bactericidal against clinical isolates obtained from 1971 to 1985.

TABLE 2. Bactericidal titers of anti-PAL antiserum versus *H. influenzae* clinical isolates

H. influenzae strains tested	Site of isolation	Titer[a]
Typable (Hib)		
Eagan	CSF[b]	160
305	NA[c]	80
MAD	NA	160
HST-23	Blood	160
HST-16	Blood	160
H101	NA	40
H146	NA	40
H148	NA	40
A712	Blood	320
A659	Blood	50
A567	Blood	160
A584	Trachea	80
Nontypable		
HST-31	Discharge	160
HST-35	Nasopharynx	160
HST-34	Nose	80
HST-30	Eye	20

[a] Reciprocal of the highest dilution of antiserum killing >50% of test bacteria. Normal rabbit serum had a bactericidal titer of 1:2 or less.
[b] CSF, Cerebrospinal fluid.
[c] NA, Data not available.

Protective activity of anti-PAL antiserum. The ability of anti-PAL antiserum to protect infant rats from a 100% lethal dose of five different clinical isolates of Hib is shown in Table 4. The antiserum protected the animals from challenge with each of the five different strains. Two of the Hib clinical isolates (H148 and HST-60) were less reactive than Hib Eagan in IFA assays with anti-PAL. The highest dilution of antiserum giving 50% or greater protection was 1:30 with a challenge of 10^4 CFU of Hib strain Eagan, >1:90 with 10^6 CFU of HST-60 or 10^5 CFU of HST-61, 1:10 with 4×10^5 CFU of H198, and 1:30 with 5×10^5 CFU of H148. Control groups sham immunized with either buffer or NRS diluted 1:10 showed no protection.

Biologic activity of mixed anti-PRP and anti-PAL antisera. The biologic activity of antisera containing a mixture of anti-PAL and anti-PRP antibodies was evaluated with both the in vitro bactericidal assay and the infant-rat model. Bactericidal effects of the pooled antisera were compared with the activity of each antiserum alone (Table 5). The dilutions shown in Table 5 are final dilutions of each antiserum. Anti-PRP at dilutions of 1:1,600 or greater or anti-PAL at dilutions of 1:160 or greater were not bactericidal for Hib strain Eagan. However, when both antisera diluted past their respective bactericidal endpoint titers were mixed together, bactericidal activity was restored. The bactericidal activity of the mixed antisera was dependent upon the concentration of each antiserum; increasing the dilution of either one removed bactericidal activity. The bactericidal activity of the mixed antisera appeared to be additive.

Protective activities of mixed anti-PRP and anti-PAL were examined in the infant-rat model (Table 6). Anti-PRP antiserum and anti-PAL antiserum were titered to obtain their protective endpoints against Hib strain Eagan as described above. For example, anti-PRP antibody protected six of six rats at a dilution of 1:1,000, but only two of six rats at a dilution of 1:2,000 (Table 6). Anti-PAL antibody protected three of six rats at a dilution of 1:90, but only one of six rats at a dilution of 1:100 (Table 6). When infant rats were passively immunized with a mixture containing protective amounts of anti-PRP (1:1,000) and nonprotective amounts of anti-PAL (1:100), six of six rats were protected. These data indicate that the anti-PAL antiserum had no blocking effect on the anti-PRP antibody. When rats were passively immunized with a mixture of both antisera diluted beyond their

TABLE 3. Geographic distribution of Hib clinical isolates tested in bactericidal assay

Location	No. of isolates killed[a]/total
Northeastern United States	62/62
Winnipeg, Manitoba, Canada	49/49
Dallas County, Tex.	39/39
Southeastern United States	24/25
Gallup Indian Reservation, Ariz.	3/3
New York City	1/1
California	2/2
Germany	1/1

[a] Killing of isolate determined as a bactericidal titer of at least fivefold over NRS, which had a bactericidal titer of 1:2 or less.

TABLE 4. Infant rat protection by anti-PAL antiserum[a]

Challenge strain	Antiserum passively transferred	Antiserum dilution	Challenge dose[b]	No. of survivors[c]/ total
HST-60	NRS	1:10	1×10^6	0/6
	PAL	1:10	1×10^6	5/5
	PAL	1:30	1×10^6	6/6
	PAL	1:90	1×10^6	6/6
HST-61	NRS	1:10	1×10^5	0/5
	PAL	1:10	1×10^5	6/6
	PAL	1:30	1×10^5	6/6
	PAL	1:90	1×10^5	3/5
Eagan	NRS	1:10	1×10^4	0/4
	PAL	1:10	1×10^4	3/5
	PAL	1:30	1×10^4	5/5
	PAL	1:90	1×10^4	0/5
H198	PCM		4×10^5	1/6
	PAL	1:10	4×10^5	4/6
	PAL	1:30	4×10^5	2/5
	PAL	1:90	4×10^5	0/5
H148	PCM		5×10^5	0/6
	PAL	1:10	5×10^5	6/6
	PAL	1:30	5×10^5	5/6
	PAL	1:90	5×10^5	2/6

[a] Protection against challenge with strain Eagan was done several times, and the results of a representative experiment are shown. Results with the other strains are from four separate experiments in which protection against strain Eagan was compared with that against each of the four strains.
[b] Challenge dose was determined by titering the bacterial strain in infant rats as described in the text.
[c] Survivors were counted at 72 h postchallenge.

protective endpoints, additive protection was observed. For example, four of six rats survived when a mixture of anti-PAL (1:100) and anti-PRP (1:4,000) antibodies was injected.

DISCUSSION

Previous investigators have demonstrated that antibody to surface-exposed epitopes of *H. influenzae* may be bactericidal or opsonic or both (2, 22). Antisera against LOS (3), PRP (6), and OMPs (2) have been shown to be bactericidal. Murphy et al. (18) have demonstrated that antibody, both monoclonal and polyclonal, directed against a 16-kilodalton OMP of nontypable *H. influenzae* recognized surface epitopes and cross-reacted with an OMP of identical molecular size in all Hib and nontypable *H. influenzae* strains tested and appeared to be antigenically conserved among all *H. influenzae*. The protein they isolated was assumed to be identical to the OMP called P6 isolated from a protein-cell wall complex with 1% SDS at 60°C by Munson and Granoff (17).

In the present study, efforts were made to purify a peptidoglycan-associated OMP free of LOS without the use of SDS or denaturing conditions as previously described (17, 18). The protein isolated was free from LOS contamination (Fig. 1B, lane 1) and did not elicit anti-LOS antibody as measured by an enzyme-linked immunosorbent assay (data not shown). The purified protein migrated as a single major band with an approximate molecular weight of 15,000 by SDS-PAGE, which is in agreement with molecular weights calculated from its amino acid composition (Zlotnick et al., in preparation) and its DNA sequence (R. Deich, B. J. Metcalf, C. W. Finn, J. E. Farley, and B. A. Green, manuscript in preparation). At present it is not certain that the 15K PAL is identical to the P6 OMP; however, based on bactericidal and protective antibody assays, anti-15K PAL antibody appears to be functionally similar to antibody produced to the P6 OMP (17, 18).

Previous studies investigating the biological activity of polyclonal anti-PAL antibody were conducted by using the homologous strain or a very limited number of strains. The results presented in this study show that polyclonal antisera against a 15K PAL from Hib is bactericidal against a wide variety of Hib clinical isolates from widely dispersed geographic locations and isolated at different dates (Table 3). Although the bactericidal titer of the antiserum varied from strain to strain, with some isolates being less sensitive and others more sensitive than the homologous strain, Hib strain Eagan, 181 of 182 clinical Hib isolates were killed.

Bactericidal activity of antibody directed against somatic antigens of *H. influenzae* has been shown to be affected by the relative amount of LOS that the strains produce. When Hib are exposed to low-molecular-weight serum components, they undergo a phenotypic shift in which the amount of LOS produced is greatly increased (10). This shift reduced, but did not eliminate, the bactericidal activity of anti-somatic antigen antisera, but not of anti-PRP antisera. Experiments to determine the ability of anti-PAL to protect infant rats against phenotypically shifted organisms have not been done. However, the organisms used in the animal protection experiments presented here were virulent; 26 of 27 control animals were killed, and the anti-PAL antiserum was protective in the infant-rat model against multiple virulent Hib clinical isolates (Table 4).

The reasons for the failure of anti-PAL to kill one of the

TABLE 5. Bactericidal activity of a mixture of anti-PAL and anti-PRP antiserum against *H. influenzae* Eagan

Anti-PAL dilution[a]	Bactericidal activity at the following anti-PRP dilutions[b]:					
	No anti-PRP	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600
No anti-PAL	−	−	−	−	−	−
1:160	−	+	+	+	+	−
1:320	−	+	+	+	+	−
1:640	−	+	−	−	−	−

[a] Anti-PAL at dilutions less than 1:100 was bactericidal.
[b] −, No bactericidal activity (>50% of bacteria survived); +, bactericidal activity (>50% reduction in CFU). Anti-PRP at dilutions less than 1:1,000 was bactericidal.

TABLE 6. Protective effect of anti-PAL and anti-PRP antiserum in the infant-rat model

Dilution of serum injected		No. of survivors[c]/total
Anti-PAL[a]	Anti-PRP[b]	
		0/6
1:90		3/6
1:100		1/6
	1:1,000	6/6
	1:2,000	2/6
1:100	1:1,000	6/6
1:100	1:3,000	5/6
1:100	1:4,000	4/6

[a] Polyclonal rabbit anti-PAL diluted in PCM.
[b] Polyclonal rabbit anti-PRP–CRM_{197} conjugate diluted in PCM.
[c] Infant Sprague-Dawley rats surviving at 72 h postchallenge with 10^4 CFU of *H. influenzae* Eagan. Representative results of one of two separate experiments are shown.

clinical Hib isolates remain unresolved. The strain has been shown to produce a 15K PAL which cross-reacts with anti-PAL antiserum, and the PAL is surface exposed as evaluated by IFA (data not shown). The strain seems to produce somewhat less PAL than most isolates when assayed by Western blotting (data not shown). Experiments are in progress to determine whether anti-PAL antiserum can protect infant rats against challenge with this strain.

Antibody directed against surface antigens of *Neisseria gonorrhoeae* has been shown to have blocking effects upon bactericidal or protective antibody (12). The vaccine potential of PAL to prevent infections caused by *H. influenzae* would be seriously compromised if antibody to it interfered with anti-PRP. The results presented here suggest that this is not a problem with anti-PAL antiserum, since both bactericidal (Table 5) and protective (Table 6) effects of anti-PRP are not blocked. The data indicate that the two antibodies may have additive effects in both assay systems. Further studies are under way to explore this possibility and to determine which epitope(s) on the PAL molecule is responsible for bactericidal and protective antibodies.

These data on the antigenicity of the 15K PAL suggest that this protein might be a useful component of a vaccine to prevent all infections caused by both Hib and nontypable *H. influenzae*. Antibodies to this protein are bactericidal against nontypable strains and Hib strains. Ongoing investigations will continue to assess the vaccine potential of this interesting protein.

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