A Recombinant Non-Fatty Acylated Form of the Hi-PAL (P6) Protein of *Haemophilus influenzae* Elicits Biologically Active Antibody against Both Nontypeable and Type b *H. influenzae*

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Received 30 March 1990/Accepted 9 July 1990

An approximately 15,000-dalton outer membrane lipoprotein of *Haemophilus influenzae*, the Hi-PAL (P6) protein, has been shown to elicit bactericidal and protective antibodies against both type b and nontypeable *H. influenzae* strains and is a vaccine candidate for these organisms. To determine whether the lipid modification of this protein is required for immunogenicity or the elicitation of biologically active antibodies, a genetic fusion was constructed that contains the sequence of mature Hi-PAL fused to the polylinker region of pUC19. This protein expressed by this clone does not contain detectable lipid and was purified to homogeneity. This recombinant fusion protein, rPAL, elicited a strong immune response when injected into rabbits, and the antisera reacted well with native Hi-PAL. The antisera was bactericidal against a number of clinical nontypeable strains, duplicating the activity of anti-Hi-PAL. The anti-rPAL antisera was also protective against type b bacteremia in the infant rat model. These results demonstrate that purified rPAL elicits antibodies with biological activities that are similar to those of anti-Hi-PAL antibodies. Thus, the lipid component of Hi-PAL is not required for either immunogenicity or elicitation of biologically active antibodies.

*Haemophilus influenzae* strains represent a major cause of morbidity and mortality in infants and elderly adults (2, 19, 24). *H. influenzae* type b (Hib) strains are the leading causative agents of neonatal meningitis (21), and nontypeable (NT) *H. influenzae* strains are among the major causative agents of acute otitis media (2). The current vaccines for Hib consist of the polysaccharide capsule from Hib, polyribosyl-ribitol phosphate or oligosaccharides derived from polyribosyl-ribitol phosphate coupled to protein carriers. The protective efficacy of anti-polyribosyl-ribitol phosphate antibody is demonstrated in the infant rat meningitis model system, in which antisera is passively transferred and the animals are challenged with virulent Hib (23). Presently, the protein-polysaccharide conjugate vaccines are licensed for use in 18-month-old children. Clinical trials of saccharide-protein vaccines are currently underway to demonstrate efficacy in infants. Although anti-polyribosyl-ribitol phosphate antibody appears to be sufficient to protect humans from Hib disease, it has no efficacy against diseases caused by NT *H. influenzae*.

Recent attempts to identify potential vaccine components that would be effective against NT *H. influenzae* have focused on surface-exposed antigens such as outer membrane proteins (8, 9, 11) and pili (4). A low-molecular-weight lipoprotein of *H. influenzae*, the Hi-PAL (P6) protein, has been the focus of intensive research efforts. This protein has been shown to be present in every NT *H. influenzae* and Hib isolate (9, 16, 18), is antigenically invariable (16, 18), and is a target for human bactericidal antibodies (17). Hi-PAL has been purified by using sodium dodecyl sulfate (SDS) (15) and nondenaturing detergents (28) and elicits bactericidal (9, 17) and protective antibodies (9, 15). The above properties make this protein attractive as a component in a vaccine against both Hib and NT *H. influenzae*. The gene encoding the Hi-PAL protein has been cloned and sequenced (7, 20), and the purified protein has been sequenced, with the exception of the blocked amino terminus (28). The Hi-PAL protein belongs to a class of gram-negative bacterial outer membrane lipoproteins, known as peptidoglycan-associated lipoproteins (PALs), which share large areas of amino acid sequence homology (5, 14, 28). Although the function(s) of these proteins is as yet unknown, the PAL protein of *Escherichia coli* appears to be essential (5).

When the Hi-PAL gene is expressed in *E. coli*, the signal sequence is recognized by signal peptidase II, and the amino-terminal cysteine residue is posttranslationally modified by the addition of a glycerol moiety containing ester-linked fatty acids and then an amide-linked fatty acid after removal of the signal sequence (for a review, see reference 26). The resulting lipoprotein is apparently identical to Hi-PAL produced in *H. influenzae*. In *E. coli* and *H. influenzae*, only small amounts of Hi-PAL are produced, possibly due to the posttranslational modification of the pal gene product. However, removal of the lipid may have deleterious effects on the stability and immunogenicity of Hi-PAL. To evaluate whether the lipid moieties are required for antigenicity and immunogenicity of Hi-PAL, especially the elicitation of biologically active antibody, we constructed a recombinant clone that expresses a nonlipidated Hi-PAL fusion protein and purified the protein to homogeneity. In this study we report that this protein elicits biologically active antibody against *H. influenzae* and that lipid is not required for antigenicity or immunogenicity of the Hi-PAL protein.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** *H. influenzae* strains used in this study were Hib strain Eagan, NT strain S-2, and clinical NT strains N90, 1939, Hst-34, Hst-35, Hst-36, and O45E. *E. coli* strains used were HB101 (3), PR13 (10), and JM101 (13). *H. influenzae* strains were grown in brain heart...
infusion (Difco Laboratories, Detroit, Mich.) supplemented with 10 μg of heme (Sigma Chemical Co., St. Louis, Mo.) per ml and 2 μg of NAD (Sigma) per ml. E. coli strains were grown in LB medium (12).

Plasmids used were pUC19 (27), pAA152 (7), pPX160, and pPX167 (this study).

Isolation of DNA. Plasmid DNA was isolated by alkaline SDS extraction followed by equilibrium banding in cesium chloride-ethidium bromide gradients (1). DNA fragments were isolated from agarose gels (FMC Corp., Marine Colloids Div., Portland, Maine) by electroelution onto NA45 membranes (Schleicher & Schuell Co., Keene, N.H.) and subsequently eluted with 1 M NaCl-10 mM Tris hydrochloride (pH 8.0)-1 mM EDTA.

DNA sequencing. DNA sequencing was carried out as previously described (7).

Protein determination. Protein was determined by the method of Lowry et al. as modified by Peterson (22).

SDS-PAGE and Western blotting. SDS-polyacrylamide gel electrophoresis (PAGE) analysis and Western immunoblot analysis were carried out as described previously (7). Rabbit antibodies were detected using peroxidase-conjugated goat anti-rabbit immunoglobulin G (Tago, Inc., Burlingame, Calif.), and mouse monoclonal antibodies were detected using peroxidase-conjugated goat anti-mouse immunoglobulin G and M antiserum (Tago).

Monoclonal antibodies. Mouse monoclonal antibodies to the Hi-PAL protein were produced as previously described (7, 9). Binding specificities of the monoclonal antibodies were determined by using a competitive binding enzyme-linked immunosorbent assay (ELISA) with Hi-PAL as the antigen. Four noncompeting monoclonal antibodies representing different epitopes of the Hi-PAL were used in this study. One of the antibodies, G204-2, recognizes a linear epitope near the amino terminus of Hi-PAL; two other antibodies, G18-3 and G56-5, do not recognize proteolytically digested Hi-PAL and thus probably recognize nonlinear determinants. G219-3 will only react with Hi-PAL or a large peptide fragment of Hi-PAL and is also probably conformational.

Restriction analysis. Restriction enzymes were purchased from New England BioLabs, Inc., Beverly, Mass., or Bethesda Research Laboratories, Inc., Gaithersburg, Md., and used according to the directions of the manufacturer. Agarose gel electrophoresis was carried out in 89 mM Tris-89 mM borate-2 mM EDTA (pH 8.3) buffer. DNA bands were visualized by ethidium bromide fluorescence.

Construction of signalless pal gene. The sequence of the Haemophilus chromosomal fragment encoding the Hi-PAL gene has previously been published (7). To express the Hi-PAL protein devoid of lipid at the amino terminus, the portion of the Hi-PAL gene encoding the mature form of the protein was fused to β-galactosidase α-peptide in pUC19. Construction of this plasmid is shown in Fig. 1. The 737-base pair BamHI-BglII fragment containing the pal gene in pAA152 (7) was subcloned into pUC19 at the BamHI site, forming pPX160. The 315-base pair PvuII-BstNI fragment of the pal gene was isolated from pPX160 and digested with AluI to obtain the 183-base-pair fragment containing the amino terminus of the pal gene. The above AluI site is located within the signal sequence, 1 amino acid upstream of the first residue of the mature form of Hi-PAL. This fragment was subcloned into pUC19 at the Smal site, and the orientation was determined by restriction analysis. One isolate with the fragment in the proper orientation (i.e., with the pal gene reading in the same direction as lac) was then digested with XmnI, and the large linear fragment was isolated. XmnI cuts once within the pal gene and once within the Amp' gene of pUC19. To complete the Amp' gene and the pal gene, pPX160 was digested with XmnI to obtain the fragment containing the 3' terminus of the pal gene and the 5' portion of the Amp' gene. These two fragments were mixed together, ligated, and transformed into E. coli JM101 with ampicillin selection. Only plasmids containing the XmnI fragment in the proper orientation will be Amp'. Amp' colonies were analyzed by Western blotting for production of PAL. Plasmids were isolated from positive colonies, and the absence of a signal sequence was confirmed by restriction analysis and sequence analysis. One isolate containing the predicted sequence was saved as pPX167.
Purification of signalless PAL. Overnight cultures of *E. coli* FR13(pFX167) were grown in LB medium containing 100 μg of ampicillin per ml at 37°C. Cells were harvested by centrifugation at 10,000 × g in a Sorvall GS-3 rotor at 4°C for 10 min. The cell pellet was washed once in 10 mM Tris hydrochloride (pH 7.5) and suspended in the same buffer at a ratio of 5 g (wet weight)/30 ml. Cells were disrupted by passage through a French pressure cell three times, with the extract kept on ice. Cell debris and whole cells were removed by centrifugation at 10,000 × g in a Sorvall SS-34 rotor for 5 min at 4°C. The supernatant was saved, and total membranes were removed by centrifugation at 120,000 × g in a Beckman 60Ti rotor for 30 min at 4°C. Signalless PAL was found primarily in the cytoplasmic supernatant fraction, and this fraction was used for further purification. The cytoplasmic fraction was passed over a DEAE-Bio-Gel A 10,000 column equilibrated with 10 mM Tris (pH 7.5). Under these conditions most of the proteins present were bound to the column. Preliminary experiments with a linear NaCl gradient from 10 to 500 mM in 10 mM Tris (pH 7.5) showed that bound recombinant PAL (rPAL) eluted from the column at NaCl concentrations ranging from 30 to 80 mM. Consequently, bound rPAL was eluted from the column with 10 mM Tris (pH 7.5) containing 80 mM NaCl. The partially purified rPAL was concentrated by precipitation with (NH₄)₂SO₄ at 60% saturation at 4°C. The pellet was collected, dissolved in 20 mM sodium phosphate buffer (pH 7.5), and dialyzed against the same buffer at 4°C to remove any residual (NH₄)₂SO₄.

Final purification of rPAL was achieved by reversed-phase chromatography. The concentrated DEAE eluate was diluted to a protein concentration of 2 mg/ml in a buffer containing 0.1% trifluoroacetic acid in distilled H₂O. The proteins were passed through a 4.6- by 15-mm C₅ reversed-phase column (Hewlett-Packard, Inc., Avondale, Pa.) on a Hewlett-Packard high-pressure liquid chromatography system. The column was run at a flow rate of 2 ml/min with the same trifluoroacetic acid buffer. Under these conditions, most of the proteins bound to the column. Bound rPAL was eluted as a single peak with a 0 to 95% acetonitrile gradient in 0.1% trifluoroacetic acid over 20 min. The large peak containing the rPAL was collected and concentrated by evaporation of the solvent. Dried rPAL was dissolved in distilled H₂O, dialyzed against 10 mM Tris (pH 7.5) containing 0.15% NaCl, and stored frozen at −20°C.

Hi-PAL was purified as described by Zlotnick et al. (28).

14Pmalic acid labeling of plasmid encoded lipoproteins. Lipoproteins in plasmid-containing cells were labeled by incubation of logarithmic-phase cells with 14Cpalmitic acid (Amersham Corp., Arlington Heights, Ill.). Overnight cultures of JM103(pPX160), JM103(pPX167), and JM103 (pUC19) were diluted 1:50 in fresh LB and grown to the early log phase. Then 50-μCi samples of 14Cpalmitic acid were added to 1-ml portions of the log-phase cultures (along with isopropyl-β-D-thiogalactopyranoside, where appropriate), and the cultures were incubated at 37°C for 1 h. Labeled cells were pelleted, washed three times in LB, and 20-μl samples were precipitated onto GCA glass filters with 5% trichloroacetic acid, followed by two washes in ice-cold 70% ethanol. Precipitated proteins were counted in a scintillation cocktail. SDS-PAGE analysis of labeled cells was performed by loading 10,000 cpm of each sample per lane of a 15% polyacrylamide gel. The gel was prepared for autoradiography as described previously (7), and an autoradiogram was made by exposure to XAR-5 X-ray film (Eastman Kodak Co., Rochester, N.Y.) for 2 weeks at −70°C.

Amino-terminal sequencing of rPAL and synthesis of fusion peptide. The amino terminus of purified rPAL was sequenced by using Edman chemistry and an Applied Biosystems model 477A protein sequencer. Analysis was performed on a reversed-phase C₁₈ high-pressure liquid chromatography cartridge column as previously described (28). A peptide consistent of the predicted amino-terminal sequence of rPAL (fusion peptide) was synthesized with an Applied Biosystems model 430A automated peptide synthesizer. The peptide was purified by reversed-phase chromatography on a C₄ column with 0.1% trifluoroacetic acid and a 0 to 60% acetonitrile gradient.

Immunization of rabbits with rPAL. New Zealand White rabbits were immunized intramuscularly with 25-μg doses of purified rPAL in physiological saline emulsified in Freund incomplete adjuvant (Difco). Rabbits were boosted on day 30 with another 25-μg dose of rPAL prepared as described above and bled on day 45 via the marginal ear vein.

ELISA for anti-rPAL and anti-Hi-PAL antibodies. Samples of 100 μl of either purified Hi-PAL (28), purified rPAL, or synthetic peptide were added to the wells of 96-well micro-dilution plates (Nunc, Thousand Oaks, Calif.) and incubated for 90 min at 37°C for antigen absorption. After five washes with phosphate-buffered saline−0.1% Tween 20, each well was filled with 50 μl of diluted antiserum and incubated for 1 h at 37°C. Unbound antibody was removed by washing five times with phosphate-buffered saline−0.1% Tween 20, and bound antibody was detected with alkaline phosphatase conjugated goat anti-rabbit immunoglobulin G (heavy and light chains) (Tago) with para-nitrophenylphosphate in diethanolamine buffer as substrate. Endpoint titers were read as the reciprocal of the highest dilution of antibody giving an optical density at 410 nm of greater than 0.2.

Bactericidal assays. Bactericidal assays were done as previously described (9) with the following modifications. For NT strains of *Haemophilus*, the complement source, precollostral calf serum, was adsorbed before use with cells from an overnight culture of the bacteria to be tested. Briefly, bacteria in 1 ml of an overnight culture of an NT strain were pelleted in a microfuge, washed in phosphate-buffered saline (pH 7.5) containing 0.15 mM CaCl₂−0.5 mM MgCl₂−0.1% bovine serum albumin (Sigma; largely immunoglobulin free), and pelleted again. The packed cells were suspended in 1 ml of ice-cold complement and held on ice for 30 min. Cells were pelleted in the cold, and the complement was adsorbed with the washed pellet from another 1 ml of overnight culture. After the second adsorption, the cells were pelleted, and the complement was sterilized by filtration through a 0.22-μm-pore-size filter.

Infant rat protection studies. Passive protection of infant rats from meningitis was performed as described by Munson and Granoff (15).

RESULTS

Construction of signalless PAL. Native Hi-PAL protein exists as a lipoprotein with both amide- and ester-linked fatty acids attached to the amino-terminal cysteine residue (7, 25). The Hi-PAL gene encodes a signal sequence that is recognized by *E. coli* signal peptidase II. This allows processing of the pal gene product by the addition of diacylglycerol via a thioether linkage to the cysteine residue, followed by cleavage of the signal sequence and addition of an amide-linked fatty acid. The processed protein is transported to the outer
membranes in *E. coli* (7, 26). To obtain nonlipidated Hi-PAL protein, we constructed a plasmid expressing the mature PAL protein lacking the signal sequence fused to an 18-amino-acid peptide derived from the beginning of the β-galactosidase α-peptide and the pUC19 multiple-cloning-site region (Fig. 1). This plasmid, pPX167, expresses the PAL fusion protein under control of the lac promoter. The absence of the signal sequence was confirmed by DNA sequencing (data not shown). The lipid in Hi-PAL can be radiolabeled in *H. influenzae* (25) and in recombinant *E. coli* clones (7) if [3H]palmitic acid is included in the growth media. When we attempted to label rPAL by incorporation of [3H]palmitic acid (Fig. 2, lane 2), we found no bands at the appropriate size for rPAL. The positive control lane containing pPX160 (which expresses lipidated Hi-PAL under control of the native *Haemophilus* promoter) shows the expected band at approximately 15,000 daltons (Fig. 2, lane 1). The negative control lane (Fig. 2, lane 3) contained pUC19 and showed no bands at the expected sizes of either Hi-PAL or rPAL.

**Purification of rPAL.** rPAL was purified from *E. coli* PR13(pPX167). After disruption of the cells with a French press, the majority of the protein was found in the cytoplasmic extract (Fig. 3A, lane 1), with little found in the membrane pellet (data not shown). The rPAL eluted over a broad range of salt concentrations from DEAE, with virtually all of the rPAL eluting between 30 and 80 mM NaCl. Elution of the DEAE column with 80 mM NaCl yielded a partially purified and concentrated fraction that contained rPAL and many contaminating proteins (Fig. 3A, lane 2). Further purification was obtained after binding to a C4 reversed-phase column and elution with an acetonitrile gradient. The rPAL was eluted in the first few fractions as a single peak. SDS-PAGE and Western blot analysis of the peak showed it to contain two protein bands, rPAL and a very small amount of another, slightly smaller band that was also reactive with anti-Hi-PAL monoclonal antibodies (Fig. 3B, lane 3). This smaller band may represent a degradation product of the rPAL. The predicted molecular size of rPAL is approximately 3,000 daltons larger than the size of mature Hi-PAL, and the rPAL band comigrates with the 18,000-dalton standard, approximately 3,000 daltons larger than the purified Hi-PAL (Fig. 3A, lanes 3 and 4). The lipopolysaccharide content of the purified rPAL was analyzed by silver staining as previously described (9) and was found to be <2.5 ng per 10 μg of protein (data not shown).

Purified rPAL was analyzed by amino-terminal sequencing (Fig. 4). This sequence agrees with the predicted sequence for the amino terminus of rPAL (shown above the actual sequence). Native Hi-PAL has a blocked N terminus (28) and cannot be sequenced by standard Edman chemistry. Since rPAL can be sequenced and is not labeled with [3H]palmitic acid, the data support the conclusion that rPAL is not lipidated.

**Reactivity of rPAL with anti-Hi-PAL polyclonal and monoclonal antibodies.** Purified rPAL was analyzed by Western blotting and ELISA for its reactivity to both a hyperimmune anti-Hi-PAL antiserum (Table 1) and monoclonal antibodies against different Hi-PAL epitopes. rPAL was highly reactive with the polyclonal antiserum (Table 1) and was recognized by all of the anti-Hi-PAL monoclonal antibodies, including the ones directed against nonlinear epitopes (Fig. 5). Under the conditions used in the Western blotting experiments, the anti-Hi-PAL monoclonal antibodies did not react quite as strongly with rPAL as they did with Hi-PAL. However, the reactions of the monoclonal antibodies directed against linear or nonlinear epitopes appeared equally as strong with rPAL (Fig. 5).

**Reactivity of polyclonal anti-rPAL antiserum.** Anti-rPAL antiserum was generated in rabbits by injection of rPAL emulsified in Freund incomplete adjuvant. We have not performed experiments designed to compare the relative immunogenicities of rPAL and Hi-PAL. The rPAL elicited a strong immune response, and the antiserum was analyzed for its reactivity with rPAL and Hi-PAL by ELISA (Table 1). Anti-rPAL antiserum reacted as well with native Hi-PAL.

Derived amino terminal sequence of rPAL

Thr-Met-Ile-Thr-Pro-Ser-Leu-His-Ala-Cys-Arg-Ser-Thr-Leu-Glu-Asp-Pro-Pro-Cys-

Amino terminal sequence of purified rPAL

FIG. 4. Amino acid sequence of the amino terminus of purified rPAL. The amino acid sequence of the NH2 end of purified rPAL, obtained as described in Materials and Methods, is shown on the bottom line. The predicted amino acid sequence of this region from the DNA sequence is shown on the top line. The amino acid sequencing method used does not detect Cys residues but allows assignment of a residue (•) in that position.
TABLE 1. Reactivity of rabbit anti-Hi-PAL and anti-rPAL antisera with PAL antigens

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Serum</th>
<th>ELISA titer versus:</th>
<th>rPAL</th>
<th>Hi-PAL</th>
<th>Fusion peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>rPAL</td>
<td>Prebleed</td>
<td>800</td>
<td>800</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>After 1 immunization&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3,200</td>
<td>2,300</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
<td>800</td>
</tr>
<tr>
<td></td>
<td>After 2 immunizations</td>
<td>250,600</td>
<td>120,800</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Hi-PAL</td>
<td>Prebleed</td>
<td>300</td>
<td>300</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Hyperimmune&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2,500,000</td>
<td>2,500,000</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Synthetic peptide representing the amino-terminal 20 amino acids of rPAL, including the 18-amino-acid sequence derived from the pUC polylinker region (see text).
<sup>b</sup> ND, Not determined.
<sup>c</sup> Immunizations with 25 μg of rPAL in incomplete Freund adjuvant as described in Materials and Methods.
<sup>d</sup> Hyperimmune antiserum after five injections of 10 μg of Hi-PAL in incomplete Freund adjuvant at 4-week intervals.

As it did with rPAL. No significant (i.e., greater than twofold) difference was observed in overall ELISA titers when either Hi-PAL or rPAL was used as the solid-phase antigen. Thus, rPAL elicited high-titer antisera that was also highly reactive with native lipidaded Hi-PAL.

The amino terminus of rPAL is a peptide encoded by the pUC vector. This peptide could be common to many recombinant proteins expressed in pUC vectors. To determine whether the fusion peptide portion of rPAL is immunogenic in animals, we analyzed the anti-rPAL antisera for any anti-fusion peptide reactivity in an ELISA. A synthetic peptide corresponding to the amino acid sequence of the fusion peptide was used as the ELISA solid-phase antigen.

The anti-rPAL antisera reacted with the 18-amino-acid fusion peptide portion of rPAL (Table 1), indicating that the fusion peptide portion of rPAL is, indeed, immunogenic in animals and elicits an immune response. The specificity of the anti-rPAL response for the peptide was shown by the failure of the anti-Hi-PAL antisera to react with the peptide (Table 1). In addition, a 20 M excess of the peptide inhibited up to 98% of the anti-rPAL binding to the peptide, whereas an internal peptide corresponding to a common region of both Hi-PAL and rPAL showed no inhibitory effect (data not shown).

**Biologic activity of anti-rPAL antisera.** Anti-rPAL antisera was tested for biologic activity against NT strains by using an in vitro bactericidal assay system to determine whether it duplicated the activity of anti-Hi-PAL antisera.

The anti-rPAL had high levels of bactericidal activity against several clinical NT *H. influenzae* isolates (Table 2). The activity of a previously described (9) anti-Hi-PAL antisera is also shown against the same strains. This anti-Hi-PAL antisera had an ELISA titer against Hi-PAL similar to that of the anti-rPAL antisera. Although the bactericidal titers of the two antisera are not directly comparable (different lots of complement were used for the two sets of assays), they demonstrate that both antisera have high levels of biologic activity against multiple NT clinical isolates.

To determine whether anti-rPAL was protective against type b *H. influenzae*, we used the infant rat bacteremia model. After passive administration of antisera, rats were challenged with type b strain Eagan. At 24 h postchallenge, the numbers of Hib cells in blood samples were determined. A hyperimmune anti-Hi-PAL antisera (Table 1) was used as positive control because of the low volume remaining of the antisera used for the BC assays. This antisera, DM-1, has previously been shown to be protective in the infant rat meningitis model when death was used as an endpoint (9). The DM-1 antisera was diluted to an ELISA titer against Hi-PAL approximately equivalent to that of the dilution anti-rPAL antisera used in the model. As expected, the anti-Hi-PAL antisera protected the rats from bacteremia (Table 3). Although the preimmune sera from the rabbits immunized with rPAL showed no protective activity, the anti-rPAL protected infant rats from bacteremia (Table 3) and thus has biologic activity against Hib.

**DISCUSSION**

The Hi-PAL protein is a leading candidate to be a component of a vaccine directed against NT *H. influenzae*. One

TABLE 2. Bactericidal activity of rabbit anti-rPAL and anti-Hi-PAL antisera against NT *H. influenzae* clinical isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Bacterial titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Bacterial titer&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Preimmune</td>
<td>Anti-rPAL&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>S-2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1:20</td>
<td>1:160</td>
</tr>
<tr>
<td>O45E</td>
<td>&lt;1:20</td>
<td>1:80</td>
</tr>
<tr>
<td>Hst-33</td>
<td>1:20</td>
<td>1:160</td>
</tr>
<tr>
<td>Hst-34</td>
<td>1:5</td>
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<td>Hst-35</td>
<td>1:10</td>
<td>1:320</td>
</tr>
<tr>
<td>1939</td>
<td>&lt;1:10</td>
<td>1:160</td>
</tr>
<tr>
<td>N10</td>
<td>1:20</td>
<td>1:1280</td>
</tr>
</tbody>
</table>

<sup>a</sup> Expressed as the highest dilution of antiserum capable of killing 50% of cells compared with the control.
<sup>b</sup> Bled at 6 weeks from animals immunized with rPAL (see text).
<sup>c</sup> Anti-Hi-PAL bleed after two immunizations with purified Hi-PAL.
<sup>d</sup> ND, Not determined.

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**TABLE 3. Passive protection of infant rats with anti-rPAL antisera**

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Anti-Hi-PAL ELISA titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. bacteremic/ no. challenged</th>
<th>Avg CFU/10 μl</th>
<th>p&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer control</td>
<td>200,000</td>
<td>5/6</td>
<td>555</td>
<td>0.0001</td>
</tr>
<tr>
<td>Anti-Hi-PAL</td>
<td>200,000</td>
<td>0/6</td>
<td>0</td>
<td>0.0001</td>
</tr>
<tr>
<td>Preimmune</td>
<td>1,600</td>
<td>5/7</td>
<td>547</td>
<td>0.009</td>
</tr>
<tr>
<td>Anti-rPAL</td>
<td>100,000</td>
<td>0/7</td>
<td>0</td>
<td>0.009</td>
</tr>
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<sup>a</sup> Antisera were diluted before injection to provide approximately equivalent anti-Hi-PAL ELISA titers.
<sup>b</sup> P values were determined by the unpaired Student t test.
potential factor limiting the usefulness of Hi-PAL is the relatively small amount of the protein present in H. influenzae and the small amount of recombinant Hi-PAL produced in E. coli. Since the signal sequence of the Hi-PAL gene is recognized in E. coli and results in fatty acylated PAL indistinguishable from the native Hi-PAL, this processing may be a limiting factor in the expression of Hi-PAL in E. coli. Removal of the signal sequence should result in nonfatty acylated protein. However, the resulting non-lipid-containing protein might not have properties equivalent to those of the native protein. For example, since lipid attachment may significantly enhance the immunogenicity of a protein (6), the nonlipidated protein might not be very immunogenic. Also, the protein might not be conformationally similar to native Hi-PAL and thus might be unable to elicit biologically active antibodies against native Hi-PAL, rendering it useless for vaccine purposes.

With these considerations in mind, this study was designed to construct a plasmid that would express Hi-PAL in a nonlipidated form and to then purify the recombinant protein. This recombinant protein (rPAL) was then used to evaluate whether lipid is necessary for the elicitation of biologically active antibody by Hi-PAL. rPAL is a genetic fusion protein that contains the peptide encoded by the pUC polyclinker fused to the mature Hi-PAL amino acid sequence. In this study, rPAL was expressed under control of the lac promoter (data not shown) and was recovered from the cytoplasmic extract (Fig. 3).

We purified rPAL from the recombinant E. coli and analyzed the purified protein to confirm that it contained the expected amino-terminal sequence (Fig. 4). Since the amino-terminal sequence of purified rPAL was obtained, there can be no amide linked fatty acids in rPAL. Confirmation of this sequence along with the failure of rPAL to be labeled with [14C]palmitic acid (Fig. 2) established that rPAL is indeed non-fatty acylated. When purified rPAL was analyzed by Western blotting with four anti-Hi-PAL monoclonal antibodies, it reacted well with all four. The rPAL was as reactive with G204-2 (which recognizes a linear epitope) as it was with the other three monoclonal antibodies, which recognize nonlinear epitopes (Fig. 5). This observation, plus the finding that rPAL was as reactive as native Hi-PAL with anti-Hi-PAL antiserum (Table 1), suggests that purified rPAL may maintain a conformation similar to that of the native Hi-PAL. When the rPAL was used to immunize rabbits, the anti-rPAL antiserum was also as reactive with native Hi-PAL as it was with rPAL (Table 1), further supporting the conclusion that rPAL has an antigenic conformation similar to that of Hi-PAL.

Antibodies against Hi-PAL have been shown to be both bactericidal and protective. To determine whether rPAL elicited functional antibodies similar to those elicited by Hi-PAL, we evaluated the bactericidal activity of the anti-rPAL antisera using the in vitro bactericidal assay and the in vivo infant rat bacteria model. The antisera were compared on the basis of activity to Hi-PAL and thus were normalized by their ELISA titer against Hi-PAL. The anti-rPAL antisera was bactericidal against a number of NT clinical isolates (Table 2) with bactericidal titers close to those of an anti-Hi-PAL antisera of similar ELISA titer. The range of bactericidal titers observed with anti-rPAL and anti-Hi-PAL is not unexpected; we have previously reported that anti-Hi-PAL BC titers against NT H. influenzae strains vary. This is probably at least partly due to the innate serum sensitivity of NT H. influenzae and may also relate to differences on the surfaces of the various NT H. influenzae strains used. When used in the infant rat model (Table 3), anti-Hi-PAL antiserum, as expected, was also protective against challenge with Hib. These results establish that purified rPAL is capable of eliciting functional antibodies with biological activities similar to those of anti-Hi-PAL antisera. They further show that posttranslational modification of Hi-PAL is unnecessary to obtain the desired antibodies.

Although the actual function of the PAL proteins in gram negative bacteria is unknown, they appear to be essential at least in E. coli (5) and H. influenzae (Green et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, D200, p. 104). In our hands, the expression of the fatty acylated form of recombinant Hi-PAL appears to be tightly regulated (data not shown). Thus, the usefulness of Hi-PAL as a vaccine candidate is restricted by the small amounts produced. The ability of the non-fatty acylated rPAL to elicit biologically active antibody should allow further development of rPAL as a vaccine candidate as overexpressing strains of E. coli are developed. These studies also represent the first use of a recombinant Haemophilus outer membrane protein to elicit biologically active antibody against Hib and NT H. influenzae.

The immunogenicity of the pUC-derived fusion peptide may be problematic for rPAL and other pUC-derived vaccine candidates. This peptide and others with similar sequences encoded by the pUC vectors may, in the future, be common to many recombinant proteins that are used in humans. Since the antibody produced to this region will cross-react with other proteins expressed as fusions in pUC18 or pUC19 (data not shown), this could conceivably cause problems for some recombinant-derived vaccine candidates or therapeutic agents. Experiments are currently in progress to address these concerns.

ACKNOWLEDGMENTS

We thank Mary Jane Del Mastro and Bob Seid for assistance with the protein sequencing work, John Farley for DNA sequencing, and Mary Vazquez for able technical support. We also thank Jim Cowell for his help in reviewing the manuscript.

A portion of this work was supported by Public Health Service SBIR grant AI25233-01 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED


