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Characterization of a Recombinant Pneumolysin and Its Use as a Protein Carrier for Pneumococcal Type 18C Conjugate Vaccines

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Pneumolysin from *Streptococcus pneumoniae* was expressed in *Escherichia coli* as a glutathione *S*-transferase fusion protein and purified by affinity and hydroxylapatite chromatography. The purified recombinant pneumolysin (rPL), with a molecular mass of 53 kDa, had a specific activity of 3×10^5 hemolytic units per mg of protein on rabbit erythrocytes and reacted identically in immunodiffusion with the antisera against native pneumolysin. The rPL was used as a protein carrier to prepare conjugate vaccine with pneumococcal type 18C polysaccharide (PS18C). The PS18C was directly coupled to rPL by reductive amination or was indirectly coupled to rPL via a spacer molecule, adipic acid dihydrazide. The conjugates were nontoxic for mice and guinea pigs at 100 μg per dose. The immunogenicity and protective efficacy of both conjugates were tested in mice. A single dose of either of the vaccines elicited a rise in immunoglobulin G antibody production; after two booster injections of the vaccines, statistically significant booster responses ($P < 0.001$) to both rPL and PS18C were produced. The sera containing the antibodies to rPL were capable of neutralizing the hemolytic activity of rPL to rabbit erythrocytes and the cytotoxicity of rPL to bovine pulmonary endothelial cells. Immunization with the conjugate vaccines conferred statistically significant protection in mice against lethal challenge with type 18C pneumococci.

Streptococcus pneumoniae is the major cause of bacterial pneumonia, bacteremia, meningitis, sinusitis, and acute otitis media (3, 5). The current pneumococcal vaccine is composed of 23 purified pneumococcal polysaccharide (PS) antigens (42). Although the vaccine covers the most-prevalent serotypes found in the population at risk, it does not prevent diseases with all pneumococcal types. Furthermore, like other bacterial PSs, pneumococcal PS antigens are processed as non-T-lymphocyte-dependent antigens, and children under 2 years of age do not produce protective levels of antibody response to the vaccine (14, 34). To achieve immunoprophylactic control of pneumococcal infection in infants less than 2 years of age, there is a need to develop a more-immunogenic vaccine based on the PS conjugated to protein carriers which are processed by T lymphocytes (2, 43).

Pneumolysin (PL), a sulfhydryl-activated cytolytic toxin, is produced by all types of *S. pneumoniae* (30) and is considered a putative virulence factor in pneumococcal infections (9). PL has been shown to detrimentally affect the activity of human phagocytes and immune cells (17, 28) and is a major pneumococcal cytotoxin to pulmonary endothelial and epithelial cells in vitro (44, 45). Genetically engineered PL-negative mutants of *S. pneumoniae* have significantly reduced virulence for mice (6, 7). Patients with pneumococcal pneumonia have been reported to elicit an antibody response to PL (25, 29). Mice injected with inactivated PL or recombinant pneumolysin toxoid exhibit enhanced survival when challenged with live *S. pneumoniae* (1, 33, 36, 37). Therefore, PL itself is a potential vaccine candidate to induce species-specific antipneumococcal immunity based on anti-PL antibody and, more importantly, may be a useful protein carrier for the preparation of the conjugate vaccine. *S. pneumoniae* organisms produce relatively low levels of PL, and purification is rather complex (27, 30, 36). To facilitate production of larger amounts of the toxin, the PL gene has been cloned and expressed in *Escherichia coli* and *Bacillus subtilis* (35, 51, 53). In our laboratory, the PL gene from *S. pnuemoniae* type 18C was cloned and overexpressed in *E. coli* as a glutathione *S*-transferase (GST) fusion protein (41). The recombinant pneumolysin (rPL) was readily purified from the GST fusion protein by a simple and efficient affinity chromatography method (50).

We report here the purification and characterization of the rPL from *E. coli*. We analyzed the amino acid composition, N-terminal amino acid sequence, and biological and immunologic reactivities of the rPL. In addition, we synthesized conjugate vaccine preparations consisting of oxidized pneumococcal PS18C conjugated to the rPL and evaluated their immunogenic response in mice. The conjugate vaccines significantly enhanced the immunogenicity of PS18C and elicited significant rises in the anti-rPL antibody titers. Protective activities of the vaccines have also been demonstrated in mice challenged with live *S. pneumoniae.*

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MATERIALS AND METHODS

Bacterial strains and plasmid. *S. pneumoniae* type 18C was obtained from G. Schiffman (State University of New York, Brooklyn) and grown in modified Holt's medium (32). *E. coli* SCS1 (*endA1 gyrA96 thi-1 hsdR17* [r_K^- m_K^+] *supE44 recA1 relA*) (20) was grown in Luria-Bertani medium (46) or Terrific Broth (52) with appropriate antibiotics. A promoter selection vector, pKK232-8 (11), and an expression vector, pGEX-2T (50), were obtained from Pharmacia (Piscataway, N.J.).

Molecular cloning of the *ply* gene. Genomic DNA was isolated from *S. pneumoniae* type 18C by the procedure described by Wilson (54). A 1.3-kb *Sal*I-*Eco*RV fragment that spans the type 20 *ply* gene (unpublished data) was labeled by mixed-primer labeling and used as a probe for Southern hybridization to identify restriction fragments containing the type 18C *ply* gene. The 2.8-kb *Bst*YI fragment was recognized to contain the gene along with the upstream and

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downstream regions. *Bst*YI fragments of genomic DNA (2.7 to ~2.9 kb) were isolated from an agarose gel, digested with *Hin*dIII, and inserted into *Bam*HI and *Hin*dIII sites of pKK232-8. *E. coli* SCS1 was transformed with the ligated mixture. Six recombinants which span the upstream region and 5′ half of the *ply* gene and three recombinants that cover the 3′ half of the gene along with the downstream region were selected by colony hybridization with the same probe as that used in Southern blotting (46).

DNA fragments from pBH1-35 and pHB2-32 were subcloned to construct the complete *ply* gene at the C terminus of the GST carrier in pGEX-2T by a conventional cloning method and PCR (46). Ampicillin-resistant transformants were screened by rabbit erythrocyte overlay (53). Eight colonies carrying the recombinant plasmid (pGEX-PL18C) showed small circular zones of hemolysis (<1 mm).

Expression and purification of rPL. An overnight culture of *E. coli* SCS1(pGEX-PL18C) (ATCC 69654) in Luria-Bertani or Terrific Broth medium containing ampicillin (100 μg/ml) was diluted 1 to 20 in the same medium. The culture was then grown at 37°C with vigorous shaking until an A_{600} of 1 was reached. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 1 mM, and incubation was continued for 2 h.

Briefly, affinity chromatography (50) was performed with minor modifications. Cells containing the overexpressed fusion protein GST-rPL were spun down at 10,000 × *g* for 10 min at 4°C, washed once with phosphate-buffered saline (PBS; 150 mM NaCl, 16 mM NaH_2PO_4, 4 mM Na_2HPO_4 [pH 7.3]), and resuspended in 1/50 volume of PBS. Triton X-100 was added to a 1% final concentration, and the cells were lysed by mild sonication. The lysate was centrifuged (10,000 × *g*, 20 min, 4°C), and cell debris was washed with the same volume of TPBS (1% Triton X-100 in PBS) once.

Supernatants were pooled and applied to a column of 50 ml of glutathione-agarose gel (Sigma) equilibrated with TPBS. The column was washed with 5 bed volumes of TPBS, 2 bed volumes of PBS, and 1 bed volume of 50 mM Tris-HCl (pH 8.0). The fusion protein was eluted with 10 mM glutathione–50 mM Tris-HCl (pH 8.0). Fractions showing hemolytic activity were pooled and dialyzed extensively in thrombin cleavage buffer (50 mM Tris-HCl [pH 8.3], 150 mM NaCl, 2.5 mM $CaCl_2$). The fusion protein after dialysis was mixed with bovine plasma thrombin (Sigma; 5 U per mg of protein) and incubated overnight. The mixture of GST, rPL, and some undigested fusion protein was centrifuged at 3,000 × *g* at 20°C with an Amicon Centriprep-10 (molecular weight cutoff, 10,000) to concentrate and change the buffer to PBS and then applied onto the glutathione-agarose column. Hemolytic fractions containing rPL were pooled. The buffer was exchanged to 10 mM sodium phosphate (pH 7.0) with a Centiprep-10 (Amicon). Thrombin was removed (4) from rPL by passing the rPL through a column of 1 ml of heparin-Sepharose gel (Pharmacia). Throughout the purification, the samples were monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by measuring the hemolytic activity (36).

The affinity-purified rPL that was contaminated with residual GST or lipopolysaccharide (LPS) was further purified with a hydroxylapatite (HA) column. HA chromatography was performed with a column (1.6 by 8.0 cm) of HA-Fast Flow (Calbiochem Corp., La Jolla, Calif.) equilibrated with 10 mM sodium phosphate (pH 6.8). A 10-ml (ca. 10-mg) portion of the rPL was applied to an HA column, and the sample was eluted with a 100-ml linear gradient of 10 to 200 mM sodium phosphate (pH 6.8). Fractions were assayed for protein content. Fractions containing the rPL were pooled and analyzed for protein concentration, hemolytic activity, and purity by SDS-PAGE. The purified rPL was stored at 4°C.

Amino acid analysis sequence determination. Amino acid analysis (Beckman 6300 amino acid analyzer) was performed on protein samples hydrolyzed in 6 N HCl in the presence of 0.2% phenol for 22 h at 110°C. The N-terminal amino acid sequence analysis of rPL was done by performing 42 cycles on a Milligen/Biosearch solid-phase sequencer (Prosequencer model 6600) with a Sequelon DITC attachment kit (Millipore Corp., Milford, Mass.) whereby the protein sample was covalently attached to the polyvinylidene difluoride membrane before sequencing.

SDS-PAGE and Western blotting (immunoblotting). Samples were prepared by mixing with an equal volume of 2× sample buffer (20 mM Tris-HCl [pH 8.0], 2.0 mM EDTA, 5% SDS, 10% β-mercaptoethanol) and heated at 100°C for 5 min. SDS-PAGE and Coomassie brilliant blue staining for protein were carried out on a Pharmacia PhastSystem or a Novex mini-gel system (Novel Experimental Technology) with precast polyacrylamide gels. Western blotting was carried out as described by Sambrook et al. (46). Proteins separated on the gel were transferred to nitrocellulose filters by the electroblotting device (Novel Experimental Technology) as described in the directions of the manufacturer. The rabbit antiserum containing antibodies to native PL was kindly donated by R. Lock (Adelaide Children's Hospital, North Adelaide, South Australia, Australia). Anti-rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase (Bio-Rad Laboratories) and an alkaline phosphatase conjugate substrate kit (Bio-Rad Laboratories) were used for the detection of the rPL on Western blots.

Hemolytic assay. The hemolytic activity of rPL was determined routinely with a microtiter plate assay similar to that described by Paton et al. (36), with a slight modification. Protein samples were activated with 10 mM dithiothreitol (DTT) for 15 min at 37°C before adding rabbit erythrocytes to a 1.7% (vol/vol) concentration. The mixture was then incubated for an additional 30 min at 37°C. After centrifugation at 150 × *g* for 5 min, the presence or absence of pellets was noted. The dilution representing 50% lysis of erythrocytes was determined visually.

Cytotoxicity assay. The cytotoxicity of rPL to bovine pulmonary endothelial cells in vitro was determined as described by Rubins et al. (44). To radiolabel intact cells, confluent cells were washed twice with PBS, trypsinized, washed twice with fresh culture medium, resuspended in 200 μl of PBS (pH 7.0) and 300 μl of ^{51}Cr (300 μCi), and incubated at 37°C with 5% CO_2 for 90 min. The cells were washed twice in PBS containing 5% bovine serum albumin (BSA) and 2% dextrose and resuspended in PBS containing 0.5% BSA and 0.2% dextrose. Cells were adjusted to 2×10^5/ml. In a 96-well microtiter plate, the radiolabeled cells (2×10^4) were mixed with rPL in 10 mM DTT and incubated at 37°C with 5% CO_2 for 2 h. After the cells were centrifuged at 150 × *g* for 3 min, the radioactivity in an aliquot of the supernatant was counted by liquid scintillation to determine the percentage of ^{51}Cr released. To determine the remaining cellular ^{51}Cr, 100 μl of 2 M NaOH was added, the solution was mixed, and the radioactivity in an aliquot was counted. The percentage of ^{51}Cr released was determined as the percentage of total counts per minute in the medium divided by the total counts per minute in the medium and the cell layer.

Synthesis of PS18C and rPL conjugates. Purified pneumococcal capsular PS18C was obtained from the bacterial vaccine production department of Lederle-Praxis Biologicals (Pearl River, N.Y.). To improve the solubility of PS18C during the conjugate reaction, the native PS18C was first partially depolymerized with acetic acid (final concentration, 1 M) treatment at 60°C for 40 h. The acid-treated material was passed through a Sepharose CL-4B column (Pharmacia) equilibrated with 10 mM PBS (pH 7.0) containing 0.01% thimerosal. The material eluted at a distribution coefficient (K_{av}) of 0.3 to 0.6 (corresponding to a molecular mass of about 30 to 500 kDa) was collected, dialyzed, and lyophilized. The oxidized PS18C was prepared from this partially depolymerized material with 2 mM sodium periodate in the dark for 10 min at room temperature. The excess sodium periodate was destroyed by reacting with ethylene glycol (final concentration, 25 mM). The reaction mixture containing the PS18C was dialyzed extensively against pyrogen-free water and lyophilized.

The PS18C was conjugated to rPL by either direct or indirect conjugation. For direct conjugation by reductive amination (19, 49), a sample of PS18C (6 mg/ml) in 0.1 M PBS (pH 7.0) was mixed with the rPL (3 mg/ml) at a PS/protein ratio of 1:0.5 by weight at room temperature with gentle stirring. After 30 min, sodium cyanoborohydride, at a final concentration of 20 mM, was added, and the mixture was incubated at room temperature with gentle stirring for 5 days. The mixture was chromatographed on a column of Sepharose CL-4B which had been equilibrated with 10 mM PBS (pH 7.0) containing 0.01% thimerosal. The eluate was assayed for protein and carbohydrate content. The first peak fractions that contained both protein and sugar were pooled, dialyzed, diafiltered on an Amicon concentrator (molecular weight cutoff, 30,000), and then used for the preparation of the vaccine.

For the coupling of PS18C to rPL by use of a spacer molecule, adipic acid dihydrazide (ADH), ADH was first coupled to rPL by carbodiimide-mediated condensation with 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDAC) (24, 47). Six hundred microliters of ADH (30 mg) and 0.1 ml of EDAC were added to rPL (12 mg) in 10 ml of 0.1 M potassium phosphate (pH 5.4) and incubated at room temperature with gentle mixing for 3 h. The reaction mixture was dialyzed extensively against 0.1 M potassium phosphate buffer (pH 7.0) at 4°C. The solution was concentrated and chromatographed on a column of Sepharose CL-4B (2.6 by 100 cm) as described above. The eluate was assayed for protein and for the adipic hydrazide (AH) content of deriviatized rPL (AH-rPL) (22). Peak fractions containing both protein and AH were pooled and lyophilized.

The coupling of AH-rPL to PS18C was performed as follows: AH-rPL (7.5 mg) in 3 ml of 0.2 M PBS (pH 8.0) and PS18C (15 mg) in 3 ml of the same buffer were mixed and incubated at room temperature with gentle mixing. After 2 h, sodium cyanoborohydride at a final concentration of 20 mM was added, and the mixture was incubated at room temperature with gentle mixing for 4 days. The PS18C(AH)-rPL conjugation solution was chromatographed on a column of Sepharose CL-4B. The first peak fractions containing the conjugate were pooled, dialyzed, diafiltered, and then used for vaccine preparations.

Analytical methods. Protein was assayed with the Bio-Rad protein microassay (10) with BSA as a protein standard. PS was assayed with the phenol-sulfuric assay (15) using homologous PS18C as a standard. The hydrazide was measured by the method of Inman and Dintzis (22). LPS (endotoxin), measured by the *Limulus* amoebocyte lysate assay, was expressed in endotoxin units related to the U.S. standard (21).

Rocket immunoelectrophoresis. Rocket immunoelectrophoresis was carried out on a Pharmacia PhastSystem as described in Pharmacia's Technical Note no. 3. Agarose gels (1%) containing antiserum to homologous PS were cast onto Gelbond (43 by 50 mm). After the gels were congealed, holes were punched in the agar with a template and a 2-mm gel puncher. Native, oxidized PS preparations or conjugates were applied to each well. After electrophoretic separation on the PhastSystem, the gels were removed from the separation bed and placed on a flat surface. Filter paper soaked in 0.9% saline was placed on top of the gel, followed by dry paper towels and a 1-kg weight. The filters were removed after 5 min, and new filter paper soaked in distilled water was applied in the same manner. The gels were then dried at 50°C for about 10 min. The dried gel was stained in the development unit of the PhastSystem with Coomassie blue.

Safety tests. The safety of rPL and conjugate vaccine preparations were tested in two mice (weighing <22 g) and two guinea pigs (weighing <400g), as described in the *Code of Federal Regulations*, 610.11. The rPL and the conjugate preparations, PS18C-rPL and PS18C(AH)-rPL, passed the safety test in mice and guinea pigs at 20- and 100-μg doses, respectively.

Vaccine preparations and immunogenicity studies. The conjugate vaccine preparations were diluted in PBS (pH 7.0) containing 0.01% thimerosal. The final vaccines were prepared by mixing the antigens with $AlPO_4$ (1 mg/ml) as an adjuvant, such that a 0.2-ml dose contained 0.2, 1, or 5 μg of the PS antigen. The rPL vaccine was partially inactivated by exposure to oxygen (36).

Groups of 5 or 10 female Swiss (CD-1) mice, age 6 to 8 weeks, were injected intraperitoneally (i.p.) with 0.2 ml of various vaccines or with PS or rPL as negative controls. The vaccines were administered three times at 2-week intervals. A small amount of blood (0.3 ml) was taken by retro-orbital venipuncture before each vaccine injection and 2 weeks following the final injection. The blood was centrifuged, and the serum was collected, inactivated at 56°C for 20 min, and stored at −20°C.

ELISA for antibodies to PS and rPL. The IgG antibodies to PS and rPL were measured by enzyme-linked immunosorbent assay (ELISA) as described previously by Koskela (31) and by Jalonen et al. (25), respectively.

Briefly, for measuring IgG antibodies to PS in the mouse antisera, 96-well microtiter plates were coated with PS18C and incubated at 37°C for 5 h. After the plates were washed, mouse sera which had been previously preabsorbed with C-polysaccharide (C-PS) (to eliminate any cross-contamination by the antibodies to C-PS) were serially diluted and incubated for 2 h at room temperature. Following washing, goat anti-mouse IgG conjugated with alkaline phosphatase (αIgG-AP; Southern Biotechnology Associates, Inc., Birmingham, Ala.) was added for 2 h at room temperature. The substrate, *para*-nitrophenylphosphate, was added to each well and incubated for 1 h at room temperature. The A_{405} of the plates was read. The titers were reported as the geometric mean of the inverse of the dilution representing 0.1 absorbance unit.

For measuring IgG antibodies to rPL in the mouse antisera, 96-well microtiter plates were coated with rPL and incubated overnight at 4°C. After washing, the plates were blocked with 10% horse serum in PBS for 4 h at room temperature. Following washing, mouse serum serially diluted in TPBS containing 1% BSA was incubated overnight at 4°C. After washing, αIgG-AP (ICN Biomedicals, Inc., Costa Mesa, Calif.) was added for 3 h at room temperature. The substrate, *para*-nitrophenylphosphate was added to each well for 15 min at room temperature. The A_{405} of the plates was read. The titers were reported as the geometric mean of the inverse of the dilution representing 0.3 absorbance unit.

Challenge of mice with *S. pneumoniae*. Groups of 40 female Swiss mice (CD-1), 8 weeks old, were injected i.p. (0.2 ml) with various vaccines three times at 2-week intervals. Two weeks after the last vaccination, the mice were injected with various doses of *S. pneumoniae* type 18C (ATCC 6318). The bacteria were grown overnight at 37°C on Trypticase soy agar plates with 5% sheep blood (BBL, Cockeysville, Md.), inoculated into Trypticase soy broth (BBL) containing 5% defibrinated sheep blood and 1% glucose, and incubated unshaken at 37°C for 6 h. The growth was diluted appropriately with Trypticase soy broth. The number of CFU per milliliter was determined by plate count. The doses of bacteria injected i.p. were calculated to be approximately 5, 25, 125, and 625 times the 50% lethal dose (LD_{50}), which was determined previously (LD_{50}, ≤4 CFU per dose). The animals were checked twice a day for up to 14 days, and deaths were recorded.

Statistics. Antibody levels were expressed as the geometric mean ELISA units. Concentrations below the sensitivity of the test were assigned one-half of that value. Comparisons of geometric means were based on a mixed-model analysis of variance by use of either paired or unpaired *t* tests.

The Mann-Whitney U test was used to analyze the significance of differences in the survival rate after challenge with *S. pneumoniae*.

RESULTS

Purification and properties of the rPL preparations. *E. coli* carrying the recombinant plasmid pGEX-PL18C was used to overexpress and purify rPL. We isolated and purified the rPL from *E. coli* lysates by affinity chromatography on a glutathione-agarose gel and finally by HA chromatography. Briefly, for the affinity purification of rPL, the following steps were involved (see Materials and Methods): (i) overexpression of GST-rPL in *E. coli*; (ii) glutathione-agarose affinity chromatography to purify GST-rPL fusion protein from cell lysates of the culture; (iii) digestion of GST-rPL with thrombin; (iv) second glutathione-agarose affinity chromatography to remove GST; and (v) heparin-Sepharose chromatography to remove thrombin. Although the procedure described above resulted in an rPL protein of high purity with respect to protein contaminants, it was observed that the purified rPL preparation still contained some contamination of LPS (endotoxin) from *E. coli*

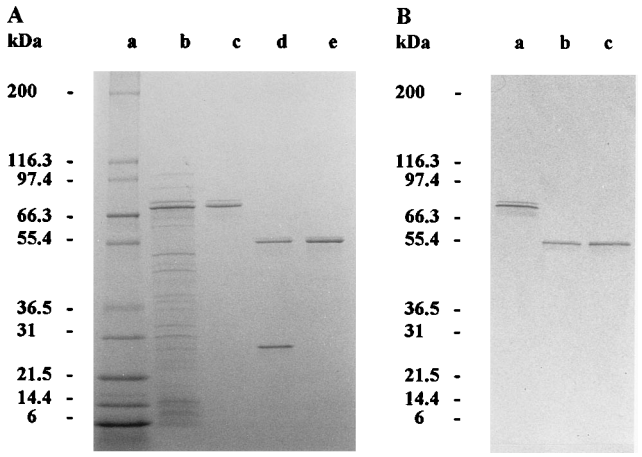

FIG. 1. SDS-PAGE (8 to 16% acrylamide) stained with Coomassie blue (A) and immunoblot (B) of rPL preparations after various purification steps. Rabbit antiserum against native PL (antiserum diluted 1:1,000) was used in Western blotting. (A) Lanes: a, molecular mass markers; b, cell lysate of IPTG-induced *E. coli*; c, purified fusion protein GST-rPL after glutathione-agarose affinity chromatography; d, mixture of GST and rPL after the thrombin digestion of fusion protein; e, purified rPL (0.5 μg of protein) free of GST and thrombin after HA chromatography. (B) Lanes: a, purified fusion protein GST-rPL; b, mixture of GST and rPL after the thrombin digestion of fusion protein; c, purified rPL after HA chromatography.

and occasionally some residual GST that was detected by the *Limulus* amoebocyte lysate assay and on stained gels by SDS-PAGE, respectively. The final step of purification with HA chromatography was found to be effective in eliminating LPS in the rPL protein. Figure 1 shows the SDS-PAGE patterns of the rPL preparation at various steps of purification. The HA-purified rPL preparation gave a single protein band with a molecular weight of 53,000 (Fig. 1A). Densitometric scans of the stained gel (5 μg of protein loaded) showed that the rPL accounted for at least 98% of the total protein. The endotoxin content in the HA-purified rPL, as estimated by the *Limulus* lysate test, was less than 40 endotoxin units per 100 μg of protein (<8 ng of LPS per 100 μg of protein). From a 1-liter culture, approximately 6 to 10 mg of rPL can be purified by this method. Immunoblot analysis of the rPL protein is shown in Fig. 1B. Both GST-rPL fusion protein and rPL reacted with the antisera containing the antibodies to native PL. Ouchterlony immunodiffusion revealed that rPL reacted identically with the anti-PL and anti-rPL antibodies. Isoelectric focusing in a polyacrylamide gel showed that the pI of the rPL protein was 4.9 to 5.2.

The purified rPL had a specific activity of 3×10^5 hemolytic units per mg of protein on rabbit erythrocytes, which is comparable to ca. 5×10^5 to 1×10^6 hemolytic units per mg of native PL (27, 30). The hemolytic activity was increased more than 100-fold by 10 mM DTT or cysteine and eliminated completely by cholesterol (10 μg/ml). The purified rPL was found to be cytotoxic to cultured bovine endothelial cells as measured by the ^{51}Cr release assay (44) (see below). Amino acid composition analysis and determination of the N-terminal amino acid sequence of the first 42 residues were performed on the purified rPL (data not shown). The N-terminal sequence of rPL is identical to that of native PL and to the predicted sequence deduced from the nucleotide sequence of the type 2 *ply* gene (53), with the exception of two additional residues (glycine and serine) which remain after thrombin cleavage at the N terminus (50). The amino acid composition of rPL agrees well with that deduced from the nucleotide sequence of

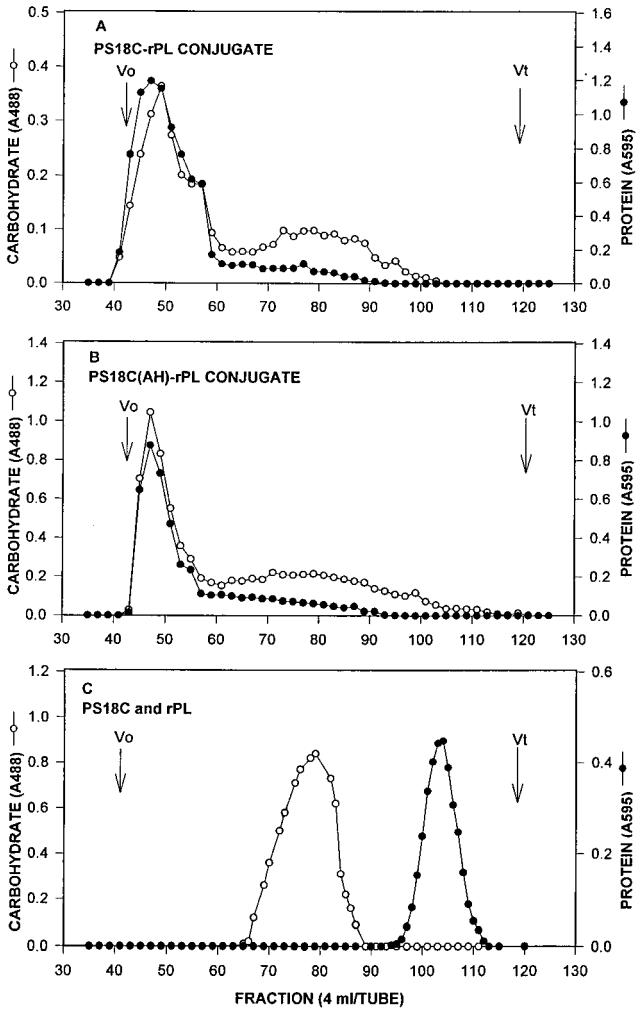

FIG. 2. Gel filtration of conjugates through a Sepharose CL-4B column. (A) PS18C-rPL; (B) PS18C(AH)-rPL; (C) unconjugated free PS18C and rPL. The phenol-sulfuric acid assay (A_{488}) (15) was used to measure PS. The method of Bradford (9) (A_{595}) was used to measure protein. Fractions having both protein and PS and eluting in the void volume, i.e., fractions 42 to 59 (K_{av} = 0.01 to 0.20) for the PS18C-rPL conjugate and fractions 45 to 55 (K_{av} = 0.02 to 0.16) for the PS18C(AH)-rPL conjugate, were pooled and subsequently used in vaccine experiments. The unconjugated free PS18C and rPL were eluted at K_{av} values of 0.30 to 0.60 and 0.61 to 0.81. Abbreviations: Vo, void volume, Vt, total volume.

the type 2 *ply* gene (53). The results indicate that the presence of the additional two N-terminal residues from GST did not affect various properties of rPL.

Characterization of PS/rPL conjugates. PS18C was coupled directly to rPL by reductive amination with cyanoborohydride or was coupled indirectly to rPL via a spacer molecule, ADH (19, 24). Figure 2A and B depict the elution profiles of the PS18C-rPL and PS18C(AH)-rPL conjugates, respectively. Both conjugates were purified over a Sepharose CL-4B column, and the peak fractions containing both protein and carbohydrate, which eluted in the void volume, were pooled. In a separate experiment, the unconjugated free PS18C and rPL were found to elute at K_{av} values of 0.3 to 0.6 and 0.6 to 0.8, respectively (Fig. 2C). To examine whether PS was covalently coupled to rPL in the conjugate preparations, the conjugates were analyzed by SDS-PAGE. It was found that the conjugate material, because of its high molecular weight, did not enter into the separating gel, which was analyzed for both protein and carbohydrate (data not shown). Ouchterlony immunodiffusion and rocket immunoelectrophoresis showed that both conjugates reacted identically with the antisera to PS, indicating that PS18C was antigenically active (data not shown). The final recovery of the purified conjugates, PS18C-rPL and PS18C(AH)-rPL, was about 20 to 30% for both rPL and carbohydrate on a weight basis.

The hemolytic activity of rPL was decreased more than 99.8% after direct coupling to PS18C and more than 99.98% after coupling to PS via ADH. Injection of 5 μg of rPL alone or 2.5 μg of conjugated protein (5 μg of PS per dose of conjugate) per mouse caused no deaths or signs of overt illness (data not shown).

Immunogenicity of PS18C/rPL conjugates. The immunogenicity of the PS18C/rPL conjugates was tested in Swiss (CD-1) mice with 0.2 and 1 μg of the vaccine (PS content for the conjugated vaccines). The antibody responses of mice immunized with PS18C/rPL conjugates are shown in Table 1. As expected, injection of either rPL or PS18C alone did not elicit PS antibodies. All PS18C/rPL conjugates elicited IgG anti-PS after the first injection. The second and third injections of these vaccines provoked significant rises in anti-PS antibodies for both conjugates ($P < 0.05$ for week 4 versus week 2 values; $P < 0.001$ for week 6 versus week 2 values). Interestingly, administration of the conjugates at doses as low as 0.2 μg of PS induced an equally great antibody response as those vaccinated with 1 μg. However, it was found that the administration of a higher dose of the conjugates (at 5 μg of PS) decreased the anti-PS antibody production (Table 2).

A single injection of rPL (1 μg) elicited anti-rPL antibodies in these mice. The second and third injection of the rPL vaccine resulted in a strong booster response to rPL antibody titers ($P < 0.001$ for week 4 and 6 versus week 2 values). All of these conjugates containing ca. 0.4 to 0.7 μg of rPL per dose elicited a significant rise in IgG anti-rPL antibodies after the first injection and marked increases in antibody titers after the second and third injections. Markedly lower levels of rPL antibodies were elicited by the low dose (ca. 0.1 μg of rPL) of the conjugate vaccine (Table 1).

Neutralizing antibodies to rPL. The neutralizing activity of antisera against rPL was tested in vitro for hemolysis of rabbit erythrocytes (Table 2). The neutralizing titers ranged from 1/4 to 1/32. The neutralizing titers elicited by the conjugates were at least one twofold step lower than those elicited by rPL alone. As expected, the sera elicited by immunization with PS18C did not exhibit any neutralizing activity. The results indicate that antibodies to rPL neutralize the capacity of rPL to lyse erythrocytes.

The neutralizing activity of antisera containing rPL antibodies was also tested for the cytotoxic effect of rPL on the cultured pulmonary endothelial cells (Table 3). The radioactive chromium release assay (^{51}Cr) was used for the measurement of rPL cytotoxicity to ^{51}Cr-labeled cells. rPL (5 ng) caused about 60% ^{51}Cr release from endothelial cells. Antisera containing antibodies to PS, to rPL, or to both PS and rPL were incubated without or with rPL and assayed for ^{51}Cr release activity. To reduce nonspecific neutralization activity from the sera, the antisera were diluted to 1/1,000 and 1/2,000 for use in these experiments. All of the antisera containing the anti-rPL antibodies completely abolished the cytotoxicity of rPL. In contrast, antisera that contained only anti-PS18C antibodies had no significant effect on rPL cytotoxicity.

Protection of i.p. vaccinated mice (challenge with *S. pneumoniae*). Groups of vaccinated and control mice (with 40 mice in each group) were inoculated i.p. with various doses of live *S. pneumoniae* type 18C cells in a total volume of 0.2 ml. Ten

TABLE 1. IgG antibody responses of mice to various vaccines

Vaccine[a]	PS18C				rPL			
	Dose (μg)	ELISA geometric mean antibody titer after wk[b]:			Dose (μg)	ELISA geometric mean antibody titer after wk[b]:		
		2	4	6		2	4	6
Expt 1								
rPL					1	176 (140–230)	15,053 (12,000–20,000)	86,110 (66,000–112,000)
PS18C	1	<50	<50	<50				
PS18C-rPL	1	185 (130–260)	603 (430–850)	7,135 (5,100–10,000)	0.68	368 (280–480)	5,840 (4,500–7,600)	24,917 (19,000–32,000)
PS18C(AH)-rPL	1	188 (130–260)	3,395 (2,400–4,800)[c]	59,767 (42,000–84,000)[c]	0.49	672 (520–870)	7,639 (5,900–10,000)	23,472 (18,000–31,000)
Expt 2								
rPL					1	199 (120–330)	21,531 (13,000–36,000)	140,025 (83,000–235,000)
PS18C	1	<50	<50	<50				
PS18C-rPL	0.2	167 (76–370)	5,238 (2,400–11,000)	31,611 (14,000–69,000)	0.08	85 (50–140)	415 (250–700)	755 (450–1,300)
PS18C-rPL	1	149 (68–330)	6,274 (2,900–14,000)	29,272 (13,000–64,000)	0.42	490 (290–820)	18,810 (11,000–32,000)[d]	53,781 (32,000–90,000)[d]

[a] Ten (experiment 1) or five (experiment 2) mice per group were injected three times (2-week intervals) with the vaccines.
[b] Values in parentheses are 25th to 75th percentiles. $P \leq 0.05$ for all week 4 titers versus week 2 titers and $P < 0.001$ for all week 6 titers versus week 2 titers, except for anti-rPL response to the PS18C-rPL vaccine containing 0.08 μg of rPL.
[c] $P < 0.05$ versus PS18C-rPL.
[d] $P < 0.01$ versus low dose (0.08 μg).

mice from each vaccinated group were challenged with 23, 115, 575 and 2,875 CFU per dose (i.e., 5, 25, 125, and 625 times the LD_{50} of pneumococci). With each vaccine, the survival results from each challenge dose were similar with respect to total numbers of survivors and times of death; the results from each group combined across challenge doses (40 mice) are shown in Fig. 3. Mice vaccinated with PS18C as the vaccine and challenged with *S. pneumoniae* died within 4 days. Mice given the rPL vaccine had a mean survival time of 4.3 days, compared with 2.5 days for control mice which were injected with PS18C (Fig. 3). When the results were analyzed by the Mann-Whitney U test, the observed differences were highly significant, with *P* values of <0.001 compared with results for mice injected with PS18C. The PS18C/rPL conjugate vaccines were effective in protecting mice against lethal infection with type 18C pneumococci. After 14 days, the survival rates for the conjugates PS18C-rPL and PS18C(AH)-rPL were 32 of 40 and 38 of 40, respectively (Fig. 3). Even at the highest challenge dose of 2.9×10^3 CFU (ca. 625 times the LD_{50}) of the live organisms, mice vaccinated with the PS18C(AH)-rPL or PS18C-rPL conjugate vaccine were protected almost totally (data not shown).

DISCUSSION

The current pneumococcal vaccine, composed of 23-valent type-specific capsular PSs, has been shown to protect adults from pneumococcal infection caused by those pneumococcal types included in the vaccine. However, it is poorly immunogenic in infants (14, 34). In view of the success of the conjugated PS vaccines in preventing *Haemophilus influenzae* diseases in children younger than 2 years of age (8, 16), there has been an interest in extending the conjugate technology to improve the immunogenicity of future pneumococcal vaccines for infant use.

Several pneumococcal conjugate vaccines have been prepared and are currently in clinical trials. In these vaccines, the type-specific capsular PSs (two to five types) have been conjugated to one of the protein carriers which has been used in *H. influenzae* type b, diphtheria toxoid, tetanus toxoid, and CRM_{197} or the outer membrane protein complex of *Neisseria meningitidis* (2, 12, 13, 18). Although these conjugates have demonstrated enhanced immunogenicity to the limited types included in the vaccine, they did not also protect against infection by other types of pneumococci. In addition, excessive use of these protein carriers in the conjugate vaccine may lead to carrier-mediated epitope suppression of the antibody response (40, 48). To induce additional protection against infection, a protein molecule, such as PL, a virulence factor produced by all types of pneumococci, might be suitable for use as the protein carrier for the new pneumococcal PS conjugate vaccine. Paton and coworkers (37) reported that mice vaccinated with a conjugate of type 19F PS and a nontoxic genetically engineered pneumolysoid induce higher antibody levels to the PS than those injected with the PS alone and also induce significant antibody titers against the protein component. In addition, immunization of the pneumolysoid or PS19F-pneumolysoid conjugate was able to confer to mice a significant degree of protection against challenge with virulent pneumococci (1, 33).

Unlike the nontoxic version of recombinant pneumolysoid used by Paton and coworkers (33, 37), the rPL used in this study is structurally and functionally equivalent to native PL. Two conjugates were synthesized in this study. The PS18C was linked to rPL directly or to rPL indirectly through a spacer molecule, ADH. Both methods resulted in the conjugates eluting as a single peak in the void volume on a Sepharose CL-4B

TABLE 2. Inhibition of hemolytic activity by anti-rPL-containing mouse sera

Vaccine	PS18C		rPL		Neutralizing activity[a] (titer)
	Dose (μg)	ELISA antibody titer after 3rd injection (wk 6) (geometric mean ± SD)	Dose (μg)	ELISA antibody titer after 3rd injection (wk 6) (geometric mean ± SD)	
PS18C	1	<100			
rPL			1	374,502 ± 0.39	+ (1:32)
PS18C-rPL	1	3,432 ± 0.95	1.39	146,113 ± 0.26	+ (1:16)
	5	654 ± 0.75	6.95	85,675 ± 0.52	+ (1:16)
PS18C(AH)-rPL	1	2,887 ± 1.40	0.69	35,478 ± 1.03	+ (1:4)
	5	2,310 ± 0.83	3.45	172,614 ± 0.55	+ (1:16)

[a] Neutralization of hemolytic activity of rPL by various antisera was examined by mixing 50 μl of rPL (20 μg/ml) with an equal volume of twofold-diluted antiserum and incubating the mixture for 15 min at 37°C; the rPL in the incubation mixture was preactivated with 5 μl of DTT (10 mM final concentration) for an additional 15 min. One hundred microliters of rabbit erythrocytes (final concentration, 0.8%) was added, the mixture was incubated at 37°C, and the hemolytic activity was determined as described in Materials and Methods.

column and did not move into the separating SDS-polyacrylamide gel, indicating that the conjugates are cross-linked, high-molecular-weight material. Although extensive cross-linking of the PS and rPL existed in the conjugates, both preparations reacted well with the antisera to PS and rPL. Interestingly, the covalent coupling of rPL to PS18C resulted in a greater-than-1,000-fold reduction in hemolytic activity of rPL, such that resulting preparations passed the safety test in mice and guinea pigs at 100 μg per dose. It remains to be investigated whether the covalent linkage of rPL to some other PSs such as types 6A, 6B, and 19F also results in the loss of hemolytic activity and whether the conjugates remain nontoxic after storage.

The immunogenicity of the vaccines adsorbed to aluminum phosphate at various immunizing doses was evaluated in mice (Table 1). PS18C alone was not immunogenic and might be considered a hapten. On the other hand, rPL alone induced antibodies to rPL after the first injection and a strong booster response after the second and third injections. We found that a single dose of the conjugate vaccines elicited anti-PS antibodies. After the second and third injections, a remarkably large booster response to PS was produced. Evaluation of the PS18C-rPL conjugate vaccine at a lower dose indicated that the vaccine at a 0.2-μg dose was still highly immunogenic in inducing the antibodies to PS. In contrast to the antibody response to PS with these conjugates, a significant increase in antibodies to rPL was produced after one injection. The rPL alone and in the conjugates induced a strong booster response after the second and third injections. A dose effect of the conjugates to the response of rPL was also found between approximately 1- and 0.4-μg doses (rPL). Unlike the antibody response to PS, significantly lower levels of rPL antibodies were elicited by the 0.08-μg dose (rPL) of the conjugate vaccine (Table 1). In short, these results indicated that the carrier protein, rPL, preserved the antigenicity of rPL and served as an excellent protein carrier which enhanced the antibody response to PS.

Effects of a spacer molecule on the immunogenicity of PSs have been investigated (23, 26, 38, 39). The introduction of a spacer, 6-aminohexanoic acid, in the conjugates containing pneumococcal PS type 4 enhanced the antibody response in mice (38, 39). However, superior antibody responses with directly coupled conjugates have also been reported (23, 26). In the present study, both types of conjugates appeared to induce equally great antibody responses for rPL; however, the PS18C(AH)-rPL conjugate appeared to elicit a higher antibody response for PS18C than the directly coupled conjugate did (Table 1).

TABLE 3. Effect of mouse antisera on the cytotoxicity of rPL in bovine pulmonary endothelial cells in vitro

Experimental condition	% ^{51}Cr release[a] (mean ± SD)
Total counts	100
Spontaneous release	9 ± 0.6
rPL (5 ng)	57 ± 0.6
rPL + anti-rPL serum (1:1,000)	11 ± 1.2
rPL + anti-rPL serum (1:2,000)	11 ± 1.0
Anti-rPL serum (1:1,000)	10 ± 0
rPL + anti-PS18C serum (1:1,000)	38 ± 1.2
rPL + anti-PS18C serum (1:2,000)	43 ± 1.5
Anti-PS18C serum (1:1,000)	11 ± 1.0
rPL + anti-PS18C-rPL serum (1:1,000)	11 ± 0
rPL + anti-PS18C-rPL serum (1:2,000)	11 ± 0.6
Anti-PS18C-rPL serum (1:1,000)	10 ± 0.6
rPL + anti-PS18C(AH)-rPL serum (1:1,000)	11 ± 0
rPL + anti-PS18C(AH)-rPL serum (1:2,000)	11 ± 0.6
Anti-PS18C(AH)-rPL serum (1:1,000)	10 ± 0

[a] The values refer to the means and standard deviations from triplicate ^{51}Cr-cell culture wells (24,000 cpm/200 μl), with 2×10^4 cells per well. The cells were incubated in the absence or presence of an agent(s) as indicated for 2 h at 37°C in the presence of 10 mM DTT. Percent ^{51}Cr release = $100 \times [2A/(A + B)]$, where A is the counts per minute in the top 100 μl and B is the counts per minute in the bottom 100 μl to which 100 μl of NaOH was added.

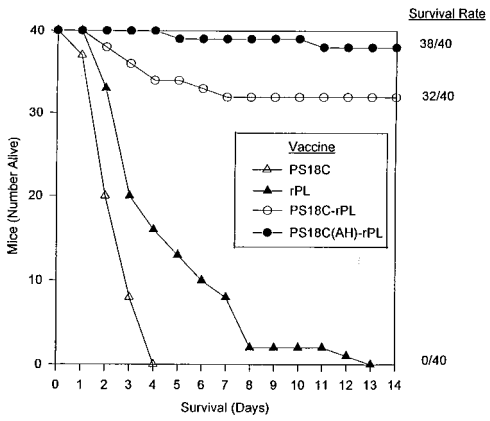

FIG. 3. Effect of vaccination with rPL and conjugate vaccines on survival of mice challenged with *S. pneumoniae*. Mice (40 per group) were immunized with the vaccine indicated. Two weeks after the last vaccination, each group of mice was challenged i.p. with 23, 115, 575, and 2,875 CFU per dose (i.e. 5, 25, 125, and 625 times the LD_{50}) of *S. pneumoniae* (ATCC 6318). The animals were checked daily for up to 14 days, and deaths were recorded.

To investigate the additional protective effect of the mouse antisera which contain the antibodies to rPL, the neutralization capacities of the antisera were examined with rabbit erythrocytes for hemolytic activity and bovine pulmonary endothelial cells for cytotoxicity. We have demonstrated that the antisera containing the antibodies to rPL neutralized the hemolytic activity of rPL to rabbit erythrocytes (Table 2). Recently, Rubins and coworkers (44, 45) reported that PL caused injury to pulmonary endothelial cells and alveolar epithelial cells. They suggested that by injuring the alveolar epithelium and pulmonary endothelium, PL may produce alveolar flooding and hemorrhage during the earliest stages of pneumococcal pneumonia. In the present study, we found that the rPL (5 ng) caused substantial injury to cultured endothelial cells by measuring the amount of ^{51}Cr release from radioactively labeled cells and was specifically inhibited by incubation with mouse antisera containing the antibodies to rPL (Table 3). In the mouse challenge studies, we obtained results in support of what has been reported by Paton and coworkers (36, 37) and demonstrated that the survival times of rPL-vaccinated mice were significantly longer than that of the control group which received PS18C vaccine. Furthermore, the conjugate vaccine containing PS18C and rPL, either through direct or indirect coupling, was capable of eliciting protection against challenge with type 18C pneumococci. These data suggest that appropriate levels of the two antibodies elicited by these conjugate vaccines may confer more protection than the conjugate with other proteins against diseases caused by encapsulated and PL-producing pneumococci.

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