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Purification and Immunogenicity of Genetically Obtained Pneumolysin Toxoids and Their Conjugation to *Streptococcus pneumoniae* Type 19F Polysaccharide

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As part of an ongoing study concerned with improving human vaccines against *Streptococcus pneumoniae*, the genes for two defined pneumolysin (PL) toxoids (pneumolysoids), Pd-A (PL with a Cys→Gly substitution at amino acid 428) and Pd-B (PL with a Trp→Phe substitution at position 433), were inserted into the high-expression vector pKK233-2 in *Escherichia coli* and the pneumolysoids were purified. Groups of mice which had been immunized with either Pd-A, Pd-B, or native PL purified from *S. pneumoniae* were then challenged either intranasally or intraperitoneally with virulent pneumococci. Mice in all immunized groups survived significantly longer than sham-immunized controls. Both pneumolysoids were more effective than PL as protective immunogens. Pneumolysoid Pd-B was conjugated covalently with pneumococcal type 19F capsular polysaccharide (19F PS), and the immunogenicities of both the protein and the PS moieties of the conjugate in mice were determined. Significant anti-PL titers were obtained, and the immunogenicity of the 19F PS moiety was markedly enhanced compared with that of unconjugated PS. Conjugation also appears to have converted the 19F PS into an antigen capable of inducing a booster effect. These results support the notion that the efficacy of human, PS-based antipneumococcal vaccines might be improved by supplementation with pneumolysoid in the form of a covalent pneumolysoid-PS conjugate.

Modern antipneumococcus vaccines for human use have all been formulated from a mixture of polysaccharides (PS) selected from the 83 serologically distinct capsular serotypes expressed by *Streptococcus pneumoniae*. The most recent commercial vaccine has been a 23-valent version designed with reference to data on the relative frequency of serotypes in pneumococcal infections, the degree of cross-reactivity of human antibodies to related serotypes, and the stability of PS in solution (7, 38).

However, PS-based vaccines have two critical shortcomings. The first is that the protection they impart is type specific. Because of this, a formulation of serotypes which is effective for one population may have a much-reduced efficacy for another if there are significant differences in serotype prevalence. For example, studies on pneumococcal isolates from Asian populations show that only 63 to 73% belong to serotypes included in the current 23-valent vaccine, as against 88 to 93% for the U.S. population (22, 38).

The second shortcoming of present vaccines is that even the protection they provide against included serotypes is by no means complete and may be very poor for certain high-risk groups such as the elderly and people with sickle-cell anemia, asplenia, multiple myeloma, nephrotic syndrome, cirrhosis, or alcoholism (6). There is evidence to suggest that people in these groups have a poorer antibody response to PS vaccines than healthy adults and are afforded less protection. For healthy adults, the initial response to pneumococcal PS antigens may last at least 5 years (31), but for immunocompromised patients the duration of protection may well be much shorter, and indeed, some high-risk groups may not be protected at all (15). Young children form a high-risk group of particular concern. In children less than 2 years old, the antibody response to most capsular types is generally poor (16). Also, a number of studies have shown that the antibody response to several important pediatric pneumococcal types (e.g., 6A, 14, 19F, and 23F) is decreased in children less than 5 years old, and this may explain the apparent lack of demonstrable clinical benefit in some vaccine trials in this age-group (10, 11, 25, 28, 37, 46).

Pneumococcal vaccines supplemented with appropriate protein antigens could possibly provide greater protection than the present PS-based formulations. Proteins are more immunogenic than PS, at least in young children who, as a group, respond well to protein-based vaccines (8). The most appropriate protein antigens would appear to be pneumococcal proteins playing an important role in the virulence of the organism. Since it seems likely that such proteins are produced by all invasive strains of the organism, a vaccine supplemented with these should have the advantage of providing a degree of protection against pneumococci of all serotypes, in contrast to the strictly type-specific protection conferred by the present formulations.

Protective pneumococcal protein antigens would also be attractive candidates for use as carriers for PS in a conjugate vaccine. Conjugation of bacterial PS to protein typically not only greatly enhances their immunogenicity but also converts them from thymus-independent to thymus-dependent antigens capable of promoting immunological memory (2, 43). Thus, there is the potential to improve existing pneumococcal vaccines by conjugating PS to a pneumococcal protein, thereby increasing the immunogenicity of the PS component as well as providing non-serotype-specific protection.

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We have evaluated the pneumococcal virulence proteins neuraminidase, autolysin, and pneumolysin (PL) as protective antigens in their own right in a mouse model of infection (3, 27, 35). Of these proteins, the most promising immunogen appeared to be PL. PL is a potent hemolytic cytotoxin, which is produced by essentially all clinically isolated strains of *S. pneumoniae* (35). Its status as an important virulence protein has been confirmed by our demonstration that genetically defined PL-negative mutant pneumococcus strains have significantly reduced virulence (4). We have also established (14, 32, 34, 36) that the native toxin has a variety of detrimental effects upon components of the human immune system in vitro.

Native PL is, of course, unsuitable for inclusion in a vaccine for humans because of its toxicity. However, cloning and sequencing of the PL gene (33, 45), as part of our ongoing studies on the structure, function, and mode of action of the toxin, has made possible a rational approach towards the generation of defined PL toxoids suitable for administration to humans. Comparison of the DNA and derived amino acid sequence of the gene for PL (45) with those for the related thiol-activated cytotoxins streptolysin O (18), listeriolysin O (29), and perfringolysin O (44) identified an 11-amino-acid sequence which was common to all and included, in each case, the only Cys residue present in each protein. The conserved sequence was thus strongly implicated in the toxic activity exhibited by this family of proteins. This region of the PL gene was a natural target for further studies involving site-directed mutagenesis (40), which revealed that the generation of certain single-amino-acid substitutions significantly reduced the hemolytic activity of the altered PL gene product.

In the present study, we examined the protective efficacy of two PL toxoids (pneumolysoids), Pd-A and Pd-B. In Pd-A, the Cys residue (at position 428) has been replaced by glycine. In Pd-B, a different residue in the 11-amino-acid conserved sequence (Trp-433) was replaced with Phe. In addition, Pd-B was conjugated to 19F PS, and the immunogenicities of both the protein and the PS moieties were determined.

MATERIALS AND METHODS

Construction of high-expression plasmids pJCP201 and pJCP202. The gene coding for Pd-A (PL Cys-428→Gly), constructed by oligonucleotide-mediated, site-directed mutagenesis of the cloned PL gene as previously described (40), was excised from its parent vector and inserted into pKK233-2, a high-expression vector containing an IPTG (isopropyl-β-D-thiogalactopyranoside)-regulated *trc* promoter (1). This was done by first introducing a unique *Nco*I restriction site into the initiation codon of the Pd-A gene by the gapped duplex mutagenesis method (19). The pneumolysoid coding sequence was excised from the parent vector by partial digestion with *Hin*dIII and digestion to completion with *Nco*I. The 1.6-kb *Nco*I-*Hin*dIII fragment containing the complete Pd-A coding sequence was then inserted into pKK233-2 which had been predigested with *Nco*I and *Hin*dIII. The resulting high-expression plasmid was designated pJCP201. High-expression plasmid pJCP202, encoding Pd-B (PL Trp-433→Phe) was constructed by replacing the distal 0.7-kb *Hin*dIII digest fragment of the pneumolysoid gene of pJCP201 with the corresponding fragment excised from replicative-form DNA prepared from an M13mp18 clone encoding the respective amino acid substitution.

Preparation of pneumolysoids. Pd-A was prepared from *Escherichia coli* JM109 harboring pJCP201, and Pd-B was prepared from *E. coli* JM109 harboring pJCP202. The pneumolysoids were purified by a modification of the procedure used previously for the preparation of PL from *S. pneumoniae* (27). Briefly, the respective *E. coli* strains were shaken overnight at 37°C in two 1-liter flasks containing 500 ml of Luria-Bertani broth supplemented with 50 μg of ampicillin per ml. These cultures were seeded into 9-liter bottles containing 7 liters of Luria-Bertani broth and 200 μl of Antifoam A emulsion (Sigma Chemical Co., St. Louis, Mo.). Incubation was continued at 37°C with aeration, and when the A_{600} of the culture reached approximately 0.3, expression of pneumolysoid was induced by adding 200 mg of IPTG. Incubation was continued for 2 h more, with the final A_{600} of the culture being about 0.5. Following concentration with an Amicon DC10LA hollow-fiber concentrator fitted with a 0.1-μm exclusion cartridge, the cells were pelleted by centrifugation and lysed by a French press. Cell debris was removed by centrifugation, and pneumolysoid was purified by serial column chromatography through DEAE-cellulose (Whatman DE-52) and Sephacryl S-200 Superfine (Pharmacia AB, Uppsala, Sweden). The presence of pneumolysoid was monitored by hemolytic assay (35), pooling fractions with activities of ≥100 hemolytic units (HU) per ml and 20 HU per ml for Pd-A and Pd-B, respectively, where 1 HU was defined as that amount of activity required to lyse 50% of a 1% (vol/vol) suspension of packed human group O erythrocytes in phosphate-buffered saline (PBS) in 30 min at 37°C. The final yield of pneumolysoid was about 2 mg per liter of original culture. The protein was ≥95% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of native PL. Native PL was prepared from *S. pneumoniae* as previously described (27).

Preparation of (Pd-B)–(19F PS) conjugate. Type 19F PS (Merck Sharp and Dohme, West Point, Pa.) was covalently linked to carrier protein Pd-B by the modified carbodiimide coupling method (26, 42). In brief, 19F PS was dissolved in 0.5 M borate buffer, pH 8.5, activated with cyanogen bromide (Sigma) at pH 10.5 for 6 min, and reacted with 0.1 M 6-aminocaproic acid (Sigma) at 4°C with gentle stirring for 24 h. The reaction mixture was dialyzed against two changes of PBS followed by deionized water and then lyophilized. The PS–6-aminocaproic acid complex and Pd-B were combined in 0.1 M sodium acetate buffer, pH 5.0, with the final concentration of each reactant being 5 mg/ml. Coupling was carried out by adding 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide–HCl (Sigma) to a final concentration of 250 mg/ml and incubating the reaction mixture for 1 h at 4°C. The mixture was fractionated by passage through a Sepharose CL-4B column with 0.2 M ammonium acetate as the eluant. The voided volume (V_0) peak was pooled, dialyzed against deionized water, and lyophilized. The concentration of the conjugate was determined by light-scattering nephelometry (21). Rabbit type 19F antiserum, obtained from the New York State Public Health Laboratories, Albany, N.Y., contained 8.16 mg of 19F antibody per ml. The antigen-antibody reaction was performed by mixing 0.6 ml of PBS in a glass tube with 42 μl of antigen sample and 42 μl of 10-fold-diluted antiserum. The scattering rate was determined by using a Beckman model 6624 Immunochemistry System Analyzer (Beckman Instruments Inc., Fullerton, Calif.) and was expressed in scattering rate units, representing the digitally filtered rate of change of the light scattering induced by the

antigen-antibody complex. Purified 19F PS was used as a positive control.

Protein assay. Protein concentrations were measured by the method of Bradford (5) with bovine serum albumin as the standard.

SDS-PAGE. SDS-PAGE was conducted essentially by the method of Laemmli (20), and gels were stained with Coomassie brilliant blue R250.

Immunization of mice with protein. Outbred, 6- to 8-week-old Quackenbush strain (Q/S) mice, were injected intraperitoneally with 0.2-ml volumes of an emulsion (1:1) containing either Pd-A, Pd-B, or PL (20 μg per antigen) in PBS and Freund complete adjuvant (Commonwealth Serum Laboratories, Melbourne, Australia). At 14-day intervals, the mice were given two additional injections of antigen emulsified in Freund incomplete adjuvant (Commonwealth). Control mice received a similar course of injections from which protein was omitted. Blood samples were collected 7 days after the last injection. Sera were tested for the presence of antibodies to PL by gel double immunodiffusion, as well as by enzyme-linked immunosorbent assay (ELISA) and an antihemolytic assay (see below).

Immunization of mice with (Pd-B)–(19F PS). Conjugate immunogen, mixed with aluminum hydroxide adjuvant (0.1 mg per dose), was administered intraperitoneally to groups of six BALB/c mice. The various immunization regimes are specified in Results.

Determination of anti-PL titers. Anti-PL titers were determined by ELISA, using native PL as antigen, as described by Jalonen et al. (17). Mouse sera were also tested for their ability to inhibit the hemolytic activity of native PL, as previously described (35). Antihemolytic titers were expressed as anti-HU per milliliter of serum.

Determination of anti-PS titers. Anti-PS antibody levels in mouse sera were determined by radioimmunoassay (41), using ^{14}C-19F PS prepared as follows. A lyophilized strain of type 19F *S. pneumoniae* was reconstituted in sterile saline, inoculated on sheep blood agar, and incubated at 37°C in humidified 5% CO_2 for 18 h. A colony was transferred to brain heart infusion medium and incubated at 37°C for 18 h. The culture was centrifuged at 10,000 × *g* for 15 min, and the pellet was washed twice with glucose-free 3% brain heart infusion–1% neopeptone medium. The washed cells were suspended in 50 ml of glucose-free brain heart infusion-neopeptone medium containing 0.5 ml of 0.5 M dibasic potassium phosphate and 1 ml (1 mCi) of uniformly labeled ^{14}C–D-glucose (Amersham Corp., Arlington Heights, Ill.) and incubated at 37°C for 15 h. The medium was adjusted to pH 7.4, and the culture was reincubated for 3 h. Sodium deoxycholate (1.0 mg/ml) was added, and the culture was left at 25°C for 1 h. The suspension of lysed pneumococci was centrifuged at 16,000 × *g* for 30 min, and the supernatant was dialyzed against three changes of deionized water at 4°C for 18 h. Sodium acetate was dissolved in the retentate to a final concentration of 5%, ethanol was added to a final concentration of 80%, and the mixture was stored at 4°C for 18 h. The PS was sedimented by centrifugation at 16,000 × *g* for 20 min, dried, and dissolved in 20 mM PBS, pH 7.4. The solution was passed through a Sepharose CL-4B column (2 by 90 cm), and the PS was eluted with 200 mM ammonium acetate. The fractions containing ^{14}C-PS were collected, dialyzed against deionized water, and lyophilized (41). The specific activity of the ^{14}C-labeled 19F PS was 10^4 to 10^5 dpm per μg of PS.

Serum samples (10 to 20 μl) were mixed with 0.5 ml of ^{14}C-19F PS (8×10^3 to 10×10^3 dpm) and incubated at 37°C for 15 min. The antigen-antibody complexes were precipitated with 0.5 ml of saturated ammonium sulfate solution at 4°C for 15 min and centrifuged at 30,000 × *g* at 4°C for 15 min. The precipitates were washed with 50% ammonium sulfate and centrifuged. The precipitates were resuspended in 50 μl of Triton X-100 (Sigma), and the radioactivity was determined by liquid scintillation after the addition of 10 ml of scintillation fluid. A reference human pneumococcal antiserum (14 valent) which contains 1,578 ng of 19F antibody N per ml was used as a standard. It was prepared by the Center for Biologics Evaluation and Research, Bethesda, Md., and the antibody levels were determined by Gerald Schiffman.

The C-polysaccharide (C-PS) content in 19F PS and (Pd-B)–(19F PS) conjugate was determined by the immunodiffusion (23) or countercurrent immunoelectrophoresis method (9). Both 19F PS and the (Pd-B)–(19F PS) conjugate contained less than 0.1% of C-PS. Some serum samples were preabsorbed with C-PS by incubating 10 to 20 μl of serum with 1 μg of C-PS for 60 min at 25°C. Preabsorption had no significant effect on the levels of 19F antibodies in these samples.

Challenge of mice. One week after bleeding, groups of protein-immunized or control Q/S mice were anesthetized and challenged intranasally with 50 μl of serum broth containing about 5×10^6 CFU of virulent type 2 *S. pneumoniae* D39, as previously described (27). This dose represents about 20 times the intranasal 50% lethal dose of D39 in Q/S mice. Alternatively, mice were injected intraperitoneally with 100 μl of serum broth culture containing about 6,500 CFU of D39. This dose represents about 20 times the intraperitoneal 50% lethal dose. The subsequent survival time of each mouse was recorded.

Statistical analysis. The results of challenge experiments were analyzed by the Mann-Whitney U test and by the χ^2 test (both one-tailed). The significance of differences in antibody levels between groups of mice was analyzed by using an unpaired, two-tailed Student's *t* test.

RESULTS

Protein antigens. After SDS-PAGE of purified PL, Pd-A, and Pd-B (not shown), all three proteins were visualized as comigrating single bands with a mobility corresponding to a molecular mass of 52 kDa. As previously reported (27), the specific hemolytic activity of PL was about 1.2×10^6 HU/mg of protein. Pneumolysoid Pd-A had a specific activity of about 6.2×10^3 HU/mg, and that of Pd-B was about 1.4×10^3 HU/mg.

Toxicity. A dose of 50 μg of active PL injected intraperitoneally in 200 μl of PBS was fatal to mice, while a similar dose of Pd-A was not. Higher doses of pneumolysoid were not tested. When protein was injected in 100 μl of PBS via a tail vein, a dose of 1 μg of native PL was tolerated, but 5 μg was lethal within 3 min. By contrast, mice survived intravenous injection of at least 25 μg of Pd-A. Again, higher doses of pneumolysoid were not tested.

Antibody responses of mice to unconjugated protein antigens. Groups of mice were immunized with 3 20-μg doses of PL, Pd-A, or Pd-B or were sham immunized as described in Materials and Methods. When tested by gel double immunodiffusion, the sera of mice which had been immunized with PL, Pd-A, or Pd-B all produced a single precipitin band when tested against PL antigen. Control sera produced no band (result not shown). Anti-PL titers of the mouse sera were also determined by ELISA, using pooled sera from

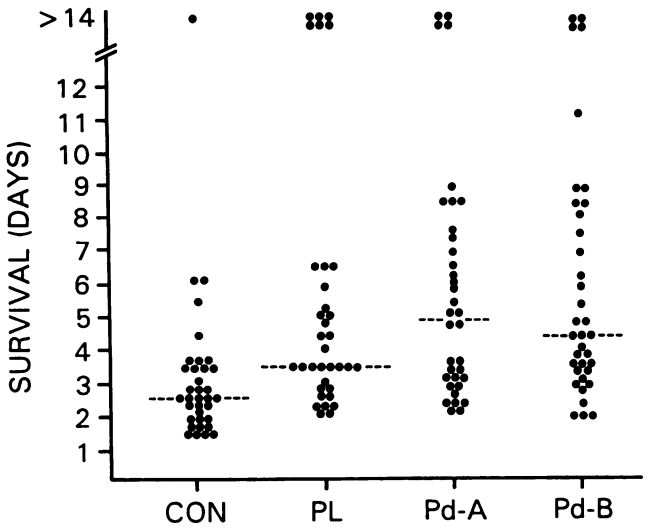

FIG. 1. Intranasal challenge of immunized mice. Groups of 36 mice were immunized with PL, Pd-A, or Pd-B or were sham immunized (CON) as described in the text. Two weeks after the last injection, mice were anesthetized and challenged intranasally with approximately 5×10^6 *S. pneumoniae* D39. The subsequent survival time of each mouse is indicated by dots, as is the number of mice which were alive and well after 14 days. Broken lines indicate the median survival time for each group.

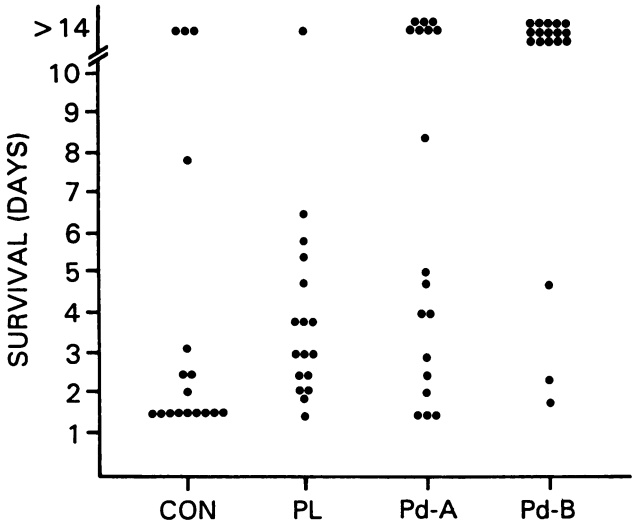

FIG. 2. Intraperitoneal challenge of immunized mice. Groups of 18 mice were immunized with the indicated antigens or were sham immunized (CON). Two weeks after the last injection, mice were inoculated intraperitoneally with 6.5×10^3 *S. pneumoniae* D39. The subsequent survival time of each mouse is indicated by dots, as is the number of mice which were alive and well after 14 days.

each group. These sera had anti-PL titers of 5,295, 5,630, and 6,420 for the PL, Pd-A, and Pd-B groups, respectively, compared with a titer of 5 for the sham-immunized control group. The capacity of sera to inhibit the hemolytic activity of PL was also measured. For control sera, the anti-HU titers were about 300, while the groups immunized with the three protein antigens gave essentially identical responses, attaining anti-HU titers of about 10,000 (result not shown). Thus, the pneumolysoids are at least as effective as PL in eliciting the production of specific antibodies, including those capable of neutralizing the hemolytic activity of the toxin.

Challenge of mice immunized with unconjugated protein antigens. To determine the protective efficacy of the various antigens, immunized and control Q/S mice were challenged intranasally with virulent type 2 *S. pneumoniae* D39, as described in Materials and Methods. Survival times for these mice subsequent to challenge are shown in Fig. 1. In a second experiment, mice were challenged intraperitoneally with D39, and their survival times are shown in Fig. 2. A statistical analysis of these results is summarized in Table 1. When challenged by either the intranasal or intraperitoneal route, mice immunized with PL, Pd-A, or Pd-B survived significantly longer than control mice. For mice challenged intraperitoneally, immunization with Pd-B, but not Pd-A or PL, also resulted in a significant increase in the overall survival rate, compared with that of the controls. The survival rate for mice immunized with Pd-B was also significantly greater than that for mice immunized with Pd-A or PL.

Preparation of (Pd-B)–(19F PS) conjugate. In view of the superior protective efficacy of Pd-B, this toxoid was chosen for conjugation to 19F-PS. Figure 3A shows the Sepharose CL-4B gel filtration chromatography profiles of unconjugated Pd-B and 19F PS. Figure 3B shows the corresponding profile obtained after conjugation of the pneumolysoid to the PS. After chromatography, the distribution coefficient (K_d) of the conjugate was estimated as 0.01 and the protein content was estimated to be 30 to 50%.

Immunogenicity of the conjugate. In a preliminary experiment, groups of six mice were immunized with a single injection of either 0.5 or 5 μg of 19F PS or similar doses of (Pd-B)–(19F PS) conjugate. Control mice received an injection of an identical volume of saline plus adjuvant. The serum anti-PS response was measured 3 weeks later. The groups of mice that received PS alone had mean (± the standard error of the means) 19F antibody levels of 0.60 (±0.04) and 1.50 (±0.06) μg/ml, for the 0.5- and 5-μg doses, respectively. Control (sham-immunized) mice had a mean level of 0.23 (±0.02) μg/ml. However, mice that received the conjugate had 19F antibody levels of 2.72 (±0.10) and 3.36 (±0.15) for the respective doses. In both cases, the conjugate elicited a significantly higher 19F antibody level than the

TABLE 1. Statistical analysis of mouse immunization and challenge results shown in Fig. 1 and 2

Immunization comparison	Significance (*P*)			
	Intranasal challenge		Intraperitoneal challenge	
	Survival time[a]	Survival rate[b]	Survival time	Survival rate
PL vs Con	<0.0001	NS[c]	<0.035	NS
Pd-A vs Con	<0.0001	NS	<0.0001	NS
Pd-B vs Con	<0.001	NS	<0.0001	<0.0005
Pd-A vs PL	NS	NS	NS	<0.05
Pd-B vs PL	NS	NS	<0.0002	<0.001
Pd-B vs Pd-A	NS	NS	<0.008	<0.02

[a] Values represent the significant difference in survival times as determined by the one-tailed Mann-Whitney U test.
[b] Values represent the significant difference in overall survival rates as determined by a one-tailed χ^2 test.
[c] NS, not significant.

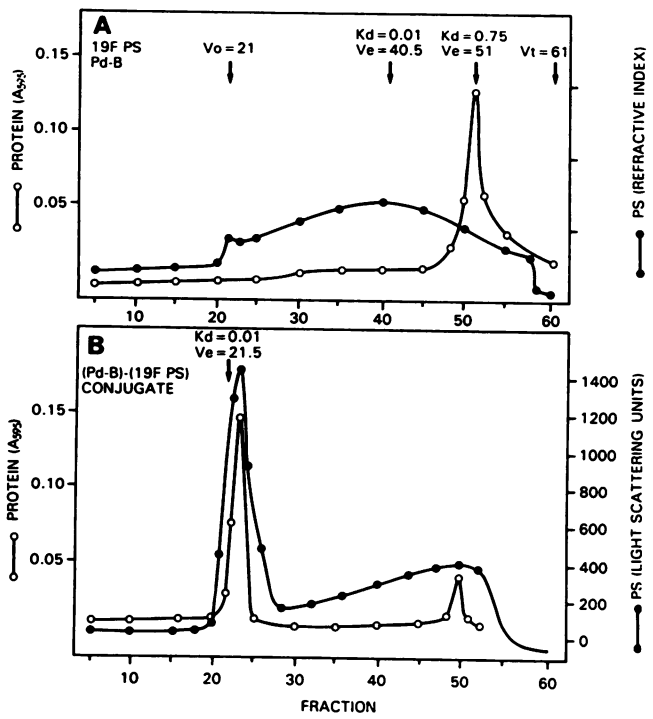

FIG. 3. Sepharose CL-4B chromatography of (Pd-B)–(19F PS). (A) Chromatographic analysis of nonconjugated Pd-B and 19F PS; (B) chromatographic analysis of the (Pd-B)–(19F PS) conjugate. Protein was monitored colorimetrically at 595 nm, according to the method of Bradford (5). PS was monitored by the refractive index or by nephelometry. Abbreviations: K_d, distribution coefficient; V_0, voided volume; V_e, elution volume; V_t, total volume.

respective dose of polysaccharide alone ($P < 0.001$ [two-tailed unpaired t test]).

In a more detailed experiment, the effect of multiple injections was investigated. The various immunization regimes used for the administration of (Pd-B)–(19F PS) to groups of six BALB/c mice are shown in Table 2, as are the anti-PS and anti-PL responses determined by radioimmunoassay and ELISA, respectively. Group 0, the control group, received three injections of saline plus adjuvant; groups I:A, I:B, and I:C received a single dose of 0.5, 5, or 50 μg of conjugate, respectively. Groups II:A and II:B received respective priming doses of 5 and 50 μg of conjugate, followed by a second (booster) dose of 5 μg, while groups III:A and III:B received the same, as well as a third dose of 5 μg of (Pd-B)–(19F PS).

TABLE 2. Immunization of mice with (Pd-B)–(19F PS) and serum antibody responses

Group	Conjugate dose (μg)[a]			Serum antibody[b]	
	1	2	3	Mean (SEM) anti-PS, μg/ml	Anti-PL titer
0				0.21 (0.03)	2.5
I:A	0.5			1.83 (0.35)	3.3
I:B	5			3.19 (0.21)	3.3
I:C	50			4.33 (0.99)	3.7
II:A	5	5		4.16 (0.81)	2.7
II:B	50	5		4.43 (0.29)	3.8
III:A	5	5	5	23.3 (3.58)	34.0
III:B	50	5	5	22.1 (3.66)	43.0

[a] The indicated doses of conjugate were administered to groups of six mice at 1-week intervals. The control group (0) received three sham immunizations of buffer plus adjuvant.

[b] Sera were collected 1 week after the final dose. Anti-PS levels of individual mouse serum were determined by radioimmunoassay and expressed as micrograms of antibody per milliliter. Values shown are group means ± the standard errors of the means. Anti-PL titers of pooled group sera were determined by ELISA.

For all the groups receiving conjugate, the anti-PS levels were significantly greater than the control (sham-immunized) group ($P < 0.001$, t test). The highest anti-PS levels (22 to 23 μg/ml) were obtained with groups III:A and III:B, which received a priming dose of either 5 or 50 μg of conjugate, followed by two booster injections of 5 μg. These anti-PS levels were 100-fold higher than that of group 0, the sham-immunized control (0.2 μg/ml). Administration of the third dose significantly increased the anti-PS level over that obtained after 2 doses ($P < 0.001$, t test).

The maximum anti-PL response also occurred in the two groups of mice which received 3 doses of conjugate, the respective anti-PL ELISA titer being approximately 20-fold greater than that of the control group.

DISCUSSION

Previous studies (4, 14, 27, 32, 34–36) have identified the toxin PL as a potential target for preventative strategies against *S. pneumoniae*. More recent work (40, 45) involving DNA sequence analysis and a study of the effects of particular DNA base mutations in the cloned PL gene on the properties of the resultant protein product has provided important information on the structure and mode of action of the toxin. This information also provided a means of overcoming one of the impediments to the use of PL as a human vaccine antigen, namely its toxicity. The aim was to generate mutations which reduced toxicity but did not impair the immunogenicity of the PL derivative. The conserved 11-amino-acid sequence in the vicinity of the unique Cys residue at position 428 was clearly important as far as the hemolytic activity of the toxin was concerned, and the respective region of the PL gene was subjected to oligonucleotide-directed mutagenesis. Amino acid substitutions were designed to be as conservative as possible in order to minimize disruption of the secondary structure of the pneumolysoid which might in turn alter its antigenicity. A Cys-428→Gly substitution significantly reduced hemolytic activity (40), as did Trp-433→Phe (unpublished data). In the present study, we examined the immunogenicity of both these pneumolysoids (Pd-A and Pd-B). The hemolytic activities of Pd-A and Pd-B were found to have been reduced to about 0.6 and 0.1%, respectively, compared with that of native PL, and limited data on Pd-A suggested that its toxicity in mice had also been substantially reduced.

Once the genes for Pd-A and Pd-B had been inserted into the high-expression vector pKK233-2 and good yields of both toxoids were readily obtainable, they were purified and used to immunize mice. Anti-PL and anti-HU titers of the resulting sera, as well as double-immunodiffusion gel analysis, indicated that both pneumolysoids were as effective as the native toxin in inducing an anti-PL immune response. Following immunization (or sham immunization of controls), groups of mice were challenged either intranasally or intraperitoneally with the virulent, wild-type pneumococcal strain D39. In the intranasal challenge experiments, the majority of mice in all groups died within 14 days of

challenge. However, the survival times of the three groups of immunized mice (median survival times were 3.55 days for PL, 4.95 days for Pd-A, and 4.70 days for Pd-B) were all increased highly significantly ($P < 0.0001$ in each case) compared with that of the control group (median survival time, 2.55 days). Statistical analysis indicated that the differences in survival times between immunized groups were not significant. Thus, both pneumolysoids were at least as protective as native PL.

In the intraperitoneal challenge experiments, the median survival times of groups of immunized mice (3.00 days for PL, 5.00 days for Pd-A, and >14 days for Pd-B) were again all significantly greater than that of the control group (1.50 days), but Pd-B was clearly much more effective as a protective immunogen than either PL or Pd-A. Indeed, 15 of the 18 Pd-B mice survived beyond 14 days after challenge, a survival rate which was itself significantly greater than that attained by mice in the other groups (3 of 17 for controls, 1 of 17 for the PL group, and 7 of 17 for Pd-A). These results identified Pd-B as the preferred protein for conjugation to pneumococcal capsular PS. The reason for the apparent superiority of Pd-B as a protective immunogen is unclear but may be related to the fact that its hemolytic activity is 5-fold less than Pd-A. If this is the case, then there may be advantages in reducing hemolytic activity even further (perhaps by mutating both the Cys-428 and Trp-433 residues), although the existence of vestigal activity provides a convenient assay for monitoring pneumolysoid during purification. A second factor is that, although the 11-amino-acid conserved region is involved in hemolytic activity and the capacity of the toxin to interfere with polymorphonuclear leukocyte functions, mutations in this region do not impair the capacity of the protein product to directly bind immunoglobulins and activate the classical complement pathway (40). Mutations in a second region of the PL gene, which has a degree of homology to human C-reactive protein, are however, capable of blocking this potentially inflammatory property. The optimum pneumolysoid, therefore, could include single or double mutations in each of the critical regions of the toxin.

Since the 1940s, the covalent conjugation of bacterial PS to proteins has been a common procedure. In most cases, the immunogenicity of the carbohydrate moiety of conjugates has been enhanced, and the carbohydrate has been converted from a thymus-independent to a thymus-dependent antigen. In addition, the protein moiety has usually remained antigenic. When the conjugate is to be used as a vaccine, there is therefore much to be gained by using a carrier protein which is itself a protective immunogen. To date, the best characterized PS-protein conjugate vaccine has been a *Haemophilus influenzae* type b PS covalently coupled to diphtheria toxoid. Such a conjugate was recently evaluated for clinical efficacy in an open trial involving 114,000 young children in Finland (12). Vaccine composed of *H. influenzae* type b capsular PS alone had previously been shown to be ineffective as an immunogen in infants. However, the rate of protection provided by 3 doses of the conjugate vaccine (administered at 3, 4, and 6 months of age) was 94% in the 18 months following the third vaccination. Studies with pneumococcal types 6B and 12F PS conjugated to either tetanus or diphtheria toxoids have also demonstrated enhanced immunogenicity of the PS moieties (13, 39).

Unlike the above studies on pneumococcal PS-protein conjugates, which used nonsense proteins as carriers, the protein carrier used in the present study is a protective antigen in its own right. It is a protein known to be produced by virtually all clinical isolates of *S. pneumoniae* (35). Moreover, sequence analysis of the PL genes cloned from unrelated serotypes (a type 1 strain isolated in Australia in 1982 [33] and a type 2 strain isolated in the United States in 1917 [45]), indicated a very high degree of conservation (a total of four base changes in the structural gene, resulting in a difference of just one amino acid residue) (30).

Type 19F PS was chosen for the present study because it is poorly immunogenic in both young children and mice (11, 24). An improvement in immunogenicity for humans is therefore particularly desirable and should be readily detected in the animal model. Following conjugation by the carbodiimide method, (Pd-B)–(19F PS) was recovered as a high-molecular-weight complex at the V_0 of the Sepharose CL-4B column and shown to have a high protein content (30 to 50%). Mice were immunized with the conjugate by a number of different regimes. Analysis of serum antibody responses to the PS moiety demonstrated clearly that it had been converted to an antigen capable of inducing a booster effect. While either a single dose of conjugate (5 or 50 μg) or such a dose followed by a 5-μg booster induced a 15- to 21-fold increase in the anti-PS response, a triple dose (5 or 50 μg followed by two boosts of 5 μg) induced a more-than-100-fold increase, with a maximum anti-19F PS level of 22 to 23 μg/ml. Mice immunized with a single (optimal) dose of 5 μg of purified 19F PS alone had 19F antibody levels of approximately 1.5 μg/ml. The observed booster effect suggests that coupling to pneumolysoid converted the PS from a thymus-independent to a thymus-dependent antigen. For this reason alone, Pd-B would appear to be an appropriate choice as a carrier protein for pneumococcal PS. It is interesting to contrast this result with those of a previous study (26) in which 19F PS was conjugated, by reductive amination, to either human immunoglobulin G, pneumococcal cell wall polypeptide, or bovine serum albumin. Secondary immunization of mice with these conjugates suppressed the immune response to the PS.

While the level of anti-PL induced by the conjugate was modest compared with that induced by 3 doses of unconjugated PL or pneumolysoid, a substantial enhancement of this response should be obtained by the inclusion of unconjugated pneumolysoid at some stage(s) in the immunization regime. Also, administration of a polyvalent pneumococcal conjugate vaccine in which several PS serotypes are conjugated to the pneumolysoid would increase the aggregate dose of protein antigen. Such studies, now in progress, are a prerequisite for the performance of trials involving challenge with virulent pneumococci of a homologous or heterologous serotype.

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REFERENCES

1. **Amann, E., and J. Brosius.** 1985. "ATG vectors" for regulated high-level expression of cloned genes in *Escherichia coli*. Gene **40**:183–190.
2. **Basten, A., and J. G. Howard.** 1973. Thymus independence. Contemp. Top. Immunobiol. **2**:265–291.
3. **Berry, A. M., R. A. Lock, D. Hansman, and J. C. Paton.** 1989. Contribution of autolysin to virulence of *Streptococcus pneumoniae*. Infect. Immun. **57**:2324–2330.
4. **Berry, A. M., J. Yother, D. E. Briles, D. Hansman, and J. C. Paton.** 1989. Reduced virulence of a defined pneumolysin-

negative mutant of *Streptococcus pneumoniae*. Infect. Immun. **57**:2037–2042.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
6. **Broome, C. V.** 1981. Efficacy of pneumococcal polysaccharide vaccines. Rev. Infect. Dis. **3**(Suppl):S82–S88.
7. **Cadoz, M., J. Armand, F. Arminjon, J.-P. Michel, M. Michel, F. Denis, and G. Schiffman.** 1985. A new 23 valent pneumococcal vaccine: immunogenicity and reactogenicity in adults. J. Biol. Stand. **13**:261–265.
8. **Chu, C., R. Schneerson, J. B. Robbins, and S. C. Rastogi.** 1983. Further studies on the immunogenicity of *Haemophilus influenzae* type b and pneumococcal type 6A polysaccharide-protein conjugates. Infect. Immun. **40**:245–256.
9. **Coonrod, J. D., and M. W. Rytel.** 1973. Detection of type-specific pneumococcal antigens by CIE. I. Methodology and immunologic properties of pneumococcal antigens. J. Lab. Clin. Med. **81**:770–777.
10. **Douglas, R. M., and H. B. Miles.** 1984. Vaccination against *Streptococcus pneumoniae* in childhood: lack of demonstrable benefit in young Australian children. J. Infect. Dis. **149**:861–869.
11. **Douglas, R. M., J. C. Paton, S. J. Duncan, and D. J. Hansman.** 1983. Antibody response to pneumococcal vaccination in children younger than five years of age. J. Infect. Dis. **148**:131–137.
12. **Eskola, J., H. Kayhty, A. K. Takala, H. Peltola, P.-R. Ronnberg, E. Kela, E. Pekkanen, P. H. McVerry, and P. H. Makela.** 1990. A randomized, prospective field trial of a conjugate vaccine in the protection of infants and young children against invasive *Haemophilus influenzae* type b disease. N. Engl. J. Med. **323**:1381–1387.
13. **Fattom, A., C. Lue, S. C. Szu, J. Mestecky, G. Schiffman, D. Bryla, W. F. Vann, D. Watson, L. M. Kimzey, J. B. Robbins, and R. Schneerson.** 1990. Serum antibody response in adult volunteers elicited by injection of *Streptococcus pneumoniae* type 12F polysaccharide alone or conjugated to diphtheria toxoid. Infect. Immun. **58**:2309–2312.
14. **Ferrante, A., B. Rowan-Kelly, and J. C. Paton.** 1984. Inhibition of in vitro human lymphocyte response by the pneumococcal toxin pneumolysin. Infect. Immun. **46**:585–589.
15. **Forrester, H. L., D. W. Jahigen, and F. M. LaForce.** 1987. Inefficacy of pneumococcal vaccine in a high-risk population. Am. J. Med. **83**:425–430.
16. **Immunization Practices Advisory Committee.** 1989. Pneumococcal polysaccharide vaccine. Morbid. Mortal. Weekly Rep. **38**: 64–76.
17. **Jalonen, E., J. C. Paton, M. Koskela, Y. Kerttula, and M. Leinonen.** 1989. Measurement of antibody responses to pneumolysin—a promising method for the etiological diagnosis of pneumococcal pneumonia. J. Infect. **19**:127–134.
18. **Kehoe, M. A., L. Miller, J. A. Walker, and G. J. Boulnois.** 1987. Nucleotide sequence of the streptolysin O (SLO) gene: structural homologies between SLO and other membrane-damaging, thiol-activated toxins. Infect. Immun. **55**:3228–3232.
19. **Kramer, W., V. Drutsa, H.-W. Jansen, B. Kramer, M. Pflugfelder, and H.-J. Fritz.** 1984. The gapped duplex DNA approach to oligonucleotide-directed mutation construction. Nucleic Acids Res. **12**:9441–9456.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. **Lee, C.-J.** 1983. The quantitative immunochemical determination of pneumococcal and meningococcal capsular polysaccharides by light scattering rate nephelometry. J. Biol. Stand. **11**:55–64.
22. **Lee, C.-J.** 1987. Bacterial capsular polysaccharides—biochemistry, immunity and vaccine. Mol. Immunol. **24**:1005–1019.
23. **Lee, C.-J., and K. Koizumi.** 1981. Immunochemical relations between pneumococcal group 19 and Klebsiella capsular polysaccharides. J. Immunol. **127**:1619–1623.
24. **Lee, C.-J., and K. T. Lin.** 1981. Studies on vaccine control and immunogenicity of polysaccharides of *Streptococcus pneumoniae*. Rev. Infect. Dis. **3**(Suppl.):S51–S59.
25. **Leinonen, M., A. Sakkinen, R. Kalliokoski, J. Luotenen, M. Timonen, and P. H. Makela.** 1986. Antibody response to 14-valent pneumococcal capsular polysaccharide vaccine in preschool age children. Pediatr. Infect. Dis. **5**:39–44.
26. **Lin, K. T., and C.-J. Lee.** 1982. Immune response of neonates to pneumococcal polysaccharide-protein conjugate. Immunology **46**:333–342.
27. **Lock, R. A., J. C. Paton, and D. Hansman.** 1988. Comparative efficacy of pneumococcal neuraminidase and pneumolysin as immunogens protective against *Streptococcus pneumoniae*. Microb. Pathog. **5**:461–467.
28. **Makela, P. H., M. Leinonen, J. Pukander, and P. Karma.** 1981. A study of the pneumococcal vaccine in prevention of clinically acute attacks of recurrent otitis media. Rev. Infect. Dis. **3**(Suppl.):S124–S129.
29. **Mengaud, J., J. Chenevert, C. Geoffroy, J. Gaillard, and P. Cossart.** 1987. Isolation of the structural gene encoding the SH-activated hemolysin of *Listeria monocytogenes*: listeriolysin O is homologous to streptolysin O and pneumolysin. Infect. Immun. **55**:3225–3227.
30. **Mitchell, T. J., F. Mendez, J. C. Paton, P. W. Andrew, and G. J. Boulnois.** 1990. Comparison of pneumolysin genes and proteins from *Streptococcus pneumoniae* types 1 and 2. Nucleic Acids Res. **18**:4010.
31. **Mufson, M. A., H. E. Krause, and G. Schiffman.** 1983. Long term persistence of antibody following immunization with pneumococcal polysaccharide. Proc. Soc. Exp. Biol. Med. **173**:270–275.
32. **Nandoskar, M., A. Ferrante, E. J. Bates, N. Hurst, and J. C. Paton.** 1986. Inhibition of human monocyte respiratory burst, degranulation, phospholipid methylation and bactericidal activity by pneumolysin. Immunology **59**:515–520.
33. **Paton, J. C., A. M. Berry, R. A. Lock, D. Hansman, and P. A. Manning.** 1986. Cloning and expression in *Escherichia coli* of the *Streptococcus pneumoniae* gene encoding pneumolysin. Infect. Immun. **54**:50–55.
34. **Paton, J. C., and A. Ferrante.** 1983. Inhibition of human polymorphonuclear leukocyte respiratory burst, bactericidal activity, and migration by pneumolysin. Infect. Immun. **41**: 1212–1216.
35. **Paton, J. C., R. A. Lock, and D. J. Hansman.** 1983. Effect of immunization with pneumolysin on survival time of mice challenged with *Streptococcus pneumoniae*. Infect. Immun. **40**:548–552.
36. **Paton, J. C., B. Rowan-Kelly, and A. Ferrante.** 1984. Activation of human complement by the pneumococcal toxin, pneumolysin. Infect. Immun. **43**:1085–1087.
37. **Riley, I. D., and R. M. Douglas.** 1981. An epidemiologic approach to pneumococcal disease. Rev. Infect. Dis. **3**:233–245.
38. **Robbins, J. B., R. Austrian, C.-J. Lee, S. C. Rastogi, G. Schiffman, J. Henrichsen, P. H. Makela, C. V. Broome, R. R. Facklam, R. H. Tiesjema, and J. C. Parke, Jr.** 1983. Considerations for formulating the second-generation pneumococcal capsular polysaccharide vaccine with emphasis on the cross-reactive types within groups. J. Infect. Dis. **148**:1136–1158.
39. **Sarnaik, S., J. Kaplan, G. Schiffman, D. Bryla, J. B. Robbins, and R. Schneerson.** 1990. Studies on *Pneumococcus* vaccine alone or mixed with DTP and on *Pneumococcus* type 6B and *Haemophilus influenzae* type b capsular polysaccharide-tetanus toxoid conjugates in two- to five-year-old children with sickle cell anemia. Pediatr. Infect. Dis. J. **9**:181–186.
40. **Saunders, F. K., T. J. Mitchell, J. A. Walker, P. W. Andrew, and G. J. Boulnois.** 1989. Pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae*, does not require a thiol group for in vitro activity. Infect. Immun. **57**:2547–2552.
41. **Schiffman, G., R. M. Douglas, M. J. Bonner, M. Robbins, and R. Austrian.** 1980. A radioimmunoassay for immunologic phenomena in pneumococcal disease and for the antibody response to pneumococcal vaccines. I. Method for the radioimmunoassay of anticapsular antibodies and comparison with other techniques. J. Immunol. Methods **33**:133–144.
42. **Schneerson, R., J. B. Robbins, J. C. Parke, Jr., C. Bell, J. J. Schlesselman, A. Sutton, Z. Wang, G. Schiffman, A. Karpas, and**

J. Shiloach. 1986. Quantitative and qualitative analyses of serum antibodies elicited in adults by *Haemophilus influenzae* type b and pneumococcus type 6A capsular polysaccharide-tetanus toxoid conjugates. Infect. Immun. **52**:519–528.

43. **Snippe, H., A.-J. van Houte, J. E. G. van Dam, M. J. de Reuver, M. Jansze, and J. M. N. Willers.** 1983. Immunogenic properties in mice of hexasaccharide from the capsular polysaccharide of *Streptococcus pneumoniae* type 3. Infect. Immun. **40**:856–861.
44. **Tweten, R. K.** 1988. Nucleotide sequence of the gene for perfringolysin O (theta toxin) from *Clostridium perfringens*: significant homology with the genes for streptolysin O and pneumolysin. Infect. Immun. **56**:3235–3240.
45. **Walker, J. A., R. L. Allen, P. Falmagne, M. K. Johnson, and G. J. Boulnois.** 1987. Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae*. Infect. Immun. **55**:1184–1189.
46. **Wright, P. F., S. H. Sell, W. K. Vaughn, C. Andrews, K. B. McConnell, and G. Schiffman.** 1981. Clinical studies of pneumococcal vaccines in infants. II. Efficacy and effect on nasopharyngeal carriage. Rev. Infect. Dis. **3**(Suppl.):S108–S112.