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Synthesis of a Conjugate Vaccine Composed of Pneumococcus Type 14 Capsular Polysaccharide Bound to Pertussis Toxin

RACHEL SCHNEERSON,[1]* LILY LEVI,[1] JOHN B. ROBBINS,[1] DOLORES M. BRYLA,[2] GERALD SCHIFFMAN,[3] AND TERESA LAGERGARD[4]

Laboratory of Developmental and Molecular Immunity[1] and Biometry and Mathematical Statistics Branch,[2] National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892; Department of Microbiology and Immunology, State University of New York, Downstate Medical Center, New York, New York 11203[3]; and Department of Medical Microbiology, University of Goteborg, Goteborg, Sweden[4]

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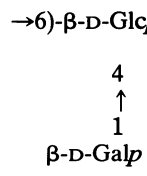
Type 14 is one of the common types isolated from patients of all ages with infections caused by *Streptococcus pneumoniae*. Its capsular polysaccharide (Pn14) is composed of a neutrally charged tetrasaccharide repeat unit. Pn14 does not elicit protective levels of antibodies in infants and children and is a less than optimal immunogen of the 23-valent vaccine for adults. Pertussis toxin (PT) is both a virulence factor and protective antigen of *Bordetella pertussis*: it is not soluble at neutral pH and forms insoluble complexes with acidic polysaccharides. Both Pn14 and PT are potential components of vaccines for infants and children. Accordingly, a synthetic scheme was devised to prepare a conjugate of Pn14 and PT. An adipic acid hydrazide derivative of Pn14 was bound to PT at pH 3.9 by carbodiimide-mediated condensation. The conjugation procedure inactivated the PT as assayed by CHO cell and histamine-sensitizing activity. The Pn14-PT conjugate elicited antibodies in mice to Pn14 at levels estimated to be protective in humans and elicited neutralizing antibodies to PT. We plan to evaluate Pn14-PT clinically.

The development of polysaccharide protein conjugates for prevention of systemic infections caused by *Haemophilus influenzae* type b serves as a precedent for making conjugates of polysaccharides of other capsulated pathogens (31). This technology has been extended to improve the immunologic properties of other polysaccharides of medically important organisms such as *Streptococcus pneumoniae* (5, 9, 10, 25, 26, 31, 38, 39). Since the objective is to provide protection against many capsulated bacterial pathogens of infants and children, investigators have used established vaccines such as diphtheria and tetanus toxoids or potentially protective antigens such as the toxoids of *Pseudomonas aeruginosa* exotoxin A and pneumococcal hemolysin (3, 7, 23, 24). These conjugates could elicit potentially protective levels of antibodies for both capsulated and toxigenic pathogens. It has been estimated that induction of protective levels of antibodies to 10 bacterial polysaccharides would result in a substantial decrease in serious infections of infants (2–4). There is a limit to the number of polysaccharides that can be bound to one protein. Accordingly, we have sought other proteins of potential prophylactic value to serve as carriers for these polysaccharides.

Pertussis toxin (PT) has been shown to be both a virulence factor and a protective antigen (9, 23, 25, 32, 36): an inactivated toxin (toxoid) is an essential component of acellular vaccines for pertussis (12, 13, 23, 24, 28, 35). PT could therefore serve as a carrier for the polysaccharide of a medically important pathogen, especially in infants.

PT has several properties which make it difficult to use as a carrier. It is mostly insoluble at pH values between 4 and 8. The addition of a negatively charged polysaccharide to PT in solution at pH <4 results in the precipitation of both (33a). The addition of a neutrally charged polysaccharide, such as pneumococcus type 14 capsular polysaccharide (Pn14), does not cause this effect.

Pneumococci are a major cause of pneumonia, meningitis, and other invasive diseases throughout the world (2–4, 6, 30, 36). Otitis media is the most common disease caused by this pathogen in infants and children (2–4, 19, 30). Several large surveys have reported that pneumococcus type 14 is one of the types isolated most frequently from patients of all ages (3, 30). Pn14 is neutral and is composed of the following repeating tetrasaccharide (17):



Pn14 is a comparatively poor immunogen among the pneumococcal capsular polysaccharides. In adults, it elicits only a fourfold rise of antibodies in ~80% of vaccinees (2, 16, 30). This property may be the reason why type 14 pneumococcus is one of the most common types isolated from adult patients immunized with the polyvalent pneumococcal vaccine (6, 36). Pn14 does not elicit protective levels of antibodies in human infants and young children. In mice, type 14 pneumococci are limited in virulence and Pn14 does not elicit serum antibodies (18, 20). In this study, Pn14 was bound to PT by a modification of the scheme for conjugating *H. influenzae* type b and pneumococcus type 6A capsular polysaccharides to tetanus toxoid (9). The resultant Pn14-PT

* Corresponding author.

TABLE 1. Serum antibodies elicited in mice by Pn14-PT conjugates[a]

Immunogen	n	Geometric mean antibody concn[b] (25th–75th centiles)					
		After first injection		After second injection		After third injection	
		Pn14	PT	Pn14	PT	Pn14	PT
Pn14	8	3.3a (1.2–5.9)	0.05f (0.05)	6.0 (4.37–10.9)	0.05f (0.05)	12.7 (8.11–16.3)	0.05f (0.05)
Pn14-PT (A)	10	188b (89.8–521)	106g (79–147)	1,772d (1,653–1,974)	965h (308–2,201)	1,642 (1,478–1,783)	1,496 (800–2,921)
Pn14-PT (B)	11	289b (111–860)	107g (69–216)	1,747d (1,553–2,074)	905h (539–1,843)	1,932 (1,813–2,187)	1,448 (1,143–1,775)
Pn14-PT (C)[c]	11	607c (512–881)	94g (68–129)	1,488e (1,302–1,636)	644h (421–1,156)	1,274 (846–1,759)	1,059 (700–1,715)

[a] Five-week-old female general-purpose mice were injected at biweekly intervals with 2.5 μg of Pn14 alone or as a conjugate and exsanguinated 14 days after the first injection or 7 days after either of the last two injections. Pn14 antibodies were measured by radioimmunoassay (nanograms of antibody nitrogen per milliliter) (33). PT antibodies were measured by ELISA and expressed as units (12, 35). Preimmune Pn14 antibody levels were 5.39 ng of antibody per ml, and PT antibodies were not detectable.

[b] $P = 0.0001$ for c and b versus a; d versus b; g versus f; and h versus g. $P = 0.002$ for e versus c.

[c] Pn14-PT (A) adsorbed onto Alhydrogel.

conjugates were found to be nontoxic and to elicit both type-specific and neutralizing antibodies to PT (antitoxin) in mice.

MATERIALS AND METHODS

Reagents. Pn14 was supplied by Dominique Schulz, Pasteur Merieux Vaccins and Serums, Lyon, France. PT was prepared as described previously (34). It was stored as a 90% ammonium sulfate precipitate at 3 to 8°C. Murine monoclonal antibodies to PT, 1015-6FX1 and 1014-3CX4, were donated by James G. Kenimer, Center for Biologics Evaluation and Research (CBER), Food and Drug Administration. Rabbit anti-pneumococcus type 14 antibody (Lederle Laboratories, Pearl River, N.Y.), cyanogen bromide (Kodak, Rochester, N.Y.), acetonitrile (high-pressure liquid chromatography grade; Fisher Scientific Co., Springfield, N.J.), adipic acid dihydrazide and 1-ethyl-1,3-(3-dimethylaminopropyl)carbodiimide (EDAC; Sigma Chemical Co, St. Louis, Mo.), and 2,4,6-trinitrobenzenesulfonic acid (Pierce Chemical Co., Rockford, Ill.) were used as reagents.

Analyses. Polysaccharide was measured by the anthrone reaction with Pn14 as a standard (10). Adipic acid hydrazide was measured by the 2,4,6-trinitrobenzenesulfonic acid reaction with adipic acid dihydrazide as a standard (9). Protein was measured by the Lowry reaction with bovine serum albumin as a standard (9).

Synthesis of Pn14-PT conjugates. Pn14 was derivatized with adipic acid dihydrazide as described previously (9): the degree of derivatization was 2.0%. The Pn14-adipic acid hydrazide was dissolved in water, and the pH was brought to 3.9 with 0.1 M HCl. PT, stored as a precipitate in ammonium sulfate, was dialyzed against 0.5 M sodium acetate (pH 3.9) at 3 to 8°C. The Pn14-adipic acid hydrazide and PT were brought to a final concentration of ~5.0 mg/ml each. EDAC was added to 0.1 M, and the pH was maintained at 3.9 for 4 h at room temperature for Pn14-PT (A) and at 3 to 8°C for Pn14-PT (B). Each reaction mixture was dialyzed against 0.5 M sodium acetate (pH 3.9) and applied to a column (1 by 90 cm) of Bio-Gel P-300 in that buffer. The void volume fractions, containing the Pn14-PT conjugate, were made 0.01% in thimerosal. The saccharide/protein ratios (wt/wt) of the three conjugates were ~5.5, and most of the PT in these preparations emerged in the void volume of a column (1 by 90 cm) of P-300 in 0.5 M sodium acetate–1% sodium dodecyl sulfate (SDS).

A portion of Pn14-PT (A) was adsorbed onto Alhydrogel (Superfos, Copenhagen, Denmark) at 0.1 mg of aluminum per 0.1 ml of conjugate containing 2.5 μg of saccharide. This adsorbed conjugate is denoted as Pn14-PT (C).

The third conjugate was treated further (see Table 2). One portion was mixed with saturated ammonium sulfate to a final concentration of 90% at 4°C, left overnight at 3 to 8°C, and centrifuged for 30 min at 15,000 × *g* and 4°C, and the precipitate (D) and the supernatant (E) were dialyzed against phosphate-buffered saline. Another portion was passed through a column (1 by 90 cm) of P-300 in 0.5 M sodium acetate–1% SDS, and the void volume (F) was used for immunization.

Serologic testing. Serum antibodies to Pn14 were measured by radioimmunoassay (33). Serum antibodies to PT were measured by enzyme-linked immunosorbent assay (ELISA) and by inhibition of the effect of the toxin on Chinese hamster ovary (CHO) cells (12, 35, 40).

Assay of PT. The histamine-sensitizing activity of PT and of the conjugates was assayed as described previously (11, 27). The in vitro toxicity of PT was assayed with CHO cells (11). We could detect 1.5 ng/ml by this method.

Immunization. Female general-purpose mice from the National Institutes of Health colony, ~5 weeks old, were injected subcutaneously at biweekly intervals three times: 30 mice were injected with each preparation. Ten animals each were exsanguinated before the second injection and 1 week after the second and third injections.

Statistics. Data analysis was performed by using the Statistical Analysis System. The logarithms of the concentrations were used for all calculations. Antibody concentrations below the sensitivity of the ELISA and the CHO assay were assigned values equal to one-half the threshold value. For comparison of the geometric means, we used the unpaired *t* test.

RESULTS AND DISCUSSION

Pneumococcus type 14 antibodies. The preimmunization serum samples of the mice contained trace or nondetectable levels of antibodies to Pn14. In the two experiments, injection of 2.5 μg of Pn14 alone did not elicit serum antibody responses. The Pn14-PT conjugates in saline, containing 2.5 μg of saccharide, elicited statistically significant antibody rises after the first two injections (Tables 1 and 2). The third injection of Pn14-PT did not elicit a rise in Pn14 antibodies. There were no differences between the levels of Pn14 antibodies elicited by the conjugate prepared at 3 to 8°C (A) and the one prepared at room temperature (B). Addition of alum to the Pn14-PT conjugates did not enhance the anti-

TABLE 2. Serum antibodies elicited in mice by Pn14-PT conjugates[a] treated with ammonium sulfate or SDS

Immunogen	n	Geometric mean antibody concn[b] (25th–75th centiles)				
		After first injection		After second injection		
		Pn14	PT	Pn14	PT	Antitoxin
Pn14	8	3.13a (1.0–1.8)	0.05	4.08a (1,128–1,494)	0.05	<10
Pn14-PT (A)	9	ND[c]	ND	1,303c (1,128–1,494)	2,928 (1,738–4,523)	346
Pn14-PT (D)	10	102b (33.3–324)	0.26 (0.05–1.00)	1,269c (1,047–1,485)	36.8 (15.7–219)	<10
Pn14-PT (E)	10	266b (128–216)	172 (122–253)	1,292c (1,125–1,524)	1,098 (833–1,897)	80
Pn14-PT (F)	10	156b (48.5–439)	0.25 (0.05–0.52)	1,237c (1,071–1,403)	1.41 (0.50–2.73)	<10

[a] Five-week-old female general-purpose mice were injected at biweekly intervals with 2.5 μg of Pn14 alone or as a conjugate and exsanguinated 7 days after either injection. Pn14 antibodies were measured by radioimmunoassay (nanograms of antibody nitrogen per milliliter) (33). PT antibodies were measured by ELISA and by CHO cell neutralization (antitoxin) and are expressed as units (35, 40). Preimmune Pn14 antibody levels were 5.39 ng of antibody nitrogen per ml, and PT antibody levels were not detectable.

[b] $P = 0.001$ for c and b versus a. $P < 0.001$ for c versus b.

[c] ND, not done.

body responses to either Pn14 or PT [Pn14-PT (C) in Table 1].

As with other polysaccharides, covalent attachment of Pn14 to a protein conferred enhanced immunogenicity and T-cell dependence (booster responses [31]). van de Wijgert et al. prepared conjugates of Pn14 with bovine serum albumin or the flagella of *Salmonella typhi* by a scheme similar to one described by us (9, 38). Injection of 100 μg of these Pn14 conjugates elicited serum antibodies in mice. Anderson and Betts synthesized a conjugate composed of Pn14 bound to the nontoxic mutant diphtheria toxin (CRM197): the periodate-treated Pn14 was bound to the protein by reductive amination (1). The resultant conjugate elicited ~sixfold rises in the level of Pn14 antibodies in three adults. These investigators did not report data from animals with this conjugate. We chose PT as a carrier because the conjugate of Pn14 with PT has the potential for inclusion among the vaccines used for routine immunization of infants (DTP) and because antibodies to Pn14 and PT confer protective immunity to pneumococcal infection and to pertussis (2, 3, 6, 12, 13, 24, 27, 40). Strains of pneumococcus type 14 have little pathogenicity for laboratory mice (inocula of $\sim 10^6$ are required to achieve a lethal effect) (20). It is probable that the presence of serum antibodies to Pn14, as well as to other pneumococcal capsular polysaccharides, is predictive of protective immunity in humans (2, 16, 30).

PT antibodies. Antibodies to PT were not detected in the preimmunization serum samples. Pn14-PT conjugates, denoted (A) and (B), elicited significant rises ($P < 0.0001$) in the level of PT antibodies (ELISA) after both the first and second injections (Tables 1 and 2): the rise after the third injection was not statistically significant. Adsorption of the Pn14-PT onto alum (Table 1) had no significant effect on the antibody responses to Pn14 or PT. Table 2 shows that precipitation of the Pn14-PT with ammonium sulfate or treatment with SDS significantly reduced the serum antibody responses to the PT but not to the Pn14 component of the conjugate. Pn14-PT (E) (supernatant of the ammonium sulfate precipitation), however, elicited similar antibody responses to the Pn14 and PT as did the untreated conjugates.

Table 2 shows the neutralizing activity of PT antibodies elicited by Pn14-PT (A), (D), (E), and (F). One injection of these conjugates did not elicit antitoxin. Following the second injection, Pn14-PT (A) elicited a geometric mean antibody concentration (GM) of 346 U. After precipitation with ammonium sulfate, the resolubilized precipitate, Pn14-PT (D), did not elicit antitoxin, whereas the supernatant, Pn14-PT (E), elicited a GM of 80 U. Pn14-PT (F), exposed to 1% SDS, also failed to elicit antitoxin. As shown in clinical studies, there was a good correlation between the levels of antitoxin and PT antibodies measured by ELISA (35). There is evidence that demonstration of neutralizing activity of pertussis antibodies by the CHO cell assay is predictive of protective immunity to pertussis (8, 12, 13, 22, 23, 28, 32, 40). It is likely that this assay will be used to standardize acellular vaccines containing PT (12, 35, 40).

PT exerts diverse pharmacologic actions including modulation of the serum antibody response to polysaccharides (14, 15). Many of these actions require the enzymatic function of its A subunit (7, 37). Our preparations of Pn14-PT were not toxic to CHO cells, which are sensitive to the A subunit (8, 37). The property of mitogenicity, however, is conferred by the B subunit of PT (6, 21). We plan to evaluate the mitogenic properties of our Pn14-PT conjugate. At 10 μg, the Pn14-PT conjugates were not active in the histamine sensitization assay, which can detect 0.1 μg of PT (27, 35). The toxicity of the PT was reduced $\sim 6 \times 10^3$-fold by the CHO cell assay (11).

The synthesis of a conjugate with PT was reported at a conference (29). An oligosaccharide, from group C CP of *Neisseria meningitidis*, was derivatized with the *N*-hydroxysuccinimide diester of adipic acid. This derivative and PT were incubated overnight in dimethyl sulfoxide and passed through a G-100 Sephadex column. Polyacrylamide gel electrophoresis (PAGE) showed two major components differing from the profile of the PT. There was no verification that the saccharide comigrated with the PT through the gel. Also, the effect of mixing PT with dimethyl sulfoxide on its migration in PAGE was not identified. Lastly, there was no mention of serum antibody responses elicited by the "conjugate." Our conclusion is that the authors did not present evidence that they had covalently bound the group C meningococcal saccharide to PT or that their preparation had enhanced the immunogenicity of the Pn14.

The method for immunization we have used, employing about 1/10th the proposed human dosage injected subcutaneously without adjuvants into young outbred mice, has been predictive of the immunogenicity of the polysaccharide component of other conjugate vaccines in human infants (31). Because the synthetic scheme yielded a nontoxic conjugate capable of eliciting antibodies to both its Pn14 and PT components, we plan to evaluate this vaccine in humans.

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