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Serum Antibody Response in Adult Volunteers Elicited by Injection of *Streptococcus pneumoniae* Type 12F Polysaccharide Alone or Conjugated to Diphtheria Toxoid

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Conjugates of an uronic acid-containing capsular polysaccharide (CP), pneumococcous type 12F (Pn12F) bound to diphtheria toxoid (DT), were studied for safety and immunogenicity in adult volunteers. In mice, these conjugates, prepared with the same lot of DT and Pn12F-40234-006, a homogenous CP of high molecular weight, or Pn12-812408, a polydisperse CP with lower-molecular-weight material, were more immunogenic than the Pn12F alone and had T-cell dependent properties (A. Fattom, W. F. Vann, S. C. Szu, A. Sutton, X. Li, B. Bryla, G. Schiffman, J. B. Robbins, and R. Schneerson, Infect. Immun. 56:2292–2298, 1988). Adult volunteers, randomized into three groups, were injected either with one of these two conjugates or with Pnu-Imune, the 23 valent pneumococcus vaccine containing 25 μg of Pn12F as one of its components. Volunteers were injected two times, 4 weeks apart, with the Pn12F-DT conjugates and once with the Pnu-Imune. Side reactions following injection of the conjugates or Pnu-Imune were mild and short-lived. At 4 weeks and at 7 months after the first injection, higher levels of Pn12F antibodies were found in the volunteers injected with the conjugates than in the Pnu-Imune group ($P < 0.001$). The conjugate prepared with the higher-molecular-weight Pn12F elicited higher levels of antibodies than the conjugate prepared with a lower-molecular-weight Pn12F preparation ($P = 0.05$). Both conjugates elicited about a 13-fold rise in DT antibodies.

We developed a synthetic scheme for derivatizing carboxyl groups of polysaccharides (PS) in order to bind them to proteins (conjugates). When injected into mice, these conjugates served to enhance the immunogenicity of and confer T-cell-dependent properties to the Vi capsular polysaccharide of *Salmonella typhi* and pneumococcus type 12F (Pn12F) (10, 26–28). Pn12F is a branched-chain copolymer composed of a hexasaccharide repeat unit (15). Its carboxylic acid is a mannosaminouronic acid (Fig. 1).

Conjugates of this capsular polysaccharide (CP) could be expected to be more immunogenic, and ipso facto more protective, in infants and in immunocompromised patients who are at higher-than-average risk for systemic diseases caused by Pn12F organisms (2, 4, 6–9, 12, 13, 17–19, 21, 24, 25, 29). The scheme was a modification of the method described for covalently binding the Vi CP and the cell wall polysaccharide of pneumococcus to proteins by using the heterobifunctional reagent *N*-succinimidyl-3-(2-pyridylthio) propionate (SPDP) (5, 26–28). Two lots of Pn12F, bound to one lot of DT, were used: (i) a polydisperse preparation with both high- and low-molecular-weight components (lot 812408); and (ii) a comparatively homogenous and high-molecular-weight CP (lot 40234-006). In mice, both conjugates were more immunogenic than the Pn12F alone and both exhibited T-cell-dependent properties (booster and carrier-priming responses), as has been observed in young children and infants with other conjugates (1, 7, 9, 23). Here, we report the safety and serum antibody responses elicited by these Pn12F-DT conjugates in adult volunteers.

MATERIALS AND METHODS

Study design. Healthy volunteers screened for human immunodeficiency virus antibodies and for pregnancy, ages 18 to 44 years, at the Clinical Center, National Institutes of Health (NIH), and at the University of Alabama at Birmingham were divided into three groups on a random basis: 81 volunteers at the NIH and 30 at the University of Alabama at Birmingham. There were 59 females and 52 males equally distributed among the three groups. A preimmunization sample of blood was taken, and the volunteers were injected intramuscularly two times, 1 month apart, with 0.5 ml of either Pn12F-812408-DT or Pn12F-40234-006-DT. The third group received only one injection of Pnu-Imune (23 valent pneumococcus polysaccharide vaccine, lot NDC 005-2309-31, Lederle Laboratories, Pearl River, N.Y.). Their temperature was taken, and the local injection site was inspected at 6, 24, and 48 h following each injection. For the volunteers at the NIH, blood samples were taken 4 weeks after the first injection and 4 weeks following the second injection of the conjugates. Blood samples were taken at 4 and 8 weeks following injection of Pnu-Imune. For the volunteers at the University of Alabama at Birmingham, additional blood samples were taken 1 week after the first and second injections. Sera were obtained from all the volunteers about 7 months after the first injection.

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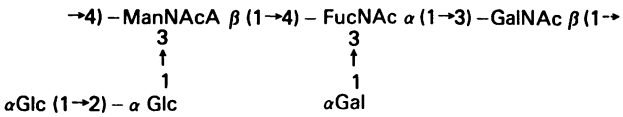

FIG. 1. The repeat unit of pneumococcus type 12F capsular polysaccharide (15).

The clinical protocol and standardization of the Pn12F-DT conjugates used in this study were reviewed by the National Institutes of Health (protocol 88-CH-157, the Institutional Review Board, University of Alabama at Birmingham, and the Office of Biologics Review and Research, Food and Drug Administration (BB-IND 2917).

Vaccines. Pnu-Imune, containing 25 μg of each type, including Pn12F, was given. As described previously (10), conjugates were prepared with DT from the Rijks Instituut voor Volksgezonheid, Bilthoven, The Netherlands. Two preparations of Pn12F were used. (i) Pn12F lot 812408 was manufactured by the Eli Lilly Co, Indianapolis, Ind., for the National Institute of Allergy and Infectious Disease. This CP had a polydisperse molecular size; most of the CP emerged through a CL-4B Sepharose column with a K_d between 0.2 and 0.6. (ii) Pn12F lot 40234006 was manufactured by Lederle Laboratories, Pearl River, N.Y., and conformed to the guidelines set for this type by the Food and Drug Administration (11). All of this Pn12F preparation emerged in the void volume of a CL-4B Sepharose column. The synthesis, composition, and immunological properties in mice of these two conjugates have been published (10). Each dose of conjugate contained 25 μg of Pn12F and about the same weight of DT. The conjugates were bottled by the NIH pharmacy, and their pyrogenicity, sterility, and toxicity tests were reviewed by the Food and Drug Administration.

Serum antibodies. Pn12F antibodies were measured by radioimmunoassay (20). DT antibodies were measured by enzyme-linked immunosorbent assay, using a human immune serum, containing 1.5 IU/ml determined by toxin neutralization, as a reference (we are grateful to Iver Heron, Statens Seruminstitut, Copenhagen, Denmark, for this serum) (10). DT antibody levels were expressed as units per milliliter.

Statistical analysis. Paired and unpaired t tests were used to compare the antibody levels between the preimmune and postimmune samples and for differences between the three vaccines.

RESULTS

Clinical reactions. No serious adverse clinical reactions were observed (Table 1). Fever was observed in one volunteer after the first injection of each conjugate and in one volunteer after the second injection of Pn12F-812408-DT; none of the volunteers injected with Pnu-Imune had fever during the 48 h after immunization. Local reactions, including erythema and induration, were observed more often in the volunteers injected with the Pn12F conjugates than in those injected with Pnu-Imune. These reactions were mild and were not detectable after 48 h.

Pn12F antibodies. Although the volunteers were randomized into groups, the preimmune geometric mean (G.M.) Pn12F antibody level of the vaccinates injected with Pn12F-812408-DT was higher (1,102 ng of antibody nitrogen [AbN] per ml) than in the other two groups (562 and 612) (Table 2). These differences, however, were not statistically significant. The Pn12F, as a component of the 23 valent vaccine, elicited a 3.1-fold increase in the G.M. of antibodies 4 weeks after immunization ($P = 0.0001$). Excluding volunteers with a preimmunization level of ≥1,500 ng of AbN/ml (1 standard deviation of the postimmunization G.M. [11]), 15 of 27 (56%) had a greater than or equal to twofold rise in Pn12F antibodies. The Pn12F-DT conjugates elicited higher levels of Pn12F antibodies than the CP alone ($P = 0.0001$). There were 4.5- and 9.7-fold increases over the preimmunization levels 4 weeks after the first immunization with Pn12F-812408-DT and Pn12F-40234006-DT, respectively. Moreover, the number of volunteers (again, excluding those with >1,500 ng of AbN/ml) with greater than or equal to twofold rise was 21 of 27 (78%) for Pn12F-812408-DT and 26 of 26 (100%) for Pn12F-40234006-DT. Higher postimmune antibody levels at the 4-, 5-, and 8-week intervals were elicited by the Pn12F-40234006-DT conjugate, compared to the Pn12F-812408-DT conjugate ($P = 0.05$). A second injection with either conjugate did not result in a booster response.

TABLE 1. Reactions observed within 48 h in healthy adult volunteers injected one time with 23 valent pneumococcus vaccine (Pnu-Imune) or two times, one month apart, with Pn12F-DT conjugates[a]

Reactions	No. of patients exhibiting symptoms after injection as follows:				
	Pnu-Imune injection 1	Pn12F-40234006-DT		Pn12F-812408-DT	
		Injection 1	Injection 2	Injection 1	Injection 2
Fever, ≤38.5°C	0	1	1	1	0
Erythema <2 cm	1	5	5	1	2
Erythema 2 to 4 cm	2	1	1	1	1
Induration <2 cm	0	3	2	3	3
Sore arm	12	5	12	4	10

[a] Healthy adult volunteers were injected with 0.5 ml of Pn12F (Pnu-Imune; $n = 35$), Pn12F-40234006-DT ($n = 35$), or with Pn12F-812408-DT ($n = 41$). Their oral temperature was taken, and the injection sites were inspected at 6, 24, and 48 h after immunization.

The G.M. Pn12F antibody levels of the two conjugate groups declined 7 months after the first injection to about one-half their maximal value. Only an ~30% decline was observed in the recipients of the Pnu-Imune. The rate of decline was inversely related to the maximal G.M. levels, i.e., the group with the highest G.M. fell proportionately more than the group with the lowest level. At this last bleeding, the rank order of antibody levels remained Pn12-40234006-DT > Pn12F-812408-DT > Pnu-Imune.

DT antibodies. Preimmune antibody levels were lower in the Pn12F-812408-DT group than in the other two groups, but this difference was not statistically significant (Table 3). Both conjugates elicited high levels of DT antibodies (13- and a 17-fold increase for Pn12F-812408-DT and Pn12F-40234006-DT, respectively). DT antibody levels were unchanged following the second immunization. No change in the level of DT antibodies was observed in the recipients of the Pnu-Imune.

DISCUSSION

As predicted from the safety and pyrogenicity assays, neither the Pnu-Imune nor the conjugates elicited serious side reactions. Local reactions following injection of the conjugates were mild and lasted for 24 to 48 h only; two of the volunteers had mild and transient fever during the 48 h following injection. Volunteers injected with the Pnu-Imune experienced similar mild reactions.

TABLE 2. Serum Pn12F antibodies elicited by Pn12F alone (Pnu-Imune) or by Pn12F conjugated to DT

Immunogen	No.	Antibody levels at following intervals after 1 injection of Pnu-Imune or 2 injections of Pn12F-DT conjugates[a]					
		Preimmune	1 week	4 weeks[b]	5 weeks	8 weeks	6 months
Pnu-Imune	35	562a (397–1,412)	1,292 (1,030–2,425)	1,702b (850–3,398)	Not done	1,654b (891–3,249)	1,297b (760–2,414)
Pn12F-812408-DT	41	1,012a (681–2,210)	1,681 (1,166–2,293)	4,525c (2,642–8,426)	4,610c (3,345–7,770)	4,359c (2,760–7,591)	2,550c (1,663–4,742)
Pn12F-40234006-DT	35	612a (233–1,587)	3,194 (2,132–4,105)	6,677d (3,604–12,973)	8,946d (5,416–14,977)	6,190d (3,531–12,980)	3,619d (2,609–6,274)

[a] Antibody levels, nanograms of AbN/ml serum, are expressed as the G.M. with the 25th to 75th percentiles in parentheses (20). Sera for antibody assay at 1 week following the first injection and at 5 weeks (1 week following the second injection) were taken from the University of Alabama at Birmingham volunteers only. b, c, and d versus a, $P = 0.0001$; c and d versus b, $P = 0.0001$; d versus c, $P = 0.05$.
[b] Second injection of the conjugates given after a blood sample was taken.

Similar to the results in mice, both Pn12F conjugates elicited higher antibody levels than the unconjugated Pn12F (the serum antibody response to one CP is unchanged by its incorporation into a multivalent vaccine) (2, 13, 19). Pn12F elicited G.M. antibody levels approximately 5-fold higher than the estimated protective level (300 ng of AbN/ml) (14, 24). The two Pn12F-DT conjugates, however, elicited 12 to 20 times higher antibody levels than the estimated protective level ($P < 0.001$). Preimmune levels >300 ng of AbN/ml were present in 29 of 35 in the Pnu-Imune group: 32 of 35 had >300 ng of Ab/ml at the 7-month bleeding. Prior to immunization, protective levels were detected in 37 of 41 in the Pn12F-812408-DT group and in 29 of 35 among the Pn12F-40234-006-DT group. All volunteers injected with the Pn12F-DT conjugates had >300 ng of AbN/ml in their 7-month sera.

The enhanced immunogenicity of Pn12F as a component of a conjugate has been demonstrated for other uronic acid-containing CP, including the Vi of *S. typhi*, pneumococcus type 3, group C meningococci, and *Klebsiella pneumoniae* type 11 in laboratory animals (3, 10, 28, 30). Recently, we have demonstrated this enhancement for conjugates of *Staphylococcus aureus* types 5 and 8 CP in mice when the synthetic scheme designed for the Vi and the Pn12F was used (9a, 27, 28). Our method of combining sulfhydryl derivatives of CP to SPDP-derivatized proteins seems to be applicable for preparing conjugate vaccines with other uronic acid-containing polysaccharides.

The Pn12F antibody levels elicited in the volunteers by the conjugates were similar to those observed in mice: higher-molecular-weight Pn12F-40234-006 was more immunogenic than the lower-molecular-weight Pn12F-812408 (10). These results are consistent with studies of *Haemophilus influenzae* type b and the Vi which showed that the molecular size of the PS, alone or as a component of a conjugate, is related to the serum antibody response, i.e., the higher-molecular-weight PS elicited higher antibody levels (1, 10, 27). Makela et al. reported that lower-molecular-weight dextrans (<10,000 kilodaltons) are more immunogenic than dextrans of 5×10^6 to 40×10^6 kilodaltons as components of conjugates (16). Our data with the Pn12F in mice and in volunteers indicate that higher-molecular-weight polysaccharides are more immunogenic components of conjugates. This discrepancy may be due to different synthetic methods for preparing conjugates or from different immunologic properties of the polysaccharides.

The second injection of either conjugate did not elicit a booster response for the Pn12F or the DT. This failure to elicit a booster response in adults has also been reported for *H. influenzae* type b, pneumococcus type 6B CP, and *Pseudomonas aeruginosa* O-specific polysaccharide conjugates (22, 23). The first two conjugates, however, elicited booster responses in infants and young children (1, 7, 9, 21, 23). To explain this age-related booster response, we postulated that the first injection of the conjugates was, in fact, a booster response, since the volunteers had natural Pn12F antibodies and had been injected previously with DT. The Pn12F and DT antibody levels in the volunteers following the first injection were maximal for each immunogen, and reinjection did not recruit additional B cells. The mechanism(s) involved in this lack of response to a second injection of polysaccharide-protein conjugates in adults may be related to the high level of vaccine-induced antibodies.

Both conjugates elicited about a 13-fold rise in DT antibodies. This indicates that our method of conjugation preserves the immunogenicity of the carrier protein.

In conclusion, this conjugation technique enhances significantly the immunogenicity of Pn12F while retaining the immunogenicity of the carrier protein, DT. These results encourage the evaluation of such vaccines in individuals at higher-than-average risk (6) as well as in patients with decreased antibody responsiveness.

TABLE 3. DT antibodies elicited in healthy adult volunteers injected one time with Pn12F (Pnu-Imune) or two times, one month apart, with Pn12-DT conjugates[a]

Immunogen	No.	Antibody levels at following intervals after injection of vaccines		
		Preimmune	4 weeks[b]	8 weeks
Pnu-Imune	25	1.09 (0.39–3.11)	1.00 (0.34–2.82)	1.14 (0.39–3.31)
Pn12F-812408-DT	31	0.63 (0.17–2.26)	7.79 (2.77–21.9)	7.03 (2.53–19.59)
Pn12F-40234006-DT	25	1.12 (0.44–2.87)	19.3 (7.15–52.0)	17.71 (7.25–43.3)

[a] DT antibodies were measured by enzyme-linked immunosorbent assay, using a human immune serum, calibrated for its antitoxin, as a reference. Results are expressed as the G.M. (25 to 75th percentiles) of units of antibody per milliliter of serum. The postimmunization G.M. DT antibody levels in the two conjugate groups were higher than the preimmune levels of the three groups and the postimmunization levels of the volunteers injected with Pnu-Imune ($P < 0.001$).
[b] Second injection of the conjugates given after a blood sample was taken.

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