INFECTION AND IMMUNITY, May 1992, p. 1826–1833
0019-9567/92/051826-08$02.00/0

Vol. 60, No. 5

Synthetic Trimer and Tetramer of 3-β-D-Ribose-(1-1)-D-Ribitol-5-Phosphate Conjugated to Protein Induce Antibody Responses to *Haemophilus influenzae* Type b Capsular Polysaccharide in Mice and Monkeys

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Received 25 November 1991/Accepted 13 February 1992

Synthetic oligosaccharides derived from the capsular polysaccharide (PRP) of *Haemophilus influenzae* type b were conjugated to carrier proteins via a thioether linkage. Conjugates were made of trimeric and tetrameric ribose-ribitol-phosphate and tetanus toxoid or diphtheria toxin. All conjugates elicited anti-PRP antibody responses with an increasing immunoglobulin G/immunoglobulin M ratio in adult mice and monkeys. Trimer conjugates elicited lower anti-PRP antibody responses compared with tetramer conjugates. Adult monkeys responded equally well to the tetrameric oligosaccharide-tetanus toxoid conjugate as to the oligosaccharide-CRM197 conjugate (HbOC), which elicits protective levels of serum antibodies in human infants after two or three injections.

Haemophilus influenzae type b (Hib) is a leading cause of meningitis in children younger than 2 years of age in both industrialized and developing countries (11, 24). Antibodies against the capsular polysaccharide (PRP), a polymer of repeating 3-β-D-ribose-(1-1)-D-ribitol-5-phosphate (RRP) units (7), are protective in both animals and humans (13, 14, 30). However, immunization with PRP induces protective amounts of anti-PRP antibodies only in children older than 2 years of age (19, 25). The immunogenicity has been improved by conjugating polysaccharides to a protein carrier, as introduced by Avery and Goebel as early as 1929 (4). At present, four different Hib conjugate vaccines are commercially available: PRP-T, a polysaccharide-tetanus toxoid conjugate vaccine (27); PRP-D, a polysaccharide-diphtheria toxoid conjugate (21); HbOC, an oligosaccharide-mutant diphtheria toxin conjugate (2); and PRP-OMP, a conjugate of polysaccharide and outer membrane proteins of *Neisseria meningitidis* (23). The four conjugates differ with respect to molecular weight, length of the saccharide molecules, carrier protein, linkage, and saccharide-to-protein ratio. In contrast to the plain polysaccharide, the conjugate vaccines are immunogenic in infants and are able to elicit booster responses upon repeated vaccination (1, 2, 6, 9, 20). However, there are differences in the immunogenicity of these vaccines. A systematic study of the relationship between the different parameters and the immunogenicity of the conjugates is of interest for these and other (e.g., pneumococcal) conjugate vaccines.

We have been investigating the immunogenic properties of conjugates of small, but well-defined, synthetic RRP oligomers. In this paper, we report the coupling of synthetic trimeric and tetrameric oligosaccharides to diphtheria toxin (DTx) and tetanus toxoid (TT) by a procedure affording stable thioether conjugates. The conjugates obtained proved to be immunogenic in mice and monkeys. Independent of the carrier protein used, tetramer conjugates were more immunogenic than trimer conjugates.

MATERIALS AND METHODS

Antigens and vaccine. The synthesis of a spacer-containing ribose-ribitol-phosphate trimer, $(RRP)_3$, was reported earlier (14, 16, 17). The fragment comprising four repeating units was also prepared by this published methodology. PRP was kindly provided by P. Anderson, University of Rochester, Rochester, N.Y. HbOC conjugate vaccine (HibTITER) was obtained from Praxis Biologics, Rochester, N.Y.

Carrier proteins. DTx and TT were the kind gifts of R. Tiesjema (Laboratory of Vaccine Production, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands). The properties of DTx and TT used in this study were similar to those used for vaccine production (33). TT was further purified by gel filtration over a Sepharose CL-6B column (Pharmacia, Uppsala, Sweden) in 0.9% NaCl solution. DTx was purified by affinity chromatography over a Reactive Green-19 agarose column (Sigma Chemical Co., St. Louis, Mo.). DTx was bound to the column material in a 0.6 M NaCl solution at pH 6.4. The elution of DTx was performed with a 2.5 M NaCl solution at pH 7.2. DTx was purified more than two times, i.e., purity, 3,200 limit flocculation units/mg of protein nitrogen.

Preparation of oligosaccharide-protein conjugates. A schematic representation of the conjugation of synthetic trimeric and tetrameric RRP to either DTx or TT via a thioether linkage is depicted in Fig. 1.

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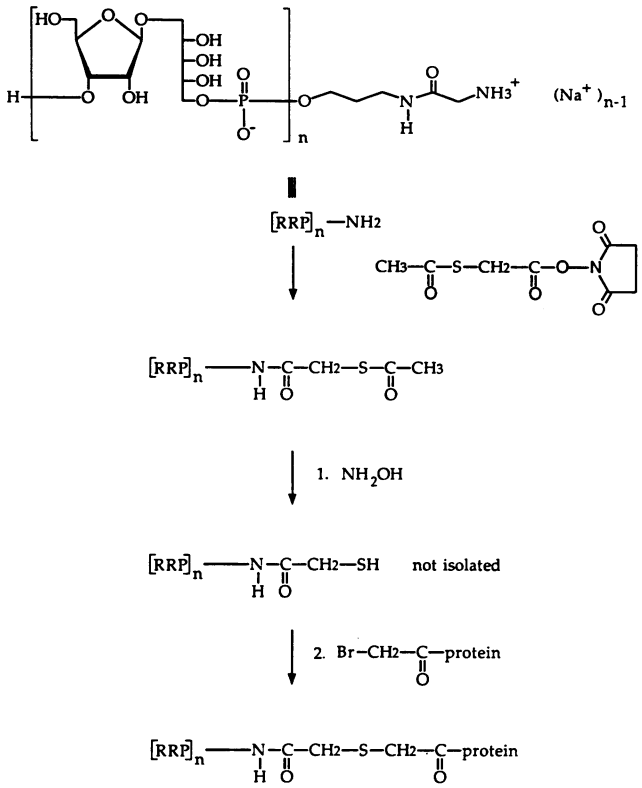

FIG. 1. Schematic representation of the conjugation of synthetic trimeric and tetrameric RRP to either DTx or TT via a thioether linkage.

The synthetic oligosaccharide (sodium salt), $(RRP)_3$ (29.4 mg; 24.2 μmol) or $(RRP)_4$ (39.4 mg; 24.8 μmol), was dissolved in 250 μl of *N*-ethylmorpholine–water–12 N hydrochloric acid (2.5:6.5:1.0, vol/vol/vol; pH 8.5), and a solution of *N*-succinimidyl *S*-acetylmercaptoacetate (28.9 mg; 125 μmol) in 250 μl of *N,N*-dimethylacetamide was added. The solutions were mixed thoroughly and left at room temperature with occasional shaking. After 1 h, acetic acid (125 μl) and acetone (5 ml) were added successively. The carbohydrate precipitate was collected by centrifugation, washed with acetone (5 ml), and dried in vacuo.

A solution of *N*-succinimidyl bromoacetate (2.36 mg; 10 μmol) in 250 μl of *N,N*-dimethylacetamide was mixed with a solution of TT (10 mg) in 1.75 ml of 0.1 M sodium phosphate buffer, pH 7.8. After 1 h, the reaction mixture was subjected to gel filtration by using a Sephadex PD-10 column (Pharmacia) and was equilibrated in 0.1 M sodium phosphate containing 5 mM EDTA, pH 6.1 (this buffer was deaerated with helium). The protein was collected in 3.5 ml of eluant. The solution (3.5 ml) of bromoacetylated TT thus obtained was added to the *S*-acetylmercaptoacetyl-modified carbohydrate [12 μmol of either $(RRP)_3$ or $(RRP)_4$] and incubated with 100 μl of 0.2 M hydroxylamine buffer (0.1 M sodium phosphate containing 5 mM EDTA, pH 6.1). After 24 h, the remaining bromoacetyl groups were blocked by the addition of 2-aminoethanethiol (2.28 mg; 20 μmol). After an additional period of 24 h, the conjugate was quickly prepurified (in two portions) over a PD-10 column in buffer, pH 6.1 (in this step, the nonconjugated carbohydrate was not removed completely). A second gel filtration was then performed over Sepharose CL-6B (Pharmacia) in a 0.9% NaCl solution (to achieve complete removal of unbound carbohydrate). The appropriate fractions were combined and stored at 4°C until used in immunological experiments.

TABLE 1. Characteristics of semisynthetic Hib oligosaccharide-protein conjugates[a]

Conjugate	Saccharide/protein ratio	
	Wt/wt	Mol/mol
$(RRP)_3$-DTx	1:7.3	9.9:1
$(RRP)_3$-TT	1:6.9	21.0:1
$(RRP)_4$-DTx	1:8.9	6.5:1
$(RRP)_4$-TT	1:20.5	5.3:1
OS_{dp20}-CRM197	1:2.5	4.3:1

[a] Synthetic trimer or tetramer of RRP was coupled to TT or DTx by a thioether linkage as described in Materials and Methods. The saccharide-to-protein ratio of the conjugates was determined by chemical assays. The protein content was determined by using a commercially available bicinchoninic acid reagent kit (Pierce Chemical Company, Rockford, Ill.) (31) with BSA as the standard. The saccharide content was determined by the phenol sulfuric assay as described by Dubois et al. (8) by using ribose as the standard. HbOC (OS_{dp20}-CRM_{197}) was prepared as described by Anderson et al. (1).

The $(RRP)_n$-DTx conjugates were prepared likewise, starting with a solution of DTx (24 mg) in 4.0 ml of 0.05 M sodium phosphate, pH 7.6. Although the coupling reaction reduced the toxicity of DTx (data not shown), a glutaric dialdehyde treatment was required for the complete elimination of toxicity. Therefore, the conjugate (0.35 μM) was incubated with 0.35 mM glutaric dialdehyde overnight at room temperature. The reaction was stopped by the addition of a glycine solution (1 M) to a final concentration of 35 mM. After incubation for 4 h at 37°C, the reaction mixture was dialyzed overnight at 4°C against a 0.9% NaCl solution and subsequently subjected to gel filtration over Sephadex G100 (Pharmacia) in 0.9% NaCl. Table 1 shows the saccharide/protein ratios of the conjugates obtained.

Adjuvants. Ten liters of an $AlPO_4$ suspension was prepared aseptically by combining 0.625 liter of 1.05 M $AlCl_3$, 5.05 liters of 0.14 M Na_3PO_4 solution, and 1 liter of distilled water. The suspension prepared in this way has an $AlPO_4$ concentration of 8 g/liter (65.6 mM) and an NaCl concentration of 11.4 g/liter (196.8 mM).

Mice and immunization. Randomly outbred female mice (Swiss Webster; Jackson Laboratory, Bar Harbor, Maine), aged 8 to 12 weeks, were kept at the National Institute of Public Health and Environmental Protection. Groups of 8 to 10 mice were immunized with 0.1-ml 0.9% NaCl solutions of synthetic oligomer $(RRP)_n$-protein conjugate containing 1 μg of saccharide. Conjugates were administered in the presence or absence of $AlPO_4$ (1 mg/ml) as an adjuvant. Four weeks after the primary immunization, mice were injected a second time with the homologous antigens. Blood samples were obtained from the tail veins prior to vaccination and at day 28 after each immunization. Sera were stored at −20°C until use.

Monkeys and immunization. Adult male and female cynomolgus monkeys (*Maccaca fascicularis*), aged 3 to 10 years, were used. Part of the monkey population consisted of randomly caught wild animals. Others were the first generation born at the National Institute of Public Health and Environmental Protection. Because of a limited availability of monkeys, only three animals could be vaccinated with the same vaccine. Monkeys were immunized intramuscularly (i.m.) with 0.5-ml 0.9% NaCl solutions of saccharide-protein conjugate containing 5 μg of saccharide. $AlPO_4$ (1 mg/ml) was used as an adjuvant through the whole study. A second immunization with the homologous conjugate vaccines was

given 4 weeks after the primary vaccination. Monkeys were bled on day 0, day 28, and day 56. The blood was allowed to clot at room temperature, and the serum was withdrawn and stored at −20°C until use.

Determination of antigenic properties. The antigenicity of trimer and tetramer oligomers and of various oligosaccharide-protein conjugates was compared with the native capsular polysaccharide in a competition enzyme-linked immunosorbent assay (ELISA). In these assays, a human hyperimmune serum with high titers (OOB; provided by J. Robbins, Office of Biologics, U.S. Food and Drug Administration) containing 70 μg of anti-PRP antibodies per ml was prediluted in phosphate-buffered saline (PBS), pH 7.4, containing Tween 80 (0.05%) and bovine serum albumin (BSA) (0.5%) to yield an absorbance of 1.00 in a PRP-specific ELISA. The diluted serum was incubated for 2 h at 37°C with fivefold serial dilutions of inhibitor (starting at a concentration of 25 μg/ml) in polyvinyl chloride microtiter plates (Flow, Irvine, United Kingdom) coated with 10 μg of tyramine-PRP per ml (26). After 2 h of serum incubation at 37°C, plates were washed and subsequently incubated for 2 h at 37°C with peroxidase-labeled rabbit anti-human immunoglobulin G (IgG) antibodies (Tago) in PBS–0.05% Tween 80–0.5% BSA. Plates were washed again and incubated at room temperature with the peroxidase substrate, 3,3',5,5'-tetramethylbenzidine (0.1 mg/ml; Sigma)–0.01% H_2O_2 in 0.11 M sodium acetate buffer, pH 5.5. After 10 to 30 min of incubation, the reaction was stopped by adding 100 μl of 2 M H_2SO_4. The A_{450} was registered on an ELISA Titertek Multiscan spectrophotometer.

Determination of antibodies by ELISA. Polyvinyl microtiter plates (Flow) were coated with TT or DT (National Institute of Public Health and Environmental Protection; 1 μg/ml in carbonate buffer, pH 9.6) overnight at room temperature and used to determine antibodies to TT and DTx. The same plates, coated under identical conditions with tyramine-PRP (10 μg/ml in PBS) (26) were used for the determination of anti-PRP antibodies. The coated plates were washed and incubated for 2 h at 37°C with twofold serial dilutions of serum samples in PBS–0.05% Tween 80–0.5% BSA. Subsequently, the plates were washed and incubated for 2 h at 37°C with horseradish peroxidase-labeled rabbit anti-human immunoglobulin (Ig), IgM, or IgG antibodies (Tago) or with horseradish peroxidase-labeled goat anti-mouse IgM or IgG antibodies in PBS–0.05% Tween 80–0.5% BSA. The plates were washed again and processed as described above for the determination of antigenic properties. Antibody titers to PRP in mouse serum samples are expressed as a percentage of a reference serum. A murine reference serum containing 40 μg of anti-PRP antibodies was prepared by immunizing mice three times with 2.5 μg of Hib polysaccharide conjugated to *H. influenzae* outer membrane protein b or c via an adipic dihydrazide spacer by use of the carbodiimide coupling procedure as described by Beuvery et al. (5). A human anti-Hib capsular polysaccharide serum containing 70 μg of anti-PRP antibodies per ml (OOB) was used as the reference serum in the ELISA determination of the anti-PRP Ig, IgG, or IgM antibody levels in monkey serum samples. Antibody titers to TT and DTx in monkey sera are expressed as the percentage of a human serum sample containing 13.6 IU of anti-DTx antibodies per ml and 5.6 IU of anti-TT antibodies per ml.

RIA. Anti-PRP antibody levels in monkey sera were also determined by a Farr-type radioimmunoassay (RIA) as described previously (22). The reference serum in all determinations was the human OOB serum (Office of Biologics, Food and Drug Administration). A correlation of 0.94 was observed between anti-PRP antibody concentrations in monkey sera as determined by RIA and ELISA (Fig. 2).

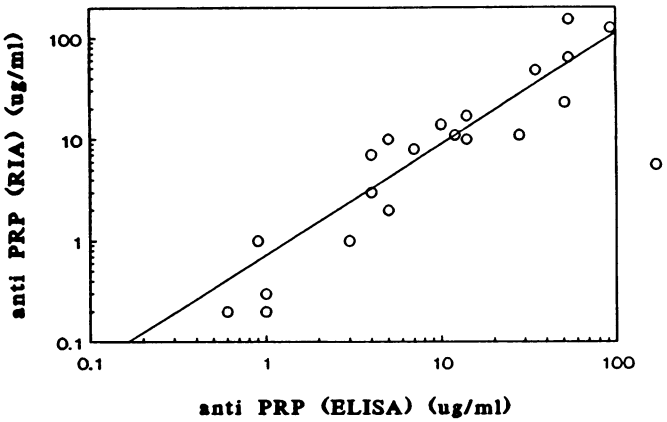

FIG. 2. Correlation of the anti-Hib antibody content in 36 monkey serum samples as determined by total Ig ELISA and RIA (correlation coefficient, 0.94).

RESULTS

Antigenic properties of synthetic trimer and tetramer of RRP. The antigenicity of a synthetic trimer and tetramer $(RRP)_n$ was compared with that of the native capsular polysaccharide studied by using a competition ELISA. As is shown in Fig. 3, PRP, $OS_{dp20}CRM_{197}$, and $(RRP)_4$-TT were able to completely inhibit the binding of anti-PRP antibodies to the coated PRP. However, larger amounts of saccharide were necessary to obtain a constant inhibition when the chain length of the saccharide decreased. Inhibition by $(RRP)_3$ and the $(RRP)_3$-TT conjugate was not complete. About 40% inhibition could be demonstrated in the presence of 25 μg of $(RRP)_3$ per ml. Free trimer and tetramer showed lower inhibition compared with the conjugate formulation. No inhibition with an irrelevant polysaccharide (meningococcal group C polysaccharide), which served as control, was observed.

Immunogenicity of synthetic trimeric and tetrameric $(RRP)_n$-protein conjugates in mice. Adult mice were immunized with 1 μg of either synthetic trimer or tetramer Hib oligosaccharide coupled to DTx or TT with and without $AlPO_4$. After primary immunization, anti-PRP IgM antibodies were induced in mice immunized with $(RRP)_4$-DTx, $(RRP)_4$-TT, and the $(RRP)_3$-TT conjugate (Table 2). The trimeric oligosaccharide coupled to DTx elicited lower amounts of anti-PRP IgM antibodies than $(RRP)_3$-TT and both $(RRP)_4$-protein conjugates. The $(RRP)_4$-protein conjugates elicited IgM antibodies in more than 75% of the immunized mice, whereas the $(RRP)_3$-DTx conjugate could only induce anti-PRP IgM antibodies in less than 25% of the mice. After the primary immunization, anti-PRP IgG antibodies could be observed in only some of the mice immunized with the $(RRP)_4$-protein conjugates (Table 2). $AlPO_4$ had no effect on the primary anti-PRP response to any of the conjugates. After booster immunization with the homologous conjugate, anti-PRP IgM antibody levels increased only in mice immunized with the $(RRP)_3$-DTx conjugate adsorbed to $AlPO_4$. Anti-PRP IgG antibody levels were increased in mice vaccinated with the tetrameric oligosaccharide-protein conjugate vaccines [$(RRP)_4$-DTx and $(RRP)_4$-TT]. $AlPO_4$

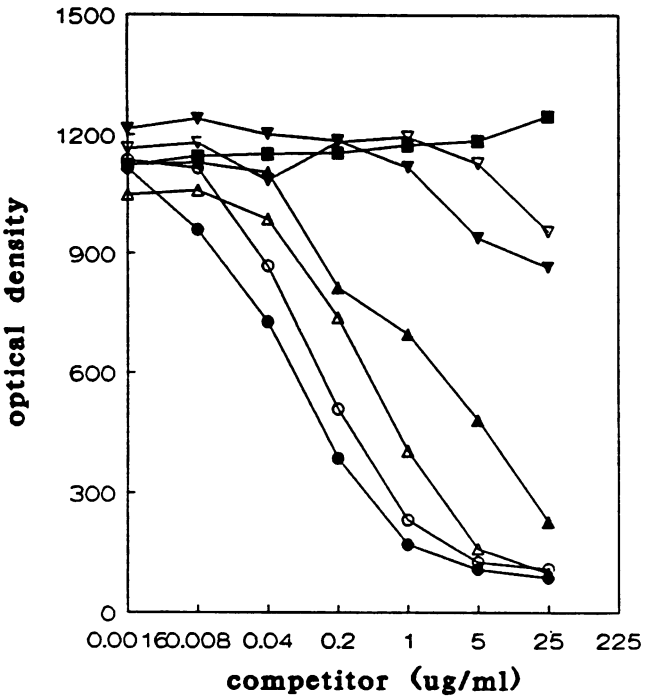

FIG. 3. Inhibition profiles of synthetic trimeric and tetrameric Hib oligosaccharides compared with that of the Hib polysaccharide. Tyramine-PRP-coated plates were incubated with a human anti-PRP serum with high titers (OOB) mixed with variable concentrations of PRP (○), $(RRP)_4$ (▲), $(RRP)_4$-TT (△), $(RRP)_3$ (▼), $(RRP)_3$-TT (▽), $OS_{dp20}CRM_{197}$ (●), or meningococcal group C polysaccharide (■). After incubation, plates were developed with horseradish peroxidase-labeled goat anti-human IgG (Tago).

had no effect on the secondary antibody response to $(RRP)_4$-protein conjugates.

Immunogenicity of synthetic $(RRP)_n$-protein conjugate vaccines in monkeys. Since anti-PRP antibodies could be induced in mice immunized with the synthetic trimer- and tetramer-protein conjugate vaccines, we wanted to compare the immunogenicity of these experimental conjugate vaccines to the immunogenicity of a commercially available Hib conjugate vaccine (HbOC) shown to be protective in infants. The immunogenicity of $(RRP)_3$-TT, $(RRP)_4$-TT, and OS_{dp20}-CRM_{197} (HbOC) was studied in adult monkeys. In adult monkeys, mean anti-PRP antibody titers increased after one dose of all conjugate vaccines. However, only two of three monkeys immunized with $(RRP)_3$-TT, $(RRP)_4$-TT, or OS_{dp20}-CRM_{197} conjugate vaccine showed more than twofold increases in the total antibody level (Fig. 4). Booster immunization with the homologous conjugate vaccines did increase mean antibody levels in all groups (Table 3 and Fig. 4). Mean antibody concentrations were highest in the groups of monkeys immunized with $(RRP)_4$-TT and OS_{dp20}-CRM_{197} conjugates. All monkeys immunized with these two conjugate vaccines showed more than twofold increases in anti-PRP antibody levels compared with prevaccination levels. In the group of monkeys immunized with $(RRP)_3$-TT, only two of three monkeys revealed more than twofold increases over prevaccination antibody levels (Table 3).

Isotype distribution of anti-PRP antibodies elicited by $(RRP)_n$-protein conjugate vaccines in monkeys. After the primary immunization, two of three monkeys vaccinated with either HbOC or $(RRP)_4$-TT showed an increase in anti-PRP IgM antibodies (Fig. 5), whereas only one of three monkeys showed an increase in anti-PRP IgM antibodies after vaccination with $(RRP)_3$-TT. Booster immunization did increase anti-PRP IgM antibody levels in two of three monkeys vaccinated with OS_{dp20}-CRM_{197}. Booster responses were not shown at the anti-PRP IgM antibody level in monkeys vaccinated with $(RRP)_4$-TT and $(RRP)_3$-TT.

All monkeys vaccinated with $(RRP)_4$-TT showed an increase in the anti-PRP IgG antibody response after the primary immunization (Fig. 6). Two of three monkeys responded with anti-PRP IgG antibodies when vaccinated with OS_{dp20}-CRM_{197} or RRP_3-TT. Anti-PRP IgG antibodies increased in all monkeys upon secondary immunization with the $OS_{dp20}CRM_{197}$ or $(RRP)_4$-TT conjugate vaccine. Only two of three monkeys vaccinated with $(RRP)_3$-TT showed booster responses upon secondary immunization with the homologous conjugate vaccine (Table 3). A comparison of the IgM and IgG antibody levels in individual monkey serum samples after primary and secondary immunizations showed that increases in IgM and IgG antibody titers were not always parallel. Anti-PRP IgA antibodies were not detectable in any of the monkey sera tested.

Anticarrier antibody response to $(RRP)_n$-protein conjugate vaccines in monkeys. Adult monkeys receiving the conjugate vaccine with CRM_{197} as a carrier protein showed an increase in the anticarrier IgG antibody level after the secondary immunization (Fig. 7), whereas only one of three monkeys showed an increase after the primary immunization. Two of

TABLE 2. Immunogenicity of synthetic trimeric and tetrameric RRP-protein conjugates in adult mice[a]

Vaccine ($AlPO_4$)	Anti-PRP antibody titers (mean $\log_{10}$ ± SD) (*n*) after the following immunization:	
	Primary	Secondary
IgM		
Saline	0.717 ± 0.276 (0/10)	0.633 ± 0.353 (0/10)
$(RRP)_3$-TT	2.205 ± 0.878 (8/9)	2.042 ± 0.506 (9/9)
$(RRP)_3$-DTx	1.026 ± 0.254 (2/9)	0.968 ± 0.414 (2/9)
$(RRP)_4$-TT	1.949 ± 0.440 (8/10)	1.650 ± 0.493 (8/10)
$(RRP)_4$-DTx	2.124 ± 0.339 (10/10)	1.803 ± 0.416 (9/10)
$(RRP)_3$-TT (+)	2.365 ± 0.724 (9/10)	2.068 ± 0.438 (9/10)
$(RRP)_3$-DTx (+)	0.782 ± 0.321 (2/10)	1.363 ± 0.238 (10/10)
$(RRP)_4$-TT (+)	1.907 ± 0.619 (8/9)	1.917 ± 0.505 (8/9)
$(RRP)_4$-DTx (+)	2.199 ± 0.409 (10/10)	1.796 ± 0.413 (9/10)
IgG		
Saline	0.398 ± 0.185 (0/10)	0.437 ± 0.211 (0/10)
$(RRP)_3$-TT	0.496 ± 0.257 (0/10)	0.805 ± 0.324 (2/10)
$(RRP)_3$-DTx	0.589 ± 0.156 (0/10)	0.478 ± 0.254 (0/10)
$(RRP)_4$-TT	0.618 ± 0.248 (0/10)	1.307 ± 0.654 (8/10)
$(RRP)_4$-DTx	1.036 ± 0.479 (4/10)	1.342 ± 0.386 (8/10)
$(RRP)_3$-TT (+)	0.764 ± 0.198 (1/10)	0.892 ± 0.434 (2/10)
$(RRP)_3$-DTx (+)	0.546 ± 0.152 (0/10)	0.484 ± 0.229 (0/10)
$(RRP)_4$-TT (+)	1.218 ± 0.982 (4/10)	1.489 ± 1.000 (8/10)
$(RRP)_4$-DTx (+)	0.955 ± 0.142 (1/10)	1.325 ± 0.449 (7/10)

[a] Groups of 8 to 10 mice each were immunized with 1 μg (on a saccharide basis) of a synthetic trimer or tetramer of RRP coupled to TT or DTx. Conjugates were injected subcutaneously in the presence (+) or absence of $AlPO_4$ as an adjuvant. A second injection with the homologous antigens was done 4 weeks after the primary immunization. Anti-Hib polysaccharide (PRP) IgM antibody titers were measured at day 8 after each immunization. Anti-PRP IgG antibody titers were measured at day 28 after the primary immunization and day 21 after the secondary immunization. Antibody titers are expressed as the mean $\log_{10}$ ± 1 SD, as a percentage of the reference serum. The number of responding mice of the total number in the experimental group is indicated (*n*). Responding mice are defined as those mice which have antibody levels more than two times higher than antibody levels in mice before immunization with the conjugate vaccines. Preimmunization levels were similar to that of the group of mice immunized with saline.

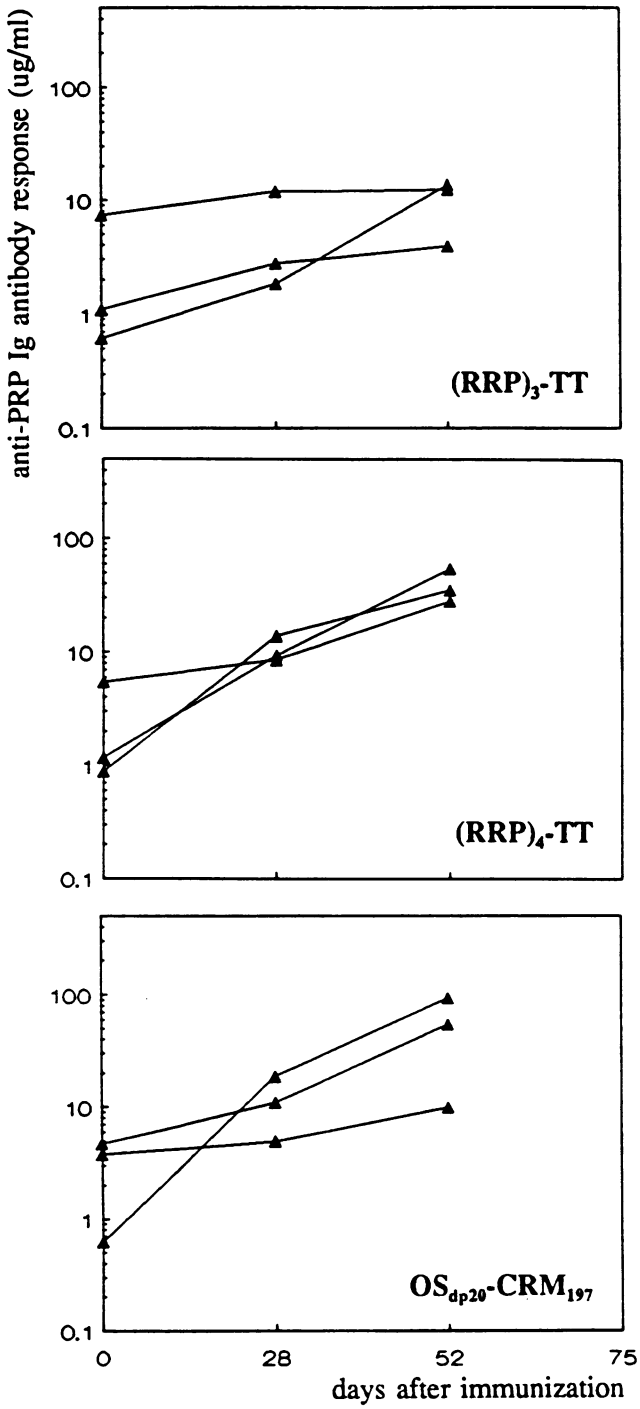

FIG. 4. Groups of three monkeys each were immunized at day 0 and day 28 with 5 μg of synthetic trimeric or tetrameric RRP-TT or -DTx conjugate vaccines or with oligosaccharide (OS_{dp20}) coupled to CRM_{197} (HbOC). Conjugates adsorbed to $AlPO_4$ were injected i.m. Anti-Hib polysaccharide (PRP) Ig antibody responses of individual monkeys are shown. Antibody responses were determined as a percentage of the reference serum.

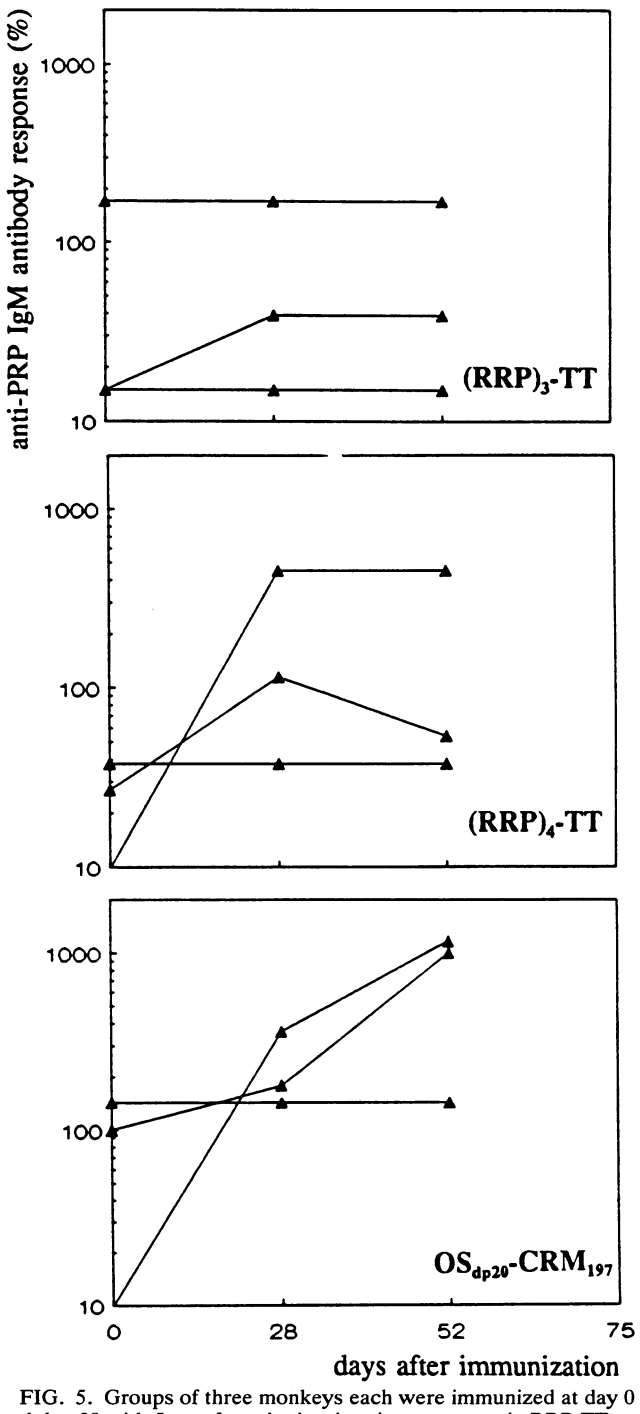

FIG. 5. Groups of three monkeys each were immunized at day 0 and day 28 with 5 μg of synthetic trimeric or tetrameric RRP-TT or -DTx conjugate vaccines or with oligosaccharide (OS_{dp20}) coupled to CRM_{197} (HbOC). Conjugates adsorbed to $AlPO_4$ were injected i.m. Anti-Hib polysaccharide (PRP) IgM antibody responses of individual monkeys are shown. Antibody responses were determined as a percentage of the reference serum.

three monkeys vaccinated with $(RRP)_4$-TT responded, whereas none of the monkeys receiving the $(RRP)_3$-TT conjugate showed an increase in the anti-TT IgG antibody response after the primary vaccination. A second injection with the homologous conjugate vaccines evoked an increase in the anticarrier IgG antibody level except for one of the monkeys vaccinated with the $(RRP)_3$-TT. In general, anticarrier antibody responses induced by the conjugate vaccines were low. No increase in anticarrier IgM antibodies could be observed (data not shown).

TABLE 3. Anti-PRP antibody responses of adult monkeys vaccinated with various synthetic trimer or tetramer RRP-protein conjugate vaccines[a]

Vaccine	Anti-PRP antibody response (mean $\log_{10}$ ± SD, μg/ml) on day:		
	0	28	52
RIA			
$(RRP)_3$-TT	0.119 ± 0.880	ND	0.835 ± 0.337
$(RRP)_4$-TT	0.539 ± 0.679	ND	1.511 ± 0.410
OS_{dp20}-CRM_{197}	0.276 ± 1.109	ND	1.806 ± 0.585
ELISA			
$(RRP)_3$-TT	0.323 ± 0.563	0.594 ± 0.427	0.942 ± 0.303
$(RRP)_4$-TT	0.249 ± 0.426	1.041 ± 0.111	1.575 ± 0.145
OS_{dp20}-CRM_{197}	0.348 ± 0.483	1.001 ± 0.289	1.565 ± 0.507

[a] Animals were vaccinated on day 0 and day 28 with 5 μg of synthetic trimer or tetramer of RRP-TT or -DTx conjugate vaccines or with oligosaccharide (OS_{dp20}) conjugated to diphtheria mutant toxin (CRM_{197}) (HbOC). Conjugates adsorbed to $AlPO_4$ were injected i.m. Total antibody responses (micrograms per milliliter) were determined by RIA or ELISA. Antibody responses of three animals per group are expressed as the mean $\log_{10}$ ± 1 SD. ND, not determined.

DISCUSSION

This study was undertaken to investigate whether synthetic trimeric and tetrameric RRP units in conjugate formulation can induce an immune response comparable to potent Hib oligosaccharide- or polysaccharide-protein conjugate vaccines. Our previous studies demonstrated that conjugates of a synthetic dimer or trimer coupled to TT by using glutaric dialdehyde lead to immunogens which can induce anti-PRP antibodies in mice and rabbits (10). Anti-PRP antibodies generated by the dimer conjugate were predominantly of the IgM isotype, whereas synthetic trimer conjugate vaccines induced both IgM and IgG anti-PRP antibodies. The glutaric dialdehyde coupling method, however, afforded conjugate vaccines with a low saccharide-to-protein ratio. Furthermore, with this coupling procedure, it is difficult to prepare consistent vaccine lots. Therefore, a coupling method which leads to conjugates with a higher incorporation of saccharides in the final conjugate and allows a better reproducibility was applied in this study. Since we found previously that the trimer conjugate revealed increased antigenicity and immunogenicity compared with the corresponding dimer, this study included a tetrameric oligosaccharide conjugate (10). In this paper, we report on the antigenic and immunogenic properties of synthetic trimeric and tetrameric RRP coupled to either TT or DTx via a thioether linkage.

Data obtained by using a competition ELISA demonstrated that both trimeric and tetrameric saccharide were able to inhibit the binding of anti-PRP antibodies. This is in agreement with the observations of Insel et al. (18) and Hetherington (15) and suggests that the antibody-combining site is complementary for a hexa- or octasaccharide; however, we found a difference: larger amounts of saccharides were necessary to obtain an inhibition when the chain length decreased from 20 to 3 repeating units. Observations with purified fragments can be influenced by the presence of low levels of larger fragments, which can be ruled out by using synthetic oligosaccharides.

A comparison of the immunogenicity of trimer and tetramer RRP conjugates in mice demonstrated that the tetramer conjugates induced a much stronger anti-PRP IgG antibody response. The trimer-TT conjugate induced IgM levels comparable to those induced by the tetramer-protein conjugates and was more immunogenic compared with the trimer-DTx conjugate. This lower immunogenicity of the DTx conjugate cannot be explained by the saccharide-to-protein ratio (Table 1) and suggests that TT is a more potent generator of

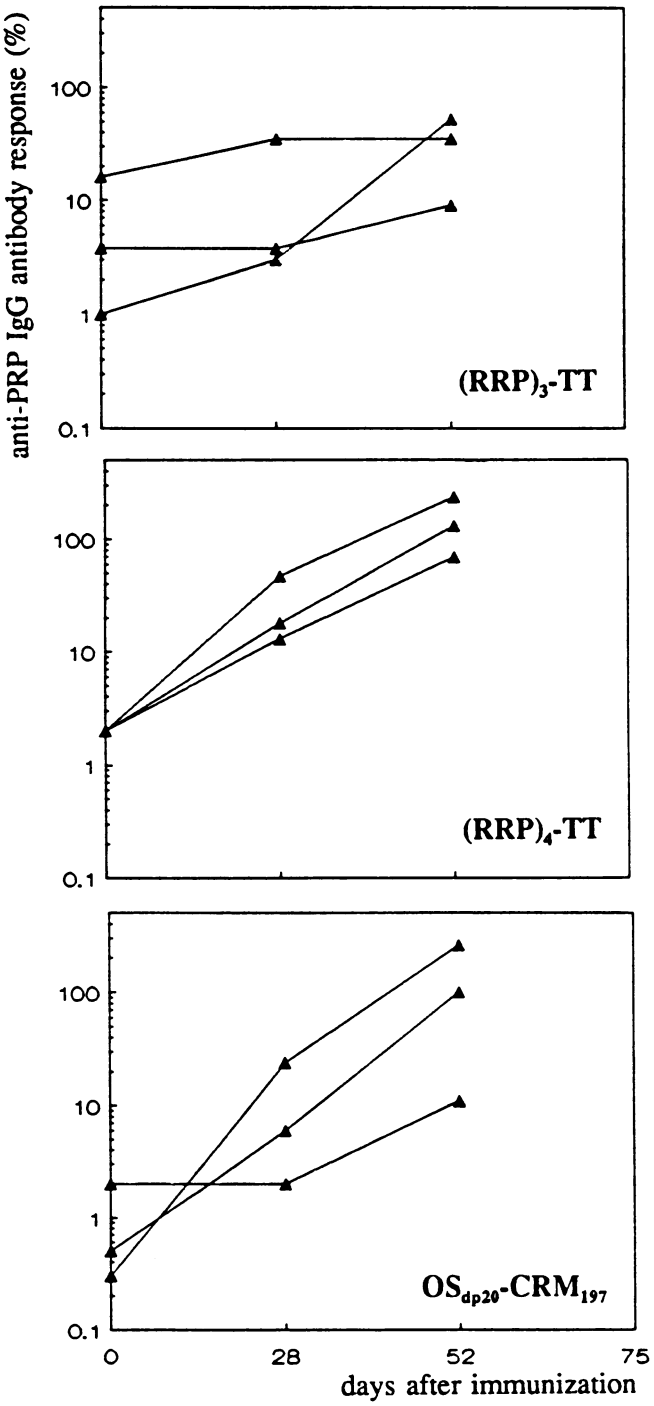

FIG. 6. Groups of three monkeys each were immunized at day 0 and day 28 with 5 μg of synthetic trimeric or tetrameric RRP-TT or -DTx conjugate vaccines or with oligosaccharide (OS_{dp20}) coupled to CRM_{197} (HbOC). Conjugates adsorbed to $AlPO_4$ were injected i.m. Anti-Hib polysaccharide (PRP) IgG antibody responses of individual monkeys are shown. Antibody responses were determined as a percentage of the reference serum.

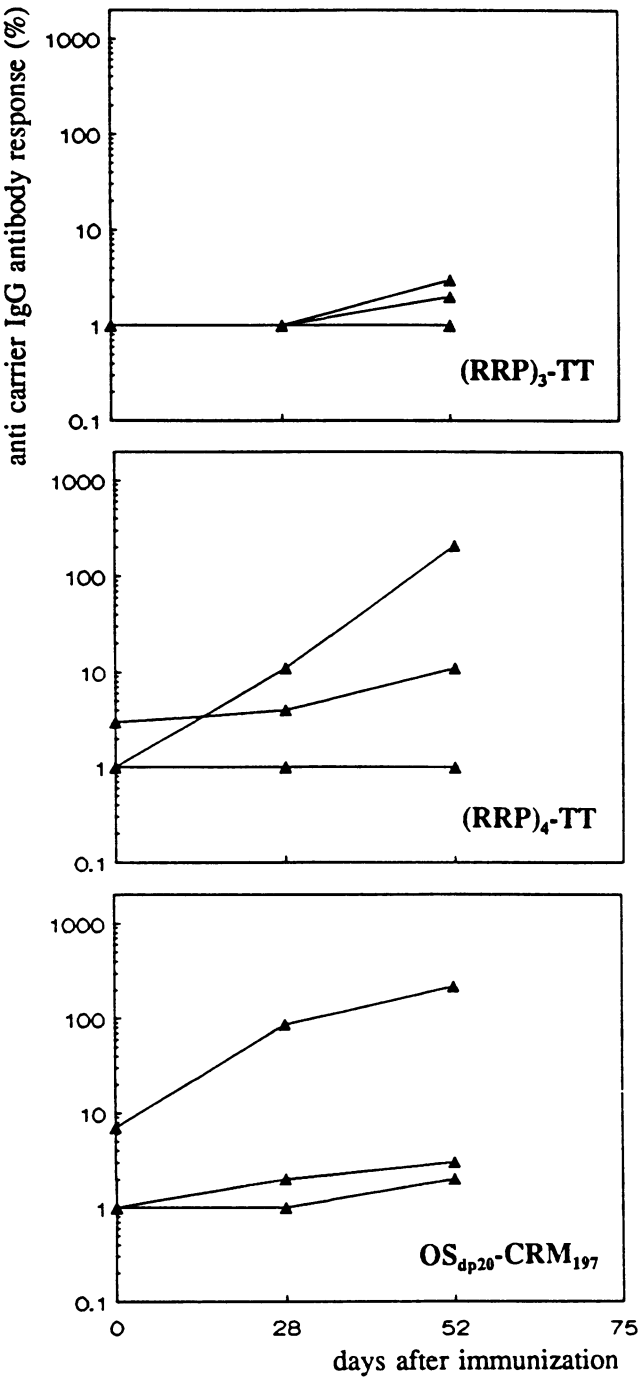

FIG. 7. Groups of three monkeys each were immunized at day 0 and day 28 with 5 μg of synthetic trimeric or tetrameric RRP-TT or -DTx conjugate vaccines or with oligosaccharide (OS_{dp20}) coupled to CRM_{197} (HbOC). Conjugates adsorbed to $AlPO_4$ were injected i.m. Anticarrier IgG antibody titers of individual monkeys were determined as a percentage of the reference serum.

T-cell help. Because of the results with mice, we decided to compare the immunogenicity of trimer and tetramer RRP-TT conjugates with a commercially available RRP conjugate that consists of RRP chain lengths with an average of 20 repeating units (HbOC).

Although individual monkeys showed considerable variation in overall anti-PRP antibody levels and a proportional contribution of anti-PRP IgG antibodies was observed, it was clear that tetramer conjugate vaccines were more immunogenic than trimer conjugate vaccines in adult monkeys. Compared with mice, monkeys are possibly more useful as a model for the prediction of the immunogenicity of Hib conjugate vaccines in humans. Both trimer and tetramer Hib conjugate vaccines were able to elicit a booster response upon secondary immunization of monkeys accompanied by an increase in the IgG/IgM ratio. It was reported by Granoff et al. (12) and Schneerson et al. (28, 29) that antibody responses to Hib conjugate vaccines are not clearly anamnestic. Adults are thought to be primed for responses to PRP through exposure to PRP and/or cross-reactive antigens in complex form on the surface of microbial flora. The secondary response in adults to those vaccines might, therefore, be the result of stimulation of previously primed B lymphocytes rather than of a recruitment of new populations of memory cells. The immune response of monkeys to $(RRP)_4$ and OS_{dp20} rather than to $(RRP)_3$ could very well represent a similar stimulation of primed B lymphocytes. It may, therefore, be concluded that a minimum chain length of eight sugars [$(RRP)_4$] is required for activation of primed B cells. $(RRP)_4$ and OS_{dp20} were equally effective in inducing an anti-PRP antibody response in adult monkeys. Whether oligosaccharides with a chain length of eight sugars are also capable of stimulating unprimed B cells of juvenile or infant monkeys equally effectively as OS_{dp20} remains to be determined. A decreased immunogenicity of saccharides with a mean chain length of eight sugars coupled to CRM_{197} compared with OS_{dp20}-CRM_{197}, prepared by reductive amination, was observed by Anderson et al. (1, 3). From these studies, it was suggested that Ig receptors need to be cross-linked in unprimed B cells before activation occurs. Additional studies will be needed to determine whether the oligosaccharide chain length or the saccharide-to-protein ratio is the most important parameter for generating optimal immune responses in naive infants.

In contrast to the observations of Vella and Ellis (32), we observed that carrier priming was not necessary to obtain an immune response to HbOC in monkeys. This contradiction might be due to the fact that we used *M. fascicularis* instead of *Rhesus* monkeys.

Although the number of monkeys in our study was not large and several other important parameters have not yet been examined, the approach of coupling synthetic oligosaccharide to carrier proteins via a stable thioether linkage was shown to be promising. It is clearly demonstrated that tetramer RRP conjugate vaccines are more immunogenic compared with trimer RRP conjugate vaccines in both mice and monkeys. Additional studies will be needed to clarify the structural requirements of conjugates for the priming of infant monkeys.

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