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A Strategy for Rational Design of Fully Synthetic Glycopeptide Conjugate Vaccines

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The present study describes a strategy to rationally design fully synthetic glycopeptide conjugate vaccines. Glycopeptide immunogens were constructed by coupling synthetic oligosaccharides comprising repeating units of synthetic 3-β-D-ribose-(1-1)-D-ribitol-5-phosphate (sPRP) to synthetic peptides containing potent T-helper cell determinants and B-cell epitopes of the *Haemophilus influenzae* type b (Hib) outer membrane proteins (OMPs) P1, P2, and P6. Rabbit immunogenicity studies revealed that some of these fully synthetic glycoconjugates were capable of eliciting high titers of both anti-PRP and anti-OMP immunoglobulin G antibodies. In addition, we systematically investigated the factors which could influence their immunogenicity. We observed that the magnitude of the anti-PRP antibody response markedly depended on the relative spatial orientation of sPRP and T-cell epitopes, the anti-PRP antibody response was enhanced when a multiple antigenic peptide was used as a carrier, the anti-PRP antibody response was optimal for three PRP repeating units, and lipidation of peptide-PRP conjugates had a minimal effect on the magnitude of the anti-PRP antibody response. The results of this study clearly demonstrate that coupling a carbohydrate hapten to a peptide can provide T-cell help and convert it into a T-cell-dependent antigen. The antisera raised against these conjugates were also found to be protective against Hib infection in the infant rat model of bacteremia.

Haemophilus influenzae type b (Hib) bacteria are protected from phagocytosis by their polysaccharide (PS) capsule, which is a polymer of polyribosyl ribitol phosphate (PRP). This T-cell-independent antigen is not immunogenic in young infants. Current licensed Hib vaccines contain PRP conjugated to a carrier protein such as diphtheria toxoid (PRP-D or ProHIBiT; Pasteur Merieux Connaught USA, Swiftwater, Pa.); tetanus toxoid (TT) (PRP-T or ActHIB; Pasteur Merieux Connaught France, Lyon, France); a mutant diphtheria toxin, CRM197 (PRP-CRM197 or HibTITER; Praxis Biologics, Rochester, N.Y.); or the outer membrane protein (OMP) complex of *Neisseria meningitidis* (PRP-OMP or PedvaxHIB; Merck & Co., West Point, Pa.). These conjugate vaccines differ by their molecular size, the length of PRP, the carrier protein, the conjugation chemistry, and their PRP-to-protein ratio and thus elicit different immune responses in infants (10, 12, 18). A systematic study of the relationship between these parameters and the immunogenicity of the resulting conjugates is critical for the design of new Hib vaccines and the development of other glycoconjugate vaccines against a variety of encapsulated bacteria. To this end, recently we have successfully developed a synthetic approach to produce immunogenic glycopeptide conjugates (15), which can be used as models to investigate the factors which influence the immunogenicity of the carbohydrate hapten. As the first step towards the rational design of a fully synthetic glycopeptide conjugate vaccine, studies were carried out to determine the importance of (i) the chain length of the oligosaccharide, (ii) the site of conjugation of sugar moieties with respect to the T-cell epitope, and (iii) the density of carbohydrate hapten in the construct. The final objective of the present study was to determine whether a glycopeptide conjugate with a built-in adjuvant could enhance humoral immune responses.

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MATERIALS AND METHODS

Peptide synthesis and purification. Peptides (Table 1) were chemically synthesized by an Applied Biosystem 430-A Peptide Synthesizer by using *tert*-butoxycarbonyl chemistry, cleaved from the resin with hydrogen fluoride, and purified by reversed-phase high-pressure liquid chromatography (RP-HPLC) using a Vydac C_4 semipreparative column (1 by 30 cm) and a 15-to-55% acetonitrile gradient in 0.1% trifluoroacetic acid developed over 40 min at a flow rate of 2 ml/min. A cysteine residue was added at either the N- or C-terminal end of the peptide for PRP conjugation purposes. The degree of purity of the peptides was assessed by analytical RP-HPLC, and their molecular weights were determined by ion spray mass spectrometry. Amino acid composition analyses were performed with a Waters Pico-Tag system. The multiple antigenic peptide (MAP) construct $(P2\text{-}8)_8$ comprised a polylysine core matrix bearing radially branched copies of peptide P2-8 (Table 1) corresponding to amino acids 193 to 219 of the Hib P2 protein. It was synthesized according to the method previously described by Tam (30) and was organized as follows: $(P2\text{-}8)_8\text{-}(K)_4\text{-}(K)_2\text{-}KGGGC$.

Purification of Hib OMPs. Hib P1, P2, and P6 OMPs were isolated from Hib strain Eagan. The chromatographic procedures for purifying P2 have previously been described (6). The purification methods for P1 and P6 are described elsewhere (8, 33).

Preparation of native PRP-carrier conjugates. Native PRP (Pasteur Merieux Connaught USA) was treated with sodium periodate (Aldrich) for 30 min in the dark at a ratio of 1:2 (wt/wt) according to the method of Jennings and Lugowski (13). The reaction was quenched by adding ethanediol (BDH). After dialysis against water (four times [4 liters each]), the periodate-oxidized PRP was lyophilized first and then dissolved in 0.2 M sodium phosphate buffer, pH 8.0, and coupled to polylysine at a molar ratio of 20 to 1 in the presence of sodium cyanoborohydride (10 mol equivalent to polylysine) at 37°C for 5 days. The polylysine-PRP conjugate was dialyzed against phosphate-buffered saline (PBS) (four times [4 liters each]) and then purified by gel filtration chromatography on a Sephadex G-50 column (1.6 by 100 cm) (15). Native PRP-peptide conjugates were prepared as described above. The PRP:peptide molar ratio in glycopeptide conjugates was calculated by using the orcinol test for ribose content and amino acid analysis for peptide content (15).

Modification of sPRP oligomers with MBS. All synthetic PRP (sPRP) oligomers were synthesized according to the method of Kandil et al. (14) and derivatized with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) at a molar ratio of 1:15 according to the method of Kandil et al. (15). Briefly, a solution of MBS (20 mg; 63.6 μmol) in tetrahydrofuran (1 ml) was added to a solution of sPRP trimer, $(PRP)_3$ (5.2 mg; 4.3 μmol), in 0.1 M phosphate buffer solution (1 ml), pH 7.5. After stirring the solution for 30 min at room temperature under

TABLE 1. Peptides used for conjugation with sPRP oligomers

Peptide	Hib OMP	Sequence	Epitope(s)[a]
P1-4[b]	P1	YAKAQVERNAGLIADSVKDNQITSALSTQ(C)	T and B
CP1-4	P1	(C)YAKAQVERNAGLIADSVKDNQITSALSTQ	T and B
P2-8	P2	(C)DIVAKIAYGRTNYKYNESDEHKQQLNG	T and B
P6-6	P6	VKGYLAGYLAGKGVDAGKLGTVSYG(C)	T and B
P24EC[c]		GPKEPFRDYVDRFYK(C)	T
TPC–P1-4[d]	P1	TPC-*SS*YAKAQVERNAGLIADSVKDNQITSALSTQ(C)	

[a] These peptides were selected to contain either a functional T-helper cell epitope or both T- and B-cell epitopes (5–8).
[b] P1-4 contains a Hib strain-specific protective B-cell epitope (NQITSALSTQ) that is recognized by the protective monoclonal antibody 7C8 (25).
[c] P24E is a potent T-helper cell epitope from the gag p24 protein of human immunodeficiency virus type 1 (5).
[d] A built-in adjuvant, TPC, was linked to peptide P1-4 with the Ser-Ser dipeptide (italicized) as a spacer.

argon, the reaction mixture was extracted with ether (four times [5 ml each]). To remove excess MBS, the aqueous layer was applied to a Sephadex G-25 (Pharmacia) column (2 by 30 cm) equilibrated with 0.1 M triethylammonium acetate buffer, pH 7.2, and eluted with the same buffer. Elution was monitored spectrophotometrically at 254 nm. The first peak (MBS-sPRP) was collected, lyophilized, and then analyzed. The amount of maleimide groups incorporated into $(PRP)_3$ was determined by a modified Ellman's method (15, 26).

Preparation of synthetic peptide-sPRP oligomer conjugates. A general protocol for preparing sPRP-peptide conjugates has been described elsewhere (15). As an example, 1 to 2 mg of individual synthetic peptides were dissolved in 0.5 ml of well-degassed water, and 0.8 ml of MBS-$(PRP)_3$ (1.6 mg) in well-degassed 50 mM $NaHCO_3$ was added. The resulting mixture was stirred overnight at room temperature under argon. The insoluble precipitate was removed by centrifugation, and the supernatant was subjected to gel filtration chromatography on a column of Sephadex G-50 (2 by 100 cm) equilibrated in 0.1 M triethylammonium acetate buffer, pH 7.2, to remove excess MBS-$(PRP)_3$. The synthetic peptide-$(PRP)_3$ conjugates were collected and analyzed by RP-HPLC for purity, the orcinol test for ribose content, and amino acid analysis for peptide content.

Preparation of sPRP-TT conjugates. Synthetic PRP oligomers (dimer and trimer) were individually coupled to TT (Pasteur Merieux Connaught Canada, Toronto) at a molar ratio of 10 to 1, using 0.1% glutaraldehyde (vol/vol). The reaction mixtures were dialyzed against PBS to remove excess glutaraldehyde and its by-products. The synthetic PRP-TT conjugates were assayed for ribose and protein contents as described above.

Preparation of fully synthetic glycopeptide conjugates with built-in adjuvant. Resin-bound peptide P1-4 (200 mg), prepared by 9-fluorenylmethoxycarbonyl chemistry was extended at the N terminus with two additional serine residues and then coupled to tripalmitoyl-*S*-glycerylcysteine (TPC) prepared according to the method of Wiesmuller et al. (32) at room temperature in the presence of *N*-hydroxybenzotriazole and dicyclohexylcarbodiimide as coupling reagents. Fourfold excesses of TPC, *N*-hydroxybenzotriazole, and dicyclohexylcarbodiimide were used. The lipopeptide TPC–P1-4 was cleaved from the resin with trifluoroacetic acid, reduced with dithiothreitol, and semipurified by gel filtration chromatography (Sephadex G-25; 1 by 30 cm). The synthetic lipopeptide (5 mg) was dissolved in 1 ml of dimethylformamide, diluted with 4 ml of 50 mM $NaHCO_3$, and then mixed with MBS-$(PRP)_3$ (4 mg in 2 ml of well-degassed 50 mM $NaHCO_3$). The mixture was stirred overnight at room temperature under argon. The insoluble precipitate was removed by centrifugation, and the supernatant was subjected to gel filtration chromatography on a column of Sephadex G-50 (2 by 30 cm) equilibrated in 0.1 M triethylammonium acetate buffer, pH 7.2, to remove excess MBS-$(PRP)_3$. The TPC–P1-4–$(PRP)_3$ conjugate was collected and analyzed by the orcinol test and amino acid analysis.

Production of anti-PRP antisera. Rabbits (three to six per group), BALB/c mice (six per group), and guinea pigs (three per group) were immunized either intramuscularly (rabbit and guinea pig) or subcutaneously (mouse) with individual PRP-carrier conjugates (10 or 50 μg of PRP equivalent) mixed either with $A1PO_4$ (1.5 mg per dose) or TPC (200 μg per dose), followed by two booster doses (half the amount of the same immunogen) at 2-week intervals. Antisera were collected every 2 weeks after the first injection, heat inactivated at 56°C for 30 min, and stored at −20°C. In the case of commercial PRP-D, three rabbits were immunized three times intramuscularly with one human dose containing 25 μg of PRP formulated in alum. For the TPC–P1-4–$(sPRP)_3$, four rabbits were immunized three times intramuscularly with 60 μg of conjugate (~10 μg of PRP equivalent) in PBS.

Protein- and peptide-specific ELISAs. Microtiter plate wells (Nunc-Immunoplate; Nunc, Roskilde, Denmark) were coated with 200 ng of purified OMPs or 500 ng of individual peptides in 50 μl of coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ [pH 9.6]) for 16 h at room temperature. The plates were then blocked with 0.1% (wt/vol) bovine serum albumin (BSA) in PBS for 30 min at room temperature. Serially diluted antisera were added to the wells and incubated for 1 h at room temperature. After removal of the antisera, the plates were washed five times with PBS containing 0.1% (wt/vol) Tween 20 and 0.1% (wt/vol) BSA. $F(ab')_2$ from goat anti-rabbit, anti-guinea pig, anti-mouse, or anti-human immunoglobulin (IgG) antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch Labs Inc.) were diluted (1/8,000) with washing buffer and added to the microtiter plates. After 1 h of incubation at room temperature, the plates were washed five times with the washing buffer. The plates were then developed with tetramethylbenzidine in H_2O_2 (Allelix Diagnostic, Inc., Toronto, Canada) as a substrate. The reaction was stopped with 1 N H_2SO_4, and the optical density was measured at 450 nm with a Titretek Multiskan II device (Flow Laboratories, McLean, Va.). Two irrelevant pertussis toxin peptides, NAD-S1 (19 residues) and S3(123-154) (32 residues), were included as negative controls in the peptide-specific enzyme-linked immunosorbent assays (ELISAs). Assays were performed in triplicate, and the reactive titer of an antiserum was defined as the dilution consistently showing a twofold increase in absorbance over that obtained with the preimmune serum.

Farr assay. Total anti-PRP antibodies in rabbit antisera were quantitatively determined by a modified radioantigen-binding assay using biosynthetically labelled [^{3}H]PRP as the antigen (2). Briefly, a tube containing 50 μl of excess tritiated antigen [^{3}H]PRP (Pasteur Merieux Connaught USA) together with ^{36}Cl ions as an internal standard (Amersham Canada Ltd.) was reacted either with various dilutions of test sera (25 μl) or with a BSA solution (negative control) overnight at 4°C. Cold saturated ammonium sulfate (75 μl) was added to each tube to precipitate antibody-PRP complexes, which were collected by 5-min centrifugations in a Beckman Microfuge. The pellets were resuspended, washed with cold distilled water, and recentrifuged. The amount of anti-PRP antibodies was calculated from the amount of ^{3}H detected in the precipitates in comparison to that from a calibrated reference antiserum (human anti-PRP antiserum, lot 1983), by using a scintillation counter (Beckman LS-6000). Assays were performed in duplicate, and the lower limit of sensitivity of the assay was 0.06 μg/ml.

ELISA for anti-PRP IgG antibody determination. The assay was performed as described for the peptide-specific ELISAs, except that the microtiter plate wells were coated with 200 ng of polylysine-PRP in 200 μl of coating buffer.

Protection studies. The protective abilities of rabbit antisera raised against peptide-sPRP conjugates were assessed in the infant rat model of bacteremia according to the method of Munson et al. (20). The Hib strain MinnA was used for challenge.

CD. Circular dichroism (CD) spectra of PRP-peptide conjugate solutions containing 100 μM peptide in PBS, pH 7.2, were recorded on a JASCO 720 CD spectropolarimeter. Measurements were performed from 300 to 180 nm (1-nm intervals) with a 0.05-cm cell, and the results of eight scans were averaged. All spectra were smoothed with a Fourier transformation algorithm supplied by the manufacturer and corrected for background response from PBS. Results were expressed as mean residue ellipticity values (θ), measured in degrees times centimeters squared per decimole.

RESULTS

Influence of the number of repeating units on the immunogenicity of PRP oligomers. To determine the minimum number of repeating units required to generate an antibody response, synthetic PRP oligosaccharides (dimer and trimer) were individually coupled to TT with glutaraldehyde. Chemical analyses revealed that the molar ratio of PRP moieties to TT was 2:1 in both sPRP-TT conjugates. Groups of six rabbits were separately immunized three times with the sPRP-TT conjugates formulated in alum. Native PRP (25 μg) and the sPRP trimer (25 μg) formulated in alum were included as controls in the study and were found to be poor immunogens (Fig. 1). After three injections, fewer than half of the rabbits immunized with the sPRP trimer developed anti-PRP antibody levels of >0.2 μg/ml (Fig. 1). Therefore, synthetic PRP oligomers appeared to be excellent model haptens to study the effect of

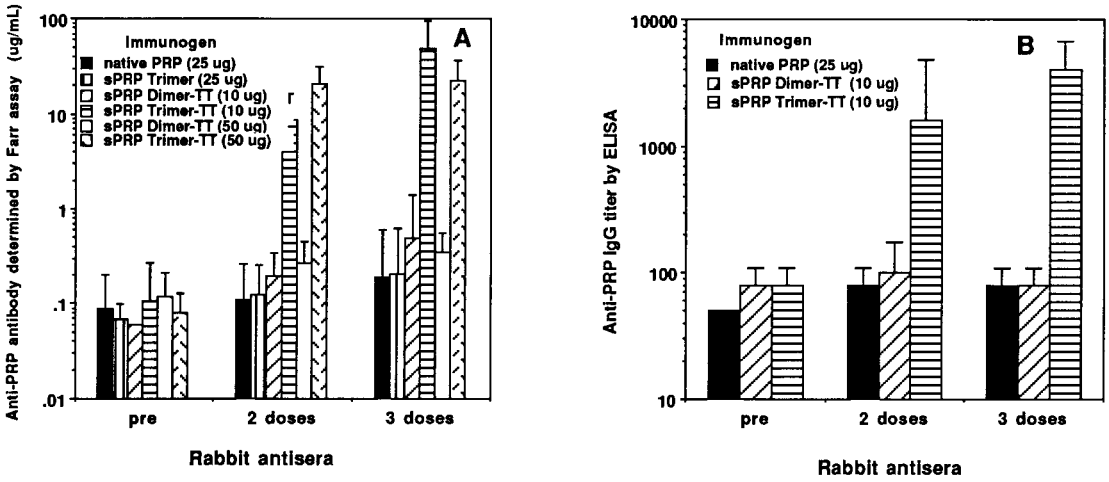

FIG. 1. Rabbit antibody responses to native PRP, PRP trimer, and sPRP dimer and trimer conjugated to TT. Levels of anti-PRP antibodies were determined by the Farr assay (A) and ELISA (B). Error bars, standard deviations.

T-cell epitopes on the immunogenicity of synthetic glycopeptide conjugates. As shown in Fig. 1, three immunizations with $(sPRP)_3$-TT elicited anti-PRP antibody responses 50- to 200-fold higher than those induced by the sPRP trimer or the $(sPRP)_2$-TT conjugate, as judged by anti-PRP IgG ELISA and the Farr assay. The geometric mean titer (GMT) by ELISA and the geometric mean anti-PRP antibody level by the Farr assay were found to be 1,020 and 8.7 μg/ml, respectively. Interestingly, a 10-μg dose of $(sPRP)_3$-TT conjugate was found to be as potent as a 50-μg dose (Fig. 1A). Anti-PRP antibody levels induced by two doses of $(sPRP)_3$-TT conjugate containing 10 μg of PRP were consistently greater than 1 μg/mL, a level considered to confer long-term protection (28). These results clearly indicated that in the rabbit model, to be highly immunogenic, a synthetic glycoconjugate requires at least three PRP repeating units. This conclusion was further supported by the results of competitive binding inhibition assays, which revealed that the binding of human anti-PRP IgG antibodies to solid-phase PRP could be efficiently inhibited by the sPRP trimer but not by the sPRP dimer (data not shown). Mouse and guinea pig immunogenicity studies were also performed with the $(sPRP)_3$-TT conjugate. In both cases, antisera were found to contain low levels of anti-PRP antibodies as determined by ELISA. Therefore, the rabbit model was selected to study the immunogenicity of synthetic glycopeptide conjugates.

Peptide carrier selection. Previous rabbit immunogenicity studies (6–8) indicated that peptides from Hib OMPs could elicit strong IgG antibody responses, suggesting that these peptides contain functional T-helper cell epitope(s) and could serve as potential carriers for PRP. In the present study, the carrier peptides (Table 1) were chosen on the basis of their T-helper cell stimulatory properties, the presence of a neutralization B-cell epitope(s), or the conservation of their sequences among *H. influenzae* strains. Peptide P1-4 was previously shown to contain both a functional T-cell epitope(s) and a Hib strain-specific protective B-cell epitope (DNQITSALS TQ) recognized by the neutralizing mouse monoclonal antibody 7C8 (22). A cysteine residue was added either at the N- or C-terminal end of P1-4 to analyze how the spatial orientation of the sPRP relative to the T-cell epitope(s) influenced the immunogenicity of the conjugates. Peptide P2-8, which contains an immunodominant murine T-cell epitope (6), is highly conserved among Hib strains (20). The cysteine residue was added at its N-terminal end, to place the PRP moiety in the conjugate at a distance from the T-cell epitope located within the C-terminal end of P2-8 (4a). Peptide P6-6 was found to be the most immunogenic P6 peptide in previous rabbit immunogenicity studies (7), and its sequence is totally conserved among Hib and nontypeable *H. influenzae* (NTHi) strains (21). Peptide P24E, derived from the gag p24 protein of human immunodeficiency virus type 1 (HIV-1), was identified as a potent T-helper epitope (5). Since results from our previous studies demonstrated that T-B tandem peptides containing B-cell epitopes linked to the C-terminal end of P24E were highly immunogenic (27), a cysteine residue was purposefully added at the C-terminal end of P24E.

Rabbit immunogenicity studies of conjugates containing linear peptide and sPRP trimer. To investigate whether peptides containing T-cell epitope(s) could function as carriers for sPRP oligomers, sPRP-peptide conjugates were prepared by coupling the sPRP trimer to the N- and C-terminal cysteines of the peptides CP1-4, P2-8, P6-6, and P24EC. The purified glycopeptide conjugates were analyzed and found to contain a 1:1 molar ratio of peptides to sPRP. The final yields were in the 30 to 60% range. Rabbits were immunized with synthetic glycoconjugates containing 10 μg of sPRP trimer adjuvanted in alum. The licensed Hib vaccine PRP-D was used as a positive control. All glycopeptide conjugates elicited good antipeptide and anti-OMP IgG antibody responses, in general similar to those obtained with the peptides alone (Table 2). As expected, the P24EC-$(sPRP)_3$ glycopeptide failed to induce antibodies against P24E, which is a pure T-cell epitope (5, 27). Glycopeptide conjugates derived from CP1-4 and P24EC elicited strong anti-PRP antibody responses in all rabbits (five out of five for CP1-4 and four out of four for P24EC). Anti-PRP IgG titers (GMT >1,000) and mean anti-PRP antibody levels (>1 μg/ml) were comparable to those obtained with PRP-D (Fig. 2). About half of the rabbits immunized with these two glycopeptide conjugates produced more than 1.0 μg of anti-PRP antibody per ml after only two injections. These results clearly indicated that both peptides had provided T-cell help and converted the sPRP determinant into a T-cell-dependent antigen. Glycopeptide conjugates derived from P2-8 and P6-6 were less potent at eliciting anti-PRP antibody responses (Fig. 2). Nevertheless, they were more immunogenic than the sPRP

TABLE 2. Rabbit antibody responses to synthetic peptides and glycopeptide conjugates[a]

Immunogen	Mean titer of IgG against:			
	Immunizing peptide	P1	P2	P6
P1-4	6,400	1,600	ND[b]	ND
$(sPRP)_3$–P1-4	6,400	1,600	ND	ND
P1-4–$(sPRP)_3$	6,400	3,200	ND	ND
P1-4–$(sPRP)_6$	3,200	1,600	ND	ND
TPC + P1-4–$(sPRP)_3$	3,200	1,600	ND	ND
TPC–P1-4–$(sPRP)_3$	12,800	3,200	ND	ND
TPC–P1-4	6,400	1,600	ND	ND
P2-8	6,400	ND	12,800	ND
$(sPRP)_3$–P2-8	12,800	ND	6,400	ND
$(sPRP)_3$-MAP	104,000	ND	25,600	ND
P6-6	6,400	ND	ND	52,600
P6-6–$(sPRP)_3$	6,400	ND	ND	3,200
P24EC	<50	ND	ND	ND
P24EC-$(sPRP)_3$	<50	ND	ND	ND

[a] Immunization protocols and the antipeptide and anti-OMPs IgG ELISAs for the determination of titers are described in Materials and Methods.
[b] ND, Not done.

trimer, since five out of six rabbits immunized with either conjugate generated more than 0.2 μg of anti-PRP antibody per ml, whereas only three out of six rabbits immunized with the sPRP trimer developed significantly lower anti-PRP antibody responses. In addition, anti-PRP IgG titers obtained with these glycoconjugates were moderately higher than those obtained with the sPRP trimer alone (Fig. 2B).

Influence of the spatial orientation of sPRP relative to the T-cell epitope(s). To study the effect of the orientation of the sPRP trimer relative to the T-cell epitope on the immunogenicity of the glycopeptide construct, a pair of P1-4 peptides were synthesized to present the hapten either at the C terminus (P1-4) or at the N terminus (CP1-4) (Table 1). The glycopeptides are designated P1-4–$(sPRP)_3$ and $(sPRP)_3$–P1-4, respectively. Two groups of six rabbits were immunized with three doses of glycopeptides containing 10 μg of sPRP trimer. Both conjugates elicited similar antipeptide and anti-P1 antibody responses (Table 2). Although two out of six rabbits immunized with P1-4–$(sPRP)_3$ generated >1 μg of anti-PRP antibodies per ml (2.29 and 1.71 μg/ml, respectively), the GMT was 0.8 μg/ml (Fig. 3). This value was significantly lower than that obtained with the $(sPRP)_3$–P1-4 conjugate (GMT = 7.1 μg/ml; $P < 0.05$). In addition, anti-PRP IgG antibody titers were found to be 1,010 and 200 for $(sPRP)_3$–P1-4 and P1-4–$(sPRP)_3$, respectively (data not shown). These results suggest that the orientation of the sugar moiety relative to the T-cell epitope can significantly influence the host immune response to the carbohydrate hapten.

Effect of the length of the sPRP oligomer. Although the sPRP trimer appeared to be the minimal immunogenic moiety, it was of interest to explore whether the immunogenicity of the hapten would increase with the number of carbohydrate repeating units. To this end, sPRP pentamers, sPRP hexamers, and the heat-sized native PRP (molecular mass, 30 kDa) were chemically coupled to either the C terminus of P1-4 or the N terminus of P2-8 to determine whether increasing the length of the oligosaccharide would enhance the weak immunogenicities of P1-4–$(sPRP)_3$ and $(sPRP)_3$–P2-8. All glycopeptides were found to contain about a 1:1 molar ratio of PRP to peptide. Rabbit immunogenicity studies showed that native PRP conjugated to either peptide failed to elicit anti-PRP antibody levels above 0.15 μg/ml, and the antipeptide IgG titers were only ~800 for both glycopeptide conjugates (data not shown). Moreover, glycopeptide conjugates containing either the sPRP pentamer or hexamer linked to P1-4, failed to elicit anti-PRP antibody responses higher than those obtained with P1-4–$(sPRP)_3$ (Fig. 3). In fact, the anti-PRP antibody levels were similar to those obtained with the uncoupled sPRP trimer (Fig. 3). Glycoconjugates containing either the sPRP pentamer or hexamer linked to the N terminus of P2-8, were repeatedly found to be poorly immunogenic and elicit low anti-PRP antibody responses (GMT ≈ 0.3 μg/ml) (data not shown).

Effect of carbohydrate density on the immunogenicity of synthetic glycopeptide conjugates. To investigate the effect of the carbohydrate density on the immunogenicity of glycopeptides, the sPRP trimer was conjugated to a MAP containing eight branched P2-8 peptides. Although eight N-terminal and one C-terminal cysteine residues were available for conjugation, only five sPRP trimer molecules could be coupled to one MAP molecule. Rabbits immunized with two doses of MAP glycoconjugate containing 10 μg of sPRP trimer formulated in alum generated about 1.0 μg of anti-PRP antibody per ml. After three doses, the mean antibody level rose to 5.1 μg/ml, compared to ~0.2 μg/ml obtained with the linear glycoconju-

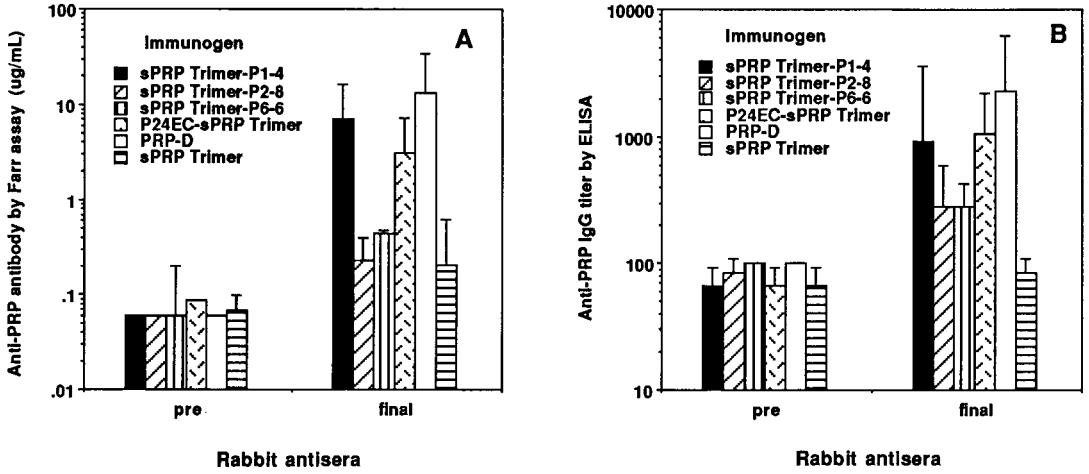

FIG. 2. Rabbit antibody responses to PRP-D, sPRP trimer, and PRP trimer conjugated to different carrier peptides. Levels of anti-PRP antibodies in preimmune and final-bleed sera (after three doses) were determined by the Farr assay (A) and ELISA (B). Error bars, standard deviations.

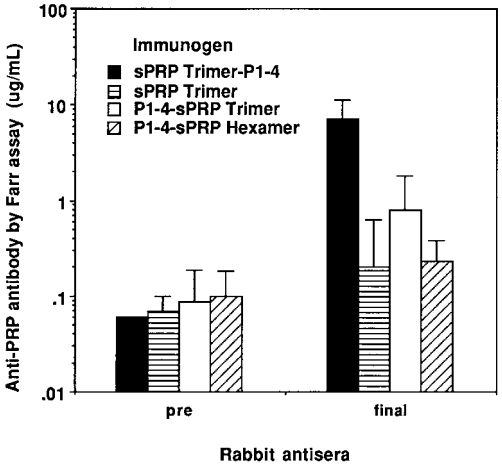

FIG. 3. Rabbit antibody responses to sPRP oligomers (trimer and hexamer) conjugated to peptide P1-4. Levels of anti-PRP antibodies in preimmune and final-bleed sera (after three doses) were determined by the Farr assay. Glycopeptide conjugates containing either the sPRP dimer or pentamer elicited antibody responses similar to those obtained with the hexamer glycopeptide conjugate. Error bars, standard deviations.

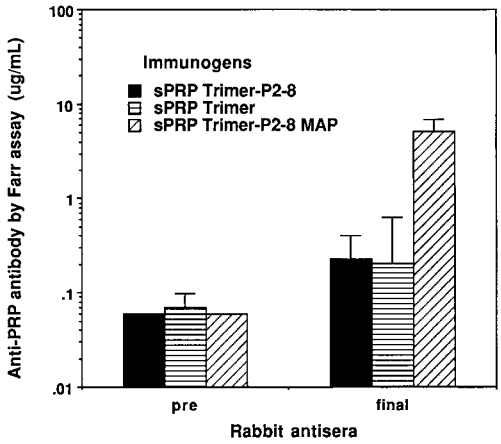

FIG. 4. Rabbit antibody responses to sPRP trimer conjugated to peptides P2-8 and P2-8 MAP. Levels of anti-PRP antibodies in preimmune and final-bleed sera (after three doses) were determined by the Farr assay. Error bars, standard deviations.

gate (Fig. 4). Anti-PRP IgG titers and anti-P2 antibody responses induced by the MAP conjugate were about 50- and 8-fold higher than those obtained with the linear peptide glycoconjugate, respectively (Table 2 and Fig. 4). These results indicate that the MAP glycoconjugate elicits faster and stronger antibody responses to both peptide and PRP determinants.

Immunogenicity of glycopeptide conjugate covalently linked to a synthetic adjuvant. We next examined whether the addition of a built-in adjuvant could induce enhanced humoral responses to a glycopeptide conjugate. A P1-4 peptide was synthesized by using 9-fluorenylmethoxycarbonyl chemistry to contain the synthetic adjuvant TPC covalently linked to its N terminus through a Ser-Ser dipeptide spacer. $(sPRP)_3$ was then conjugated to the TPC–P1-4 lipopeptide via a thioester bond. This fully synthetic lipoglycopeptide conjugate was purified by gel filtration chromatography and used to immunize rabbits without adjuvant. The P1-4–$(sPRP)_3$ conjugate formulated in TPC and the TPC–P1-4 lipopeptide in PBS were included as controls. In two sets of experiments, TPC–P1-4–$(sPRP)_3$ failed to elicit anti-PRP antibody responses higher than those obtained with sPRP trimer adjuvanted in alum after three injections (Fig. 5), whereas P1-4–$(sPRP)_3$ formulated in TPC elicited levels of anti-PRP antibodies (GMT = 0.6 μg/ml) similar to those obtained with P1-4–$(sPRP)_3$ adjuvanted in alum (GMT = 0.8 μg/ml) (Fig. 5). However, rabbit anti-TPC–P1-4–$(sPRP)_3$ antisera reacted well with native P1 in the ELISA. Antipeptide IgG titers of 12,800 (Table 2) were higher than those induced by TPC–P1-4 (6,400) and P1-4–$(sPRP)_3$ mixed with TPC (3,200).

Protection studies. Fifteen antisera containing more than 0.2 μg of anti-PRP antibodies per ml were randomly selected from rabbit antisera raised against glycoconjugates obtained by coupling sPRP to either P1-4, P2-8, P6-6, or TPC–P1-4 and were tested (0.1 ml) in the infant rat model of bacteremia. All antisera conferred protection against challenge with 250 CFU of live Hib strain MinnA (data not shown). As controls, preimmune sera from the same rabbit were tested and found to be nonprotective. These results indicate that glycopeptide conjugates can elicit protective antibodies against Hib.

Structure-function relationship of synthetic glycopeptide conjugates. CD studies were performed on P1-4, sPRP trimer, P1-4–$(sPRP)_3$, and $(sPRP)_3$–P1-4 to assess whether the conjugation of sPRP affected the conformation of the carrier peptides (Fig. 6). Analysis of the far-UV CD spectra shown in Fig. 6A revealed that P1-4 in PBS assumed a largely unordered structure (~5% α-helix) and that addition of sPRP trimer had little effect on its conformation. However, when the sPRP trimer was covalently linked to P1-4 either at its N terminus [$(sPRP)_3$–P1-4] or C terminus [P1-4–$(sPRP)_3$], significantly different CD spectra were observed (Fig. 6). The CD spectra of the conjugates were similar between 180 and 205 nm but were almost mirror images between 205 and 250 nm (Fig. 6B). Since the sPRP trimer alone exhibited weak ellipticity in these regions, these results suggest that the conformation of P1-4 was affected by glycosylation. Moreover, the local restriction imposed on the peptide conformation depended on the site of conjugation. Although both glycopeptide conjugates elicited similar anti-P1-4 antibody responses (Table 2), we investigated whether these antibodies differed in their specificities. Two small peptides, P1-4A (GLIADSVKDNQITSALSTQ) and P1-4B (YAKAQVERNAGLIADSV), representing the C- and N-

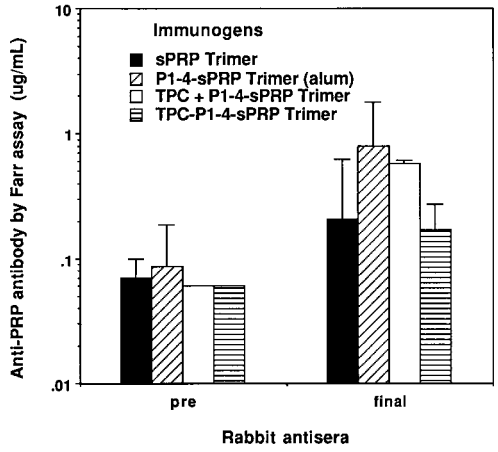

FIG. 5. Rabbit antibody responses to sPRP trimer conjugated to carrier peptides P1-4 and lipopeptide TPC–P1-4 formulated in different adjuvants (TPC or alum). Levels of anti-PRP antibodies in preimmune and final-bleed sera (after three doses) were determined by the Farr assay. Error bars, standard deviations.

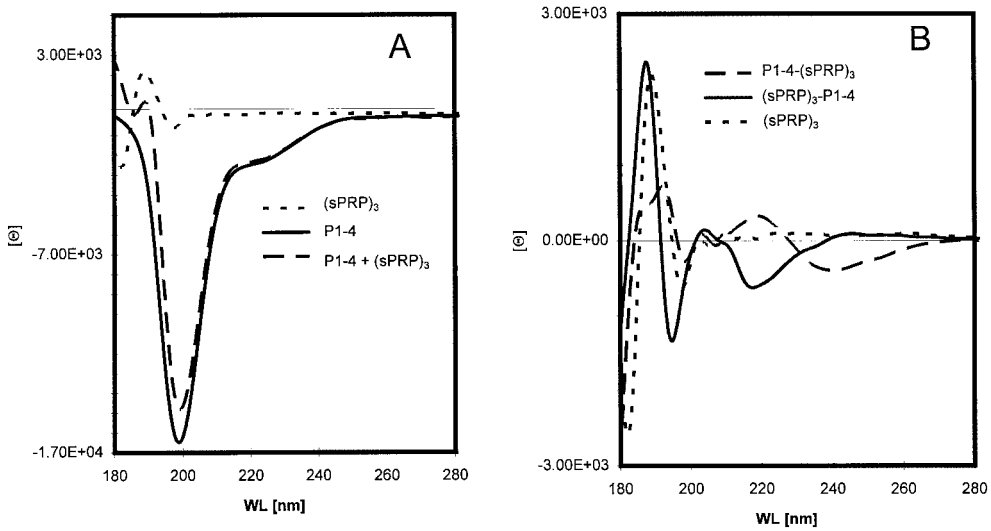

FIG. 6. CD spectra of P1-4 glycopeptide conjugates, sPRP trimer, and P1-4. (A) Far-UV CD spectra of P1-4, $(sPRP)_3$, and P1-4 + $(sPRP)_3$; (B) far-UV CD spectra of P1-4–$(sPRP)_3$, $(sPRP)_3$, and $(sPRP)_3$–P1-4. Θ, ellipticity value.

terminal regions of P1-4, respectively, were synthesized and used as target antigens in peptide-specific ELISAs. The results (Table 3) confirmed that the glycopeptide conjugates induced antibodies with different specificities. Rabbit anti-P1-4–$(sPRP)_3$ and anti-P1-4–$(sPRP)_6$ antisera reacted well with both peptides P1-4A and P1-4B, whereas anti-$(sPRP)_3$–P1-4 antisera predominantly recognized peptide P1-4B.

DISCUSSION

The development of well-defined, synthetic or biosynthetic immunogens capable of eliciting protective immunity against disease is one of the major challenges in vaccinology. We performed immunogenicity studies of rabbits with synthetic glycopeptide conjugates to better understand the factors which can modulate the immune responses to both their carbohydrate and peptide components. The present study demonstrates that coupling synthetic oligosaccharides to a synthetic T-cell epitope(s) yields constructs which acquire the typical characteristics of T-cell-dependent antigens, provided that their components are arranged in a correct spatial orientation. The $(sPRP)_3$–P1-4 glycopeptide elicited 5- to 10-fold-higher anti-PRP antibody responses than its positional isomer P1-4–$(sPRP)_3$. This large difference in antibody responses is consistent with earlier results emphasizing the importance of the relative orientation of the T- and B-cell epitopes on the immunogenicity of chimeric synthetic immunogens (peptides or glycopeptides) (1, 9, 17, 23, 27). Interestingly, Alonso De Velasco et al. (1) observed that a PS conjugated to the N terminus of a T-cell epitope (PS-cPN) elicited an anti-PS antibody response significantly higher than that elicited by the PNc-PS glycoconjugate. They concluded from T-cell proliferation experiments that the difference in immunogenicity between PS-PNc and PS-cPN was due to the inability of antigen-presenting cells to efficiently cleave the T-cell epitope (PNc) from PS and present it in the context of major histocompatibility complex (MHC) class II molecules to helper T cells.

The results from the far-UV CD spectra analyses and antipeptide antibody specificity studies of $(sPRP)_3$–P1-4 and P1-4–$(sPRP)_3$ conjugates suggest that these glycopeptides adopt different conformations which modulate the exposure of their epitopes. These structural differences might also be responsible for eliciting different titers of antibody against PRP and P1-4 B-cell epitopes. These results also suggest that P1-4 may contain more than one functional T-cell determinant and that the specific epitope which provides T-cell help to sPRP may be less accessible to processing and presentation by antigen-presenting cells when sPRP is conjugated to the C terminus of P1-4. This hypothesis is supported by a study performed by Mouritsen et al. (19), who showed that the site of attachment of an oligosaccharide to a peptide antigen can modulate the T-cell responses to the glycopeptides. Moreover, conformational constraints imposed by the sPRP trimer may hinder the presentation of P1-4–$(sPRP)_3$ to B-cell antigen receptors and result in lower antibody responses. In any event, the present study supports the notion that the magnitude of antihapten antibody responses markedly depends on the relative spatial orientation of the hapten and T-cell epitope(s).

We have also demonstrated that an optimal number of repeating units was required to induce maximum anti-PRP antibody responses, and this number appeared to be three. We and others (24) have shown that the sPRP dimer is a poor antigen as well as a poor immunogen regardless of whether or not it is conjugated to synthetic peptides or carrier proteins (TT or CRM197). Surprisingly, glycopeptide conjugates containing either native PRP or five or six PRP repeating units did

TABLE 3. Rabbit antibody responses to synthetic peptides and glycopeptide conjugates[a]

Immunogen	Mean titer of IgG against:			
	P1-4	P1-4A[b]	P1-4B[c]	NAD-S1
P1-4	12,800	1,600	6,400	<50
$(sPRP)_3$–P1-4	6,400	800	3,200	<50
P1-4–$(sPRP)_3$	6,400	3,200	1,600	<50
P1-4–$(sPRP)_6$	3,200	1,600	1,600	<50

[a] Immunization protocols and antipeptide IgG ELISAs for the determination of titers are described in Materials and Methods. NAD-S1 was used as a negative control.

[b] P1-4A consists of the C-terminal end sequence of P1-4, GLIADSVKDNQITSALSTQ.

[c] P1-4B consists of the N-terminal end sequence of P1-4, YAKAQVERNAGLIADSV.

not elicit anti-PRP antibody responses higher than those obtained with glycoconjugates of the sPRP trimer. Anderson et al. (3) and Peeters et al. (24) reported that anti-PRP IgG antibody responses in monkeys and humans increased with the number of PRP repeating units (2- to 20-mers) linked to CRM197 or TT. The fact that we used ~30-amino-acid peptides as carriers in our rabbit immunogenicity studies may explain this discrepancy. Because of their sizes, native PRP as well as the sPRP pentamer and sPRP hexamer may prevent proper antigen processing and thus preclude the binding of the T-cell epitope(s) to MHC class II molecules. Steric hindrance may also explain the low titers of antipeptide antibody in rabbit antisera raised against conjugates containing native PRP, which may mask the accessibility of both B- and T-cell determinants. In contrast, CRM197 and TT are large proteins containing several functional T-cell epitopes that would not be sterically hindered by PRP oligomers.

One of the objectives of the present study was to investigate new conjugation technologies to prepare highly immunogenic glycopeptide conjugates. Several studies (4, 29, 30) have shown that presentation of multiple copies of a peptide antigen on a polylysine backbone (MAP system) could enhance its immunogenicity. For conjugation purposes, the MAP system provides the following advantages: (i) multiple carbohydrate haptens can be attached to increase the carbohydrate density on the carrier, and (ii) multiple T-cell epitopes can be incorporated into the construct to overcome MHC class II genetic restriction. We have demonstrated that the immunogenicity of glycopeptides could be enhanced when a MAP system was used as the carrier instead of a linear peptide. In addition, quicker and stronger responses were elicited against both the peptide and PRP. The MAP-$(sPRP)_3$ synthetic conjugate is potentially a good vaccine candidate, since it elicited protective anti-PRP IgG titers (GMT = 4,800) comparable to those obtained with native PRP coupled to diphtheria toxoid (GMT = 2,262).

Toyokuni et al. (31) reported that a small synthetic carbohydrate antigen could induce an immune response against a tumor-associated carbohydrate antigen in the absence of a macromolecular carrier or adjuvant. To enhance the immunogenicity of sPRP, a lipoglycopeptide conjugate [TPC–P1-4–$(sPRP)_3$] with a built-in adjuvant (TPC) was designed, synthesized, and tested in rabbits. However, this construct was not more immunogenic than the sPRP trimer itself. Further studies are needed to optimize the lipoglycopeptide construct, its dose, and the immunization schedule.

Recent studies (29) have indicated that effective immunization against *Bordetella pertussis* respiratory infection in mice required both antibodies and cell-mediated immunity. Little is known about cellular immune responses to Hib infection, and the precise identification and location of human T-cell epitopes in Hib OMPs have not yet been reported. However, a recent study with rats by Kyd et al. (16) indicated that an increase in P6-specific antibody and T-helper cell responses, following an intratracheal boost with P6, correlated with an accelerated recruitment of phagocytic cells and enhanced clearance of both homologous and heterologous NTHi in the lungs. Thus, it should be possible to design a strategy which would use synthetic immunodominant epitopes from OMPs as additional antigens and carriers for PRP oligomers to enhance protective antibody responses and induce cell-mediated immunity. We have shown that glycopeptide conjugates are capable of eliciting good levels of antibodies against Hib OMPs (P1, P2, or P6) and their peptides. These results indicate that T-helper cells recognizing OMP T-cell epitopes were activated and that functional memory T cells could be recalled by subsequent Hib or NTHi infections.

In conclusion, several simple synthetic glycoconjugates were constructed. Critical factors affecting the immunogenicity of the carbohydrate hapten were identified. The generic glycoconjugate technology described in the present study can be applied to the preparation of efficacious synthetic glycopeptides capable of conferring protection against infection by bacteria expressing protective polysaccharidic antigens. The technology could also be used to produce glycoconjugates to elicit antibodies against tumor-specific oligosaccharides and design future therapeutic cancer vaccines.

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