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Chemical synthesis of *Haemophilus influenzae* glycopeptide conjugates

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A simple procedure for conjugating synthetic fragments of the capsular polysaccharide of *Haemophilus influenzae* type b, poly-3-β-D-ribose-(1, 1)-D-ribitol-5-phosphate (sPRP) to linear peptides is described. The procedure consists of (i) reacting the amino group of amino-heptyl sPRP with m-maleimidobenzoyl-N-hydroxysuccinimide (MBS) in phosphate buffer, pH 7.5; (ii) selectively coupling the MBS-modified sPRP to the sulfhydryl group of the cysteine residue of peptides containing functional T-helper cell epitope(s). The glycopeptide conjugates were purified by gel filtration chromatography, biochemically characterized, and elicited protective level of anti-PRP antibody responses in rabbits.

***Keywords*: Synthetic glycopeptide conjugates, *Haemophilus influenzae* type b, synthetic vaccines**

***Abbreviations*: PRP, poly-3-β-D-ribose-(1, 1)-D-ribitol-5-phosphate; sPRP, synthetic oligo-3-β-D-ribose-(1, 1)-D-ribitol-5-phosphate; Hib, *Haemophilus influenzae* type b; MBS, m-maleimidobenzoyl-*N*-hydroxysuccinimide; PEG, polyethylene glycol monomethyl ether; CRM 197, a non-toxic diphtheria toxin mutant; TT, tetanus toxoid; DT, diphtheria toxoid; OMP, outer membrane protein; RP-HPLC reverse phase high pressure liquid chromatograph**

Introduction

One of the most significant advances in pediatric healthcare during the last decade has been the introduction of *Haemophilus influenzae* type b (Hib) capsular polysaccharide-protein conjugate vaccines [1]. At present, four different Hib conjugates are commercially available: PRP-D, PRP-T, PRP-CRM 197 and PRP-OMP, which were all shown to be efficacious in infants [2–4]. The success of Hib-polysaccharide conjugates has greatly stimulated interest in the development of other polysaccharide-protein conjugates for the protection of children and adults against a variety of encapsulated bacteria, including *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Escherichia coli*, *Shigella dysenteriae*, and *Salmonella typhi*. However, the mechanism responsible for the increased immunogenicity of PRP is not fully understood. Because these conjugates differ with respect to molecular size, length of the PRP, carrier protein, linkage, and PRP to protein ratio, they elicited different immune responses in infants [4–6]. Approximately 88% of PRP-CRM197 vaccine failures occured after the first and second dose [7] and these vaccinees consistently lacked anti-PRP antibody responses after the first two doses of vaccines. The antibody response to PRP-T in infants was similar to that to PRP-CRM197, with little antibody response until after the second or third dose [8]. PRP-OMP elicited strong antibody response in infants after one dose, but little or no increases in antibody level were seen with the subsequent two doses [9]. A systematic study of the effect of different parameters (carrier protein, conjugation chemistry, size of carbohydrate antigen and the ratio of protein to carbohydrate) on the immunogenicity of the glycoconjugates is of interest for the development of other glycoconjugate vaccines against a variety of capsulated bacteria. Biosynthetic glycoconjugate vaccine candidates using synthetic PRP oligomers chemically linked to a carrier protein (TT and DT) had been investigated and were found to induce a protective level of anti-PRP antibody response in monkeys [10]. An anti-PRP antibody level at 0.15 $\mu g\,ml^{-1}$ induced by immunization, is considered the minimum protective level against Hib disease and at 1 $\mu g\,ml^{-1}$ is considered to be the long-term protective level [11]. Similarly, coupling a T-cell epitope of diphtheria toxin to native PRP oligomers had previously been reported to induce higher antibody level than unconjugated PRP [12]. However, the disadvantage of using TT and DT as carrier proteins has been demonstrated by a recent report that the antibody response to a hapten coupled to a carrier protein can be inhibited when vaccinees have been previously immunized with the carrier protein [13]. Such carrier-induced epitope suppression has also been demonstrated to occur with other hapten-protein conjugates [14]. Therefore, instead of using these conventional heterologous protein carriers, synthetic peptides containing immunodominant T-cell epitopes from

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0282–0080

Hib OMPs as carriers, may enhance the protective ability of the conjugate and ensure autologous T-cell priming.

Such small synthetic PRP-peptide conjugates could offer several advantages compared to the conventional protein-polysaccharide conjugate vaccines. Firstly, these products have well defined chemical structures, can be purified to homogeneity and are chemically stable. Secondly, their structures can be modified systematically to achieve the optimum immune responses. Thirdly, peptides containing autologous T-cell epitopes can be selected from different Hib proteins to enhance cellular immune responses. Finally, synthesis can be performed reliably using either solution- or solid-phase chemistries [15].

This study was undertaken to develop a synthetic model of carbohydrate-peptide conjugate to investigate the factors which regulate the immunogenicity of carbohydrate antigen. We utilized a stable bifunctional cross-linker to couple synthetic oligosaccharides to different T-cell epitopes. The efficiency of antigen presentation by different T-cell epitopes was assessed using rabbit immunogenicity studies. Results from these studies will provide some vital information for rational design of synthetic carbohydrate-based conjugate vaccines.

Materials and methods

Peptide synthesis

Three linear peptides derived from Hib OMPs P1, P2 and P6 (Table 1) were synthesized on an ABI 430A peptide synthesizer using optimized t-Boc chemistry, then cleaved from the resin by hydrofluoric acid. A cysteine residue was added at either the N- or C-terminus for PRP conjugation purposes. The peptides were purified by RP-HPLC on a Vydac C_4 semi-preparative column (10×300 mm) using a 15 to 55% acetonitrile gradient in 0.1% trifluoroacetic acid (TFA) developed over 40 min at a flow rate of $2\,\text{ml}\,\text{min}^{-1}$. All synthetic peptides used in biochemical studies were $>95\%$ pure as judged by analytical HPLC. The amino acid composition of each peptide was determined on a Waters Pico-Tag amino acid analyser system.

PRP oligomer synthesis

Synthetic PRP oligomers (dimer, trimer, pentamer and hexamer) containing an aminoheptyl group were manually assembled by the phosphoramidite chemistry using polyethylene glycol monomethyl ether (average mol wt 5000) as soluble polymeric support as previously described [16]. The efficiency of each elongation cycle was found to be higher than 90% as determined by the amount of dimethoxytrityl anion released using tricholoroacetic acid after each elongation cycle. Synthetic PRP oligomers were released from the support, deprotected by hydrogenation using Pd/C as catalysts and purified with a combination of flash column chromatography and gel permeation chromatography using Sephadex LH-20 gel. Overall yields ranged from 25 to 35%, depending on the length of the oligomer.

Activation of oligosaccharides

m-Maleimidobenzoyl-N-hydroxysuccinimide (Pierce) (20 mg; 63.6 mmol) in tetrahydrofuran (1 ml) was added to a solution of synthetic PRP_3-$(CH2)_7$-NH_2 (4.3 mmol) in 0.1 M phosphate buffer (1 ml), pH 7.5. The reaction mixture was stirred for 30 min at room temperature under argon, then extracted with ether (4×5 ml) to remove excess MBS. The resulting aqueous layer was applied to a Sephadex G-25 column (20×300 mm) equilibrated with 20 mM ammonium bicarbonate buffer, pH 7.2, and eluted with the same buffer. Elution was monitored by absorbance at 280 nm, and the eluted peak was pooled and lyophilized to produce the desired MBS activated PRP. The elution profile of PRP_3-MBS is shown in Fig. 1. The number of maleimide groups incorporated into the oligomers was determined by adding excess 2-mercaptoethanol to the activated PRP_3-MBS and back-titrating the excess using a modified Ellman's method [17].

PRP-Peptide conjugation

A general protocol for synthetic PRP-peptide conjugates is schematically illustrated in Fig. 2. Briefly, individual synthetic peptides ($1\,\text{mg}\,\text{ml}^{-1}$) in degassed PBS buffer, pH 7.5, mixed with PRP_3-MBS (1.6 mg). The reaction mixture was stirred overnight at room temperature under argon atmosphere. Excess *N*-ethyl-maleimide (Aldrich) was added to quench the reaction, and stirring continued for an additional hour. The insoluble precipitate was filtered off, and the filtrate was subjected to gel filtration chromatography using a Sephadex G-25 column (Fig. 1). The PRP_3-peptide conjugate was collected and its purity analysed by RP-HPLC. The molar ratio of synthetic PRP to peptide was determined by using amino acid analysis and the orcinol test [18] for peptide and carbohydrate contents, respectively.

Table 1. Synthetic peptides used for sPRP-peptide conjugation.

Peptide	*OMP*	*Sequence*
CP1-4	P1 (165–193)	CAKAQVERNAGLIADSVKDNQITSALSTQ
CP2-8	P2 (193–219)	CDIVAKIAYGRTNYKYNESDEHKQQLNG
P6-6	P6 (90–114)	VKGYLAGYLAGKGVDAGKLGTTVSYGC

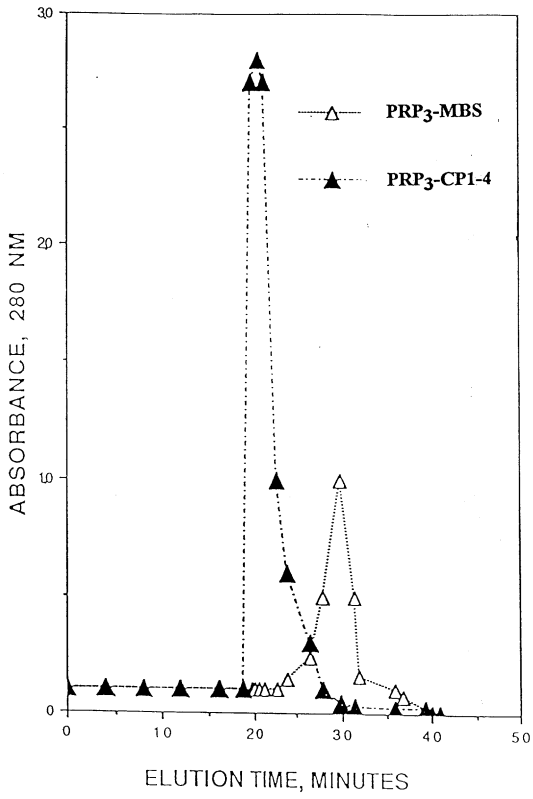

Figure 1. Gel filtration chromatograms of PRP_3-MBS and PRP_3-P1-4. Chromatographic conditions: Sephadex G-25 column (10 × 1000 mm); mobile phase 20 mM NH_4HCO_3, pH 7.2; flow rate was 1.5 ml min^{-1}, detection by absorbance at 280 mm.

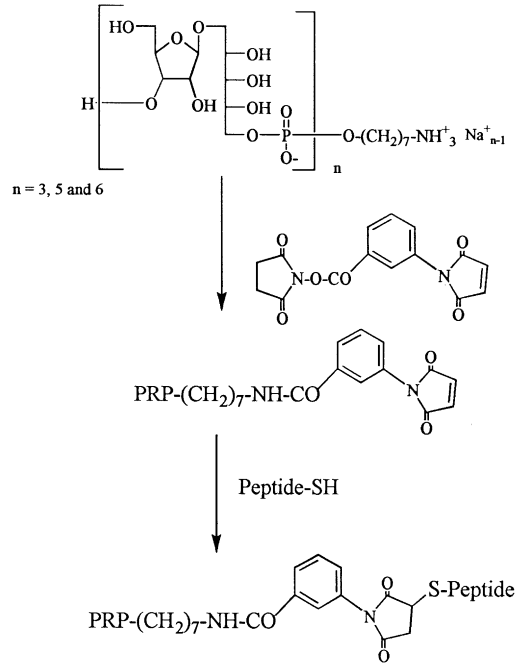

Figure 2. Scheme for producing synthetic PRP glycopeptide conjugates.

Rabbit immunogenicity studies

Rabbits (two to three animals per group) were immunized intramuscularly with 500 µl solutions of individual sPRP-peptide conjugates in PBS (5–10 µg sPRP equivalent) containing $AlPO_4$ (3 mg ml^{-1}) as adjuvant. Animals later received two booster doses (half 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.

Radioimmunassay, RIA

Total anti-Hib-PRP antibodies in serum obtained from rabbit immunized with synthetic PRP-peptide conjugates were assayed by a modified radioantigen-binding assay (RABA/Farr) using PRP antigen intrinsically labeled with tritium [19]. Briefly, excess tritiated antigen was reacted with various dilutions of test sera together with $Na^{36}Cl$ as volume marker (Amersham Canada Ltd) overnight at 4 °C. The latter was added to take account of possible inconsistencies in recovery. Samples were precipitated with 50% saturated ammonium sulfate at 4 °C, centrifuged and counted in a liquid scintillation counter. The amount of radiolabel precipitated was quantified in comparison with the standard (human anti-PRP serum, lot 1983). The lower limit of sensitivity of this assay was 0.06 µg ml^{-1}.

Results and discussion

In this study we prepared a prototype fully synthetic glycopeptide conjugates for analysing the factors which may affect the immunogenicity of carbohydrate antigens and to serve as a model system for the rational design of glycopeptide conjugate vaccines. The synthesis of PRP oligomers (trimer, pentamer, and hexamer) was carried out by the sequential coupling of protected ribose-ribitol phosphoramidite repeating units to the growing chain using polyethylene glycol monomethyl ether as the soluble polymeric support [16]. After assembling PRP subunits of desired chain lengths, the synthesis was terminated by coupling a protected amino-heptyl linker at the 5′-terminus of the ribitol moiety of the synthetic PRP oligomer. This linker was selected to enhance accessibility of the PRP, moiety, avoid harmful effects due to carrier proximity, reduce steric hindrance and provide an amino group which can either be coupled directly to a carrier or modified to generate alternative functional groups, such as sulfhydryl or aryl azide.

Synthetic peptides (Table 1) for this study were derived from Hib outer membrane proteins P1, P2 and P6. Granoff and Munson [20] had reported that antibodies directed against P1, P2 and P6 were protective in the infant rat model of bacteremia. These peptides (Table 1) to be coupled to sPRP oligomers were chosen on the basis of either their potential T-helper cell stimulatory properties or their potential protective ability or their sequences conservation that

would be important for memory T-cell recall. CP1-4 had previously been identified to contain both functional T-cell epitope(s) and a Hib strain-specific protective B-cell epitope which was recognized by a P1-specific MAb 7C8 [21]. A cysteine residue was purposely added at N-terminal end of P1-4 to be away from the B-cell neutralization epitope. P2-8 was identified to contain the immunodominant murine T-cell epitope and its sequence was highly conserved among Hib and nontypeable *H. influenzae* (NTHi) [22]. A cysteine residue was added at the N-terminal end of P2-8, so that the B-cell epitope, sPRP, would be away from the T-cell epitope which was located at the C-terminal end of P2-8 [Chong *et al*., unpublished results]. P6-6 was the most immunogenic peptide among P6 peptides in rabbit immunogenicity studies [23] and its sequence was totally conserved among Hib and NTHi [24]. It is our intention to utilize these synthetic peptides as additional antigens and as carriers for sPRP to develop the first generation of fully synthetic PRP-peptide conjugate vaccines with enhanced protective ability and autologous T-cell priming. All three peptides listed in Table 1 were synthesized using t-Boc solid-phase peptide synthesis chemistry and purified by HPLC. The purity of each peptide was found to be $>95\%$ by RP-HPLC analyses. Their compositions were confirmed by amino acid analyses (data not shown). The primary amino group of the heptyl linker of the synthetic PRP trimer was first activated by reacting with m-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) in aqueous buffer. The PRP_3-MBS was purified by gel permeation chromatography using G-25 column (Fig. 1). MBS was chosen as a bifunctional cross-linker since we and others [25] found that homobifunctional cross-linker such as glutaric dialdehyde suffered from both low loading of heptan to the protein carriers and formation of TT-homodimers and other unwanted products. The maleimide group of PRP-MBS could easily form a stable thioether linkage with the sulfhydryl group of the peptide as shown in Fig. 2. In order to have a better yield of the conjugation, threefold excess of activated PRP_3-MBS was reacted with individual synthetic peptide overnight under argon. The glycopeptide (sPRP-CP1-4) conjugate was then purified by gel-filtration (Fig. 1) and its purity were assessed to be about 95% by RP-HPLC. The molar ratio of peptide to sPRP in each conjugates was determined to be about 1:1, and the overall yield of conjugation ranged from 30 to 50%. To improve the yield, we are currently investigating whether sPRP-MBS will react better with resin-bound peptides.

The immunogenicity of various $sPRP_3$-peptide conjugates ($sPRP_3$-CP1-4, $sPRP_3$-CP2-8, and P6-6-$sPRP_3$) formulated in alum were examined in a rabbit model. Rabbits were used in the present study because currently no animal model reliably predicting the immunogenicity of Hib conjugate vaccines. We and others [11, 26] had observed that both mice and guinea pigs did not produce consistently $>1\ \mu g\,ml^{-1}$ of anti-PRP antibody responses when they were immunized either with a commercially available Hib conjugate vaccine PRP-D (ProHibit, Connaught Laboratories Inc. PA, USA) or experimental PRP-TT conjugate vaccines. Both sPRP and PRP-D were included in the immunogenicity studies as controls. Results of rabbit immunogenicity studies are summarized in Table 2, which indicate that sPRP mixed with alum were not immunogenic and elicited very low level of anti-PRP antibody responses as judged by Farr assay. Its titre was $0.08\ \mu g\,ml^{-1}$, just above the assay background ($0.06\ \mu g\,ml^{-1}$). Whereas, all three synthetic glycopeptide conjugates formulated in alum were immunogenic and elicited anti-PRP antibody responses (Table 2). A rabbit eliciting $>0.15\ \mu g\,ml^{-1}$ of anti-PRP antibodies is defined as a responder since anti-PRP antibody level at $0.15\ \mu g\,ml^{-1}$ induced by immunization is considered to be the minimum protective level against Hib disease [11]. Therefore, all three sPRP-peptide conjugates elicited protective level of anti-PRP antibody responses because their anti-PRP titres ranged from 0.27 to $21.11\ \mu g\,ml^{-1}$. The glycopeptide conjugate $sPRP_3$-CP1-4 was the most immunogenic glycopeptide among the three sPRP-peptide conjugates, and its anti-PRP antibody titre (geometric mean titre = 16.94) was comparable to those obtained with the commercially available Hib vaccine, PRP-D (geometric mean titre = 13.13).

Table 2. Immunogenicity of synthetic PRP_3-peptide conjugates in rabbits.

Conjugate	*Pre-Immune*	*Anti-PRP antibody titre ($\mu g\,ml^{-1}$)*[a]	
		Geometric mean titre ($\mu g\,ml^{-1}$)[a]	*Titre range*[d]
Native PRP	<0.06	0.07	0.06– 0.10
PRP_3	<0.06	0.08	0.06– 0.08
PRP-D[c]	<0.06[d]	13.13	4.94–35.07
PRP_3-CP1-4	<0.06	16.94	10.07–21.66
PRP_3-CP2-8	<0.06	0.35	0.27– 0.46
P6-6-PRP_3	<0.06	0.43	0.41– 0.47

[a]Total anti-PRP antibody responses ($\mu g\,ml^{-1}$) were determined by Farr assay.
[b]Antisera were obtained from rabbits immunized three times with the conjugate vaccines.
[c]PRP-D is defined as poly-3-β-D-ribose-(1,1)-D-ribitol-5-phosphate chemically conjugated with diphtheria toxoid and was obtained from Connaught Laboratories Inc. (Swiftwater, PA).
[d]The lower limit of sensitivity of this assay was $0.06\ \mu g\,ml^{-1}$, and this value was assigned to all measurements $<0.06\ \mu g\,ml^{-1}$.

Conclusion

A synthetic approach was successfully used to produce immunogenic glycopeptide conjugates. These small, well defined, synthetic PRP-peptide conjugates elicited protective level of anti-PRP antibody responses in rabbits. Using

these sPRP-peptide conjugates as models, studies are in progress to examine other factors that could affect the immunogenicity of carbohydrate antigens. Results from the current studies in progress will be vital to the rational design of synthetic carbohydrate-based conjugate vaccines.

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