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The preparation of neoglycoconjugates containing inter-saccharide phosphodiester linkages as potential anti-*Leishmania* vaccines

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The *Leishmania* express complex glycoconjugates containing phosphosaccharide repeat units at all stages of their life-cycle. One of these molecules, lipophosphoglycan (LPG) has been suggested to be a vaccine candidate. To assess the immunological properties of *Leishmania* phosphosaccharides, we have prepared neoglycoproteins and neoglycolipids containing synthetic *Leishmania* phosphosaccharide repeats. The coupling procedure uses the dec-9-enyl spacer of previously synthesised phosphosaccharides for linkage to protein and phospholipid. This alkene moiety is converted by ozonolysis to an aldehyde which is then attached to protein and phospholipid amino groups by reductive amination. The procedure produces neoglycoconjugates in good yield and without compromising the labile phosphodiester linkages within the phosphosaccharide chains.

Keywords: ***Leishmania*****, lipophosphoglycan, neoglycolipid, neoglycoprotein, phosphosaccharide, vaccine**

Abbreviations: **LPG, Lipophosphoglycan; PPG, proteophosphoglycan; BSA, Bovine serum albumin; RNAase A, Ribonuclease A; TetC, Tetanus toxin fragment C; ES-MS, Electrospray-mass spectrometry; MALDI-MS, Matrix assisted laser desorption ionisation-mass spectrometry.**

Introduction

Neoglycoproteins were first constructed for raising antibodies. The method was based on diazo-coupling using *p*-amino-phenyl glycosides [1,2]. Later, more diverse procedures for the conjugation of carbohydrate to proteins, lipids or solid matrices were developed [3,4] and the application of such derivatives expanded to include the study of protein–carbohydrate interactions, the isolation and characterisation of lectins and the targeting of drugs. Carbohydrate vaccine technology is one area that has clearly benefited from these developments. Conjugation of a polysaccharide to a protein may convert it into a T-cell dependent antigen with enhanced immunogenicity. Thus, unlike traditional polysaccharide vaccines, the new generation of neoglycoprotein vaccines against bacterial infections are immunogenic in infants and induce long lasting immunological memory resulting in a boostable response [5]. Recently a fully synthetic hexasaccharide conjuguated to keyhole limpet hemocyanin was also shown to be an effective vaccine against prostate cancer [6].

Lipophosphoglycan (LPG) is a complex glycophospholipid present on the surface of *Leishmania* parasites. These parasites cause various tropical diseases in humans, collectively called the leishmaniases, which can be lethal if left untreated. *Leishmania* are digenetic organisms that alternate between an insect vector and a mammalian host, where it resides in the macrophages. LPG, present on the surface of the insect stage of *Leishmania spp,* is an important virulence factor and is crucial for the survival of the parasite in the insect vector and for the infectivity of the parasite when it enters the mammalian host [7,8 and references therein]. It is made of phosphodi-saccharide repeat units of -6Galβ1-4Manα1-PO_4- that can be substituted by monosaccharides or oligosaccharides in a species-specific manner [9,10]. The same repeat unit is also found in proteophosphoglycans (PPGs) secreted by the intracellular amastigote stages, as well as the promastigote stages, of the parasites [11,12]. Previous studies described the protective effect of purified LPG on mice challenged with

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Leishmania major [13–15]. However, it was later argued that protein that copurified with LPG contributed to this effect [16, 17]. One way to address this is to prepare synthetic vaccines.

We previously reported the synthesis of different oligophosphosaccharides representing various LPG sub-structures, linked to a dec-9-enyl aglycone [18–20]. The hydrophobicity of the dec-9-enyl chain has been exploited for assaying the α-mannosylphosphate transferase involved in LPG biosynthesis [21]. We now report the transformation of the dec-9-enyl spacer into an aldehyde (8-carbonyloctyl) group and its coupling to a protein or lipid carrier by reductive amination. The methods reported here have been designed to preserve the phosphodiester linkages and retain the immunological properties of the phosphosaccharides. The synthetic neoglycoconjugates will be used to assess the immunological properties of LPG and its potential as a vaccine molecule.

Materials and Methods

Materials and general procedures

1,2-Di-O-hexadecyl-*sn*-glycero-3-phosphatidylethanolamine was purchased from Larodan Fine Chemicals, Sweden. An OZ06 ozone generator from Peak Scientific Ltd, Scotland was used for ozonolysis. Tetanus toxin fragment C (TetC) was a generous gift from Professor C. Watts (University of Dundee). Bovine serum albumin (BSA) and Ribonuclease A (RNAase A) from bovine pancreas (EC 3.1.27.5) were from Sigma. Oligosaccharide concentrations were determined by phenol sulphuric assay performed in triplicate [22] or by quantification of methylglycosides trimethylsilyl derivatives by GC-MS [23]. Protein concentrations were determined by the BCA assay (Pierce) according to the manufacturers instructions.

Ozonolysis of dec-9-enyl phosphosaccharides

Synthetic phosphosaccharide (500 µg) were dissolved in methanol as their triethylammonium salts, cooled to −78°C and O_3 was bubbled into the solution via a sintered-glass bubbling tube until a pale blue colour persisted. Oxygen was then bubbled through the solution until it was colourless to eliminate excess O_3. Dimethylsulfide (10 µl) was added and the solution allowed to warm to room temperature and left for 2 h to reduce the ozonide. The products (8-carbonyloctyl phosphosaccharides) were dried twice from methanol and used immediately for coupling.

Reduction of ozonolysed phosphosaccharides

Approximately 50 µg of ozonolysed phosphosaccharide was reduced to the corresponding alcohols with 200 µl 500 mM NaB^2H_4 for 1 h. The reaction was stopped by addition of excess acetic acid and products desalted by passage through 200 µl AG50X12 (H^+) using 3×500 µl H_2O as eluant, followed by drying and re-drying 3 times from methanol. The products were analysed by electrospray mass spectrometry (ES-MS).

Coupling to Ribonuclease A, Bovine serum albumin and Tetanus toxin fragment C

Ribonuclease A (RNAase A), bovine serum albumin (BSA) and tetanus toxin fragment C (TetC) were repurified by gel filtration on a column of Superdex 200 HR 10/30 using 100 mM sodium phosphate buffer pH 7.4 as eluant at a flow rate of 0.25 ml/min. Proteins were detected by UV absorption at 280 nm. Ozonolysed phosphosaccharide (about 500 µg) and $NaCNBH_3$ were dissolved in 100 mM phosphate buffer pH 7.4 and added to the protein solution to obtain a molar ratio of phosphosaccharide : protein amino-group : $NaCNBH_3$ of 1 : 5 : 50 for coupling to RNAase A or TetC and 1 : 10 : 50, 1 : 5 : 50 or 1 : 1 : 50 for coupling to BSA. The mixture was incubated at 37°C for 72 h. The neoglycoproteins obtained were immediately purified by gel filtration on a column of Superdex 200 HR 10/30 using 100 mM ammonium bicarbonate as eluant at a flow rate of 0.25 ml/min. Alternatively the BSA-phosphosaccharide conjugates were directly analysed by anion exchange chromatography as described below. Neoglycoproteins were detected both by UV absorption at 280 nm and by orcinol staining of aliquots spotted onto silica TLC plates.

Coupling to phosphatidylethanolamine

1,2-Di-O-hexadecyl-*sn*-glycero-3-phosphatidylethanolamine (PE) was dissolved at a concentration of 10 mg/ml in $CHCl_3 : CH_3OH$ (1 : 1, v/v). 8-carbonyloctyl phosphosaccharides were dissolved in water and added to the lipid solution to obtain a molar ratio of phosphosaccharide : PE of 1 : 20 in a mixture of $CHCl_3 : CH_3OH : H_2O$ (10 : 10 : 3, v/v/v). The mixture was sonicated and incubated at 60°C for 2 h in a teflon sealed tube. $NaCNBH_3$ was then added to give a 50 fold molar excess over the phosphosaccharides and the solution was left at 60°C for a further 16 h. Samples were dried under a stream of N_2, resuspended in water and the excess PE extracted 3 times with butanol saturated with water. Ammonium acetate (1 M) was added to the water phase to obtain a final concentration of 100 mM and this was applied onto a 2 ml column of octyl-Sepharose previously washed with 50% propan-1-ol and equilibrated in 5% propan-1-ol in 100 mM ammonium acetate. After 30 min, the column was washed with 10 ml 5% propan-1-ol in 100 mM ammonium acetate and the products eluted with 10 ml 50% propan-1-ol in 100 mM ammonium acetate. Propan-1-ol was evaporated and the residue freeze-dried. The neoglycolipids were redissolved in $CHCl_3$:CH_3OH (4 : 1, v/v) and applied to a 1.5 ml Kieselgel 60 column that had been washed with $CHCl_3$:CH_3OH:0.5% triethanolamine (10 : 10 : 3, v/v/v) and equilibrated in $CHCl_3 : CH_3OH$: (4 : 1, v/v). After washing with 10 ml $CHCl_3 : CH_3OH$ (4 : 1, v/v), the products were eluted from the column with 15 ml $CHCl_3$:CH_3OH:0.5% triethanolamine (10 : 10 : 3, v/v/v). Fractions of 1 ml were analysed by HPTLC.

Analysis of the neoglycoproteins by anion exchange chromatography

Neoglycoproteins were analysed using a BioCad Sprint HPLC system. Samples were injected onto an anion exchange column (Poros 20-PI, 4.6 mm × 100 mm) directly after conjugation and eluted with a NaCl gradient (0–1 M over 15 column volumes) in Tris/HCl 50 mM pH 7.5. The column was eluted 10 column volumes of 1 M NaCl prior to re-equilibration. A flow rate of 5 ml/min was used throughout and proteins were detected with a UV detector at 280 nm.

Analysis of the neoglycoproteins, by SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed using 15% gels for native and coupled RNAase A and 8% gel for native and coupled TetC using the discontinous buffer system of Laemmli [24]. Gels were stained with Coomassie blue to detect protein bands.

Analysis of the neoglycolipids by HPTLC

Samples were applied in 5 µl 40% propan-1-ol to aluminium backed silica gel 60 HPTLC plates, which were developed with $CHCl_3 : CH_3OH : 0.25\%$ KCl (10 : 10 : 3 v/v/v). PE was detected by staining with I_2 vapour. Glycans were detected by orcinol staining.

Electrospray ionisation mass spectrometry

Mass spectra were recorded on a Micromass Quattro triple-quadrupole mass spectrometer. Phosphosaccharides, ozonolysed-reduced phosphosaccharides and neoglycolipids were dissolved in 50% methanol at a concentration of 10 µM. Glycoproteins were dissolved at the same concentration using 50% acetonitrile containing 1 mM ammonium acetate. Samples were introduced into the electrospray source at 10 µl/min using a Michrom solvent delivery module. Protein and glycoprotein spectra were recorded in positive ion mode. Phosphosaccharide, lipid and neoglycolipid spectra were recorded in negative ion mode. For daughter-ion tandem mass spectrometry, the pseudomolecular ions were accelerated into a collision cell containing argon (2.3×10^{-3} millibar) through a potential difference of between 70 and 110 V. All spectra were processed using MassLynx software.

Results and Discussion

Selection of synthetic phosphosaccharides and ozonolysis of the functional group

Synthetic phosphosaccharides **1–3**, based on the structure of *Leishmania* LPG, and dec-9-enyl α-D-mannoside **4** (Figure 1) were prepared as described by Nikolaev and co-workers [18–20]. Compound **1** contains unsubstituted phosphodisaccharide repeat units as found in all *Leishmania* species and is representative of the completely unsubstituted LPG from *L. donovani* that causes lethal visceral leishmaniasis. The glucose substituent present in compound **2** is characteristic of LPG from *L. mexicana* that causes mucocutaneous leishmaniasis. Compound **3** contains the dimannosyl cap typical of LPG and PPG molecules from all species. Finally, compound **4** is designed as a control for the immunological studies. Since polysaccharide epitopes are usually encoded within the structure of 1–3 repeat units and since recent work indicated that LPG phosphosaccharides with 5 repeat units or more can inhibit the production of IL-12, and therefore lead towards an unwanted Th-2 response [25], we chose a length of 3 repeat units (as in **1–3**) in this study.

All of the compounds end in a dec-9-enyl group, used as an anomeric or phosphodiester blocking group during the synthesis, that can be converted to an aldehyde by ozonolysis of the double bond for further coupling to a carrier (Figure 2). Negative ion electrospray mass spectra of compound **3** before and after ozonolysis and NaB^2H_4-reduction of the aldehyde are presented (Figure 3). Compound **3** has a monoisotopic

Compound

1 Galβ1-4Manα-PO_4H-6Galβ1-4Manα-PO_4H-6Galβ1-4Manα-O-$(CH_2)_8CH{=}CH_2$

2 Galβ1-4Manα-PO_4H-6(Glcβ1-3)Galβ1-4Manα-PO_4H-6Galβ1-4Manα-PO_4H-$(CH_2)_8CH{=}CH_2$

3 Manα1-2Manα-PO_4H-6Galβ1-4Manα-PO_4H-6Galβ1-4Manα-PO_4H-6Galβ1-4Manα-O-$(CH_2)_8CH{=}CH_2$

4 Manα-O-$(CH_2)_8CH{=}CH_2$

Figure 1. Structures of phosphosaccharides used in this study.

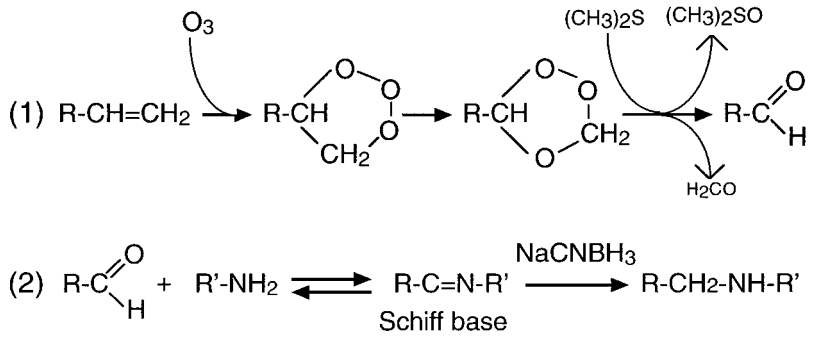

Figure 2. Reaction scheme of ozonolysis (1) and reductive amination (2).

molecular weight of 1692.5 Da and gives rise to ions at m/z 563.2, corresponding to the triply charged $[M\text{-}3H]^{3-}$ ion and m/z 845.2, corresponding to the doubly charged $[M\text{-}2H]^{2-}$ ion (Figure 3A). After ozonolysis and deutero-reduction the monoisotopic molecular mass of the product should increase by 5 Da, and this can be seen from the mass shift of 1.6 and 2.5 Da of the triply and doubly charged ions to m/z 564.8 and m/z 847.7, respectively (Figure 3B).

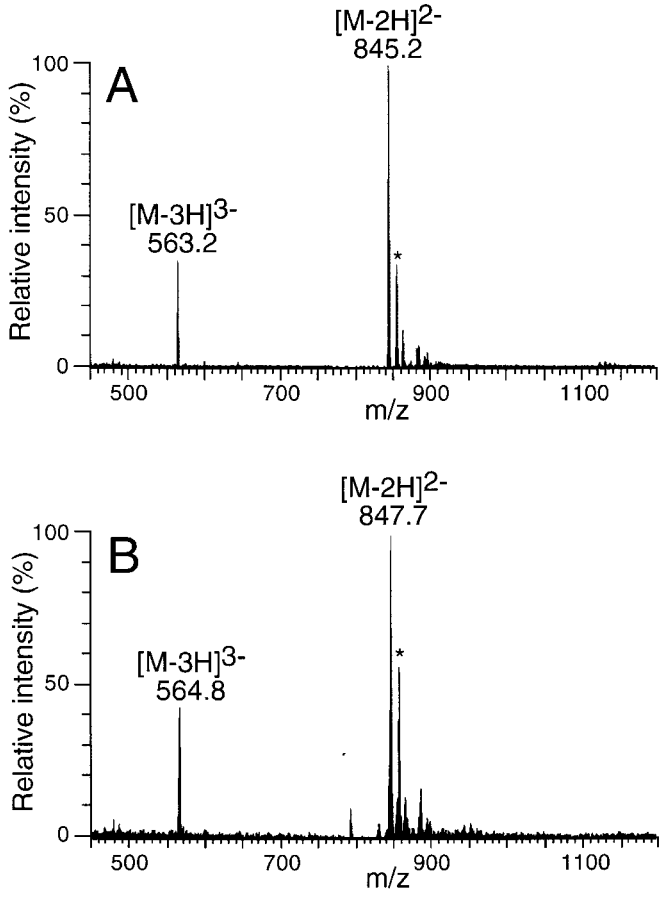

Figure 3. Electrospray mass spectra of compound **3** before (A) and after ozonolysis and reduction (B). Spectra were recorded in negative ion mode. Doubly and triply ($[M\text{-}2H]^{2-}$ and $[M\text{-}3H]^{3-}$) charged ions are observed in each spectra. * indicates the $[M\text{-}3H + Na]^{2-}$ doubly charged ion.

The conversion of **3** into a single product illustrates the efficiency of the ozonolysis technique. HPTLC analysis of the same material after ozonolysis showed 2 bands by orcinol staining (data not shown). However, after reduction with NaB^2H_4, the lower band (representing less than 5% of the products) disappeared and a single band migrating slightly slower than the aldehyde was observed. The minor band observed prior to reduction probably corresponds to the unreduced ozonide intermediate. No starting material was observed by mass spectrometry or HPTLC.

In summary, the conversion of the dec-9-enyl phosphosaccharide compounds to their corresponding 8-carbonyloctyl derivatives is complete and proceeds without destruction of the phosphodiester linkages. The aldhehyde group thus obtained can be coupled to various carriers such as protein, peptide, lipid or synthetic polymers.

Coupling conditions

Reductive amination is frequently used for the preparation of glycoconjugates to be used as vaccines [5,6]. It is usually performed by condensation of the carbonyl group of the carbohydrate with the amino group of the carrier, usually the ε-amino groups of lysine residues. The schiff-base obtained is selectively reduced with sodium cyanoborohydride to form a stable C-N linkage. The method described here uses the aldehyde group generated by ozonolysis of a dec-9-enyl group for the formation of phosphosaccharide-protein and phosphosaccharide-lipid conjugates.

RNAase A was chosen as a model protein to test the coupling conditions because of its small size (13690 Da) which makes it easy to analyse by ES-MS (Figure 4A). In this experiment, coupling was carried out in a phosphate buffer at pH 7.4 using a molar ratio of phosphosaccharide to protein of 2 : 1 (equivalent to a phosphosaccharide : protein amino-group ratio of 1 : 5). Figure 4B shows that under these conditions some RNAase A is still uncoupled, while the majority is coupled to a single phosphosaccharide chain (mass 15368 Da) and some is coupled to 2 phosphosaccharides (mass 17047 Da). These 3 main products are also seen by SDS-PAGE analysis of the products (Figure 5, lane 2). Importantly, no intra-phosphosaccharide chain degradation products was

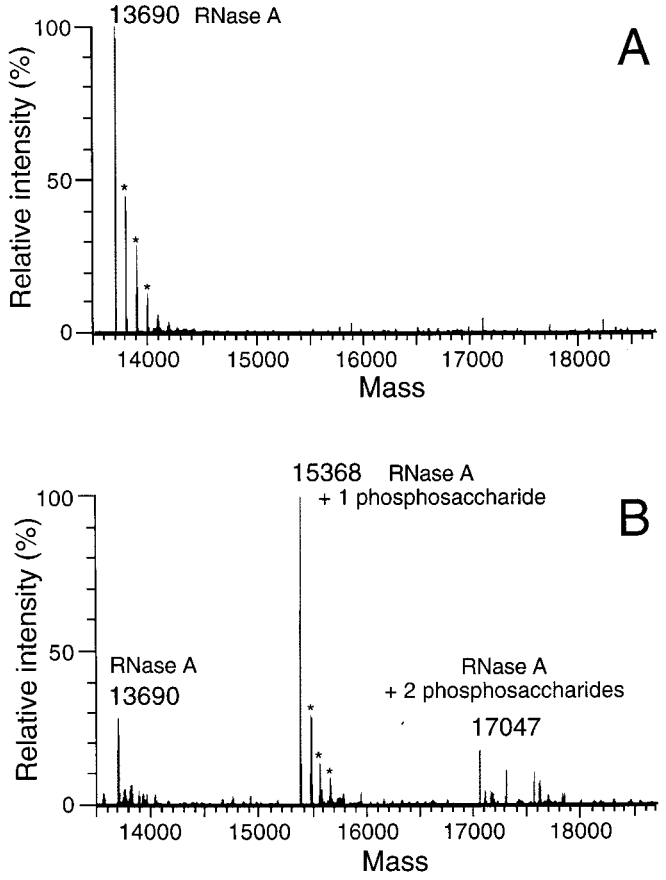

Figure 4. Transformed electrospray mass spectra of RNAase A before (A) and after coupling with compound **3** (B). * indicates aducts of the starting material, still observed after coupling.

apparent by either analytical technique. The percentage of each population can be estimated by integration of the peak areas (Figure 4B). In this case, the data indicated that 16% of the RNAase was unsubstituted, 73% was mono-substituted and 11% was di-substituted with phosphosaccharide chains. The average of 1.1 phosphosaccharide chains per RNAase A molecule (i.e. 0.8 phosphosaccharide chains per 10 kDa of protein) was supported by measurement of the carbohydrate and protein concentrations before and after coupling and purification. The latter analyses indicated a final yield of 55%, in terms of the proportion of input phosphosaccharide recovered as neoglycoprotein.

The influence of the pH and the time of the reductive amination on the yield of the reaction was studied. At pH 8.0 coupling efficiency improves slightly. However, some hydrolysis of the phosphodiester bonds could be observed by ES-MS analysis (data not shown). The same observations were made when the time of the reaction was increased from 72 h to 120 h (data not shown).

Finally, the influence of the carbohydrate : protein ratio was also studied using BSA as a carrier and the ozonolysed phosphodisaccharide Galβ1-4Manα-PO_4-dec-9-enyl. The products were analysed by anion exchange chromatography. Note: attempts to analyse phosphosaccharides coupled proteins by

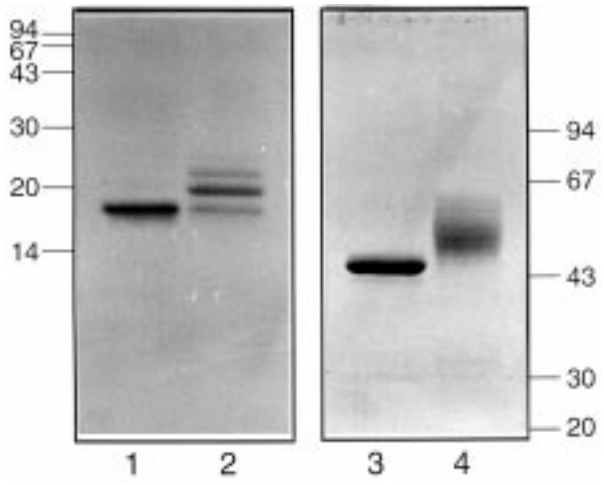

Figure 5. SDS-PAGE analysis of RNAase A and TetC before and after coupling with compound **3**. RNAase A (lane 1), coupled RNAaseA (lane 2), TetC (lane 3) and coupled TetC (lane 4). RNAase A was analysed with a 15% SDS-polyacrylamide gel while an 8% gel was used for TetC. The positions of molecular weight markers (in kDa) are indicated.

MALDI-MS were unsuccessful because the acidic matrix causes decomposition of the phosphodiester bonds. Increased coupling of phosphosaccharide to BSA increases the negative charge of the BSA neoglycoprotein, resulting in increased retention on the anion exchange column. When the carbohydrate: protein amino-group ratio was changed from 1 : 10 to 1 : 5 and 1 : 1, the average number of phosphosaccharide chains coupled to BSA increased, as expected (Figure 6). However, when a ratio of 1 : 1 is used the yield of the reaction (in terms of the proportion of input phosphosaccharide recovered as neoglycoprotein) decreased from 66% (83% when accounting for losses during purification) to 18%. This can be partly explained by the fact that not all lysine residues are accessible, such that at a ratio of 1 : 1 the phosphosaccharide aldehydes are in excess over *available* amino groups.

The average number of phosphosaccharides coupled per molecule of BSA achieved here was about 8 (i.e. about 1.2 phosphosaccharide chains per 10 kDa of protein), which is around 3-fold lower than that reported for oligosaccharides using similar reductive-amination techniques [26,27]. However, in the latter cases, a large molar excess of oligosaccharide over BSA amino groups was used (approximatley 200 : 1) making the yield, in terms of the proportion of input oligosaccharide recovered as neoglycoprotein, rather low (<5%). Since the synthetic phosphosaccharide precursors are rather precious, and since a relatively low phosphosaccharide : protein ratio may be desirable from an antigen-presentation point of view, we prefer the conditions used in this paper for our particular needs. Ozonolysis and reductive amination have also been used for the synthesis of sialyl Tn-KLH conjugate [28]. These authors obtained a low conjugation efficiency but could improve it by introducing a 4-(4-N-maleimidoethyl) cyclohexane-1-carboxyl hydrazide linker arm. In our method, the high reactivity of the aldehyde group

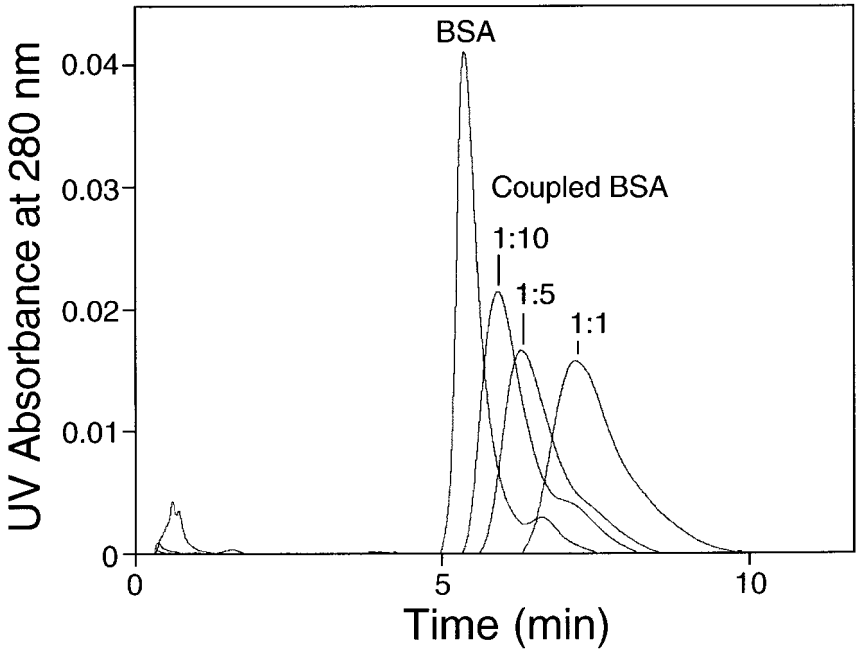

Figure 6. Elution profiles of native and coupled BSA on an anion exchange column. The phosphosaccharide: protein amino group ratio used for the coupling reaction was varied from 1:10 to 1:5 and 1:1, as described in materials and methods. The increased negative charge and retention time on the column corresponds to increased phosphosaccharide coupling.

introduced at the end of the dec-9-enyl spacer gives satisfactory yield without further manipulations.

Preparation of TetC neoglycoproteins for immunological studies

Different factors affecting the immunogenicity of a neoglycoconjugate have to be considered when designing a vaccine. These are the nature of carbohydrate, the nature of the carrier, the linkage between them and the load of carbohydrate per carrier protein. Tetanus toxin fragment C (TetC) has been engineered so that it does not retain any toxicity but retains immunogenicity [29] and is, therefore, safe for vaccine production. Already tested on the vaccine market [30,31], tetanus toxoid has also the advantage of being presented to the immune system by MHC-class II molecules and, therefore, of inducing a Th-1 response that is necessary for the killing of *Leishmania* parasites, reviewed in [32,33]. The various phosphosaccharides chosen for this study have been coupled to this protein using a molar ratio phosphosaccharide : protein amino group of 1 : 5 as described in materials and methods. Analysis by SDS-PAGE of the products obtained by coupling compound **3** to TetC is shown as an example (Figure 5, lane 4). Thus, we obtained a range of neoglycoproteins carrying, on average, 4.3 phosphosaccharides per molecule, as estimated from the protein and carbohydrate composition. The yield of the reaction, with respect to, phosphosaccharide recovery, was typically about 70%. Thus, the conjugation of phosphosaccharide to TetC is efficient and produces neoglycoproteins with approximately 1 phosphosaccharide per 10 kDa of protein, a desirable density for antigen presentation.

Preparation of neoglycolipids for immunological studies

Phosphosaccharides were also coupled to 1,2-di-O-hexadecyl-*sn*-glycero-3-phosphatidylethanolamine (PE). The purity of the neoglycolipids obtained was checked both by HPTLC (data not shown) and ES-MS after purification. The mass spectrum of the neoglycolipid generated by coupling of phosphosaccharide **3** to PE is shown in (Figure 7A). Three ions at m/z 1170, 780 and 585, carrying 2, 3 and 4 negative charges, respectively, were observed. The fragments obtained by collision of the triply charged ion at m/z 780 confirmed the structure of the conjugate (Figure 7B). No residual PE or phosphosaccharide was observed by mass spectrometry or HPTLC. Anhydrous conditions usually give a high coupling efficiency [34]. However, the large and charged phosphosaccharides used in this study are insoluble in $CHCl_3 : CH_3OH$ 1 : 1 v/v. Thus, the necessary addition of water in the solvent system, and the need for chromatographic purification of the product, resulted in only a moderate yield of neoglycolipid (36% of the input phosphosaccharides).

For the immunological studies, the neoglycolipids will be incorporated into liposomes that act as a carrier and as an adjuvant. This requirement influenced the choice of the lipid. The dihexadecylglycerol component is both chemically stable and ideal for stable incorporation into liposomes.

Neoglycolipids may have some advantages as vaccine candidates. The work of Beckman et al. [35] showed that *Mycobacterium leprae* lipids and glycolipids are recognised and presented to the immune system by CD1 molecules in a MHC-independent fashion. Since this discovery, targeting the CD1 pathway with neoglycolipids has been seen as an

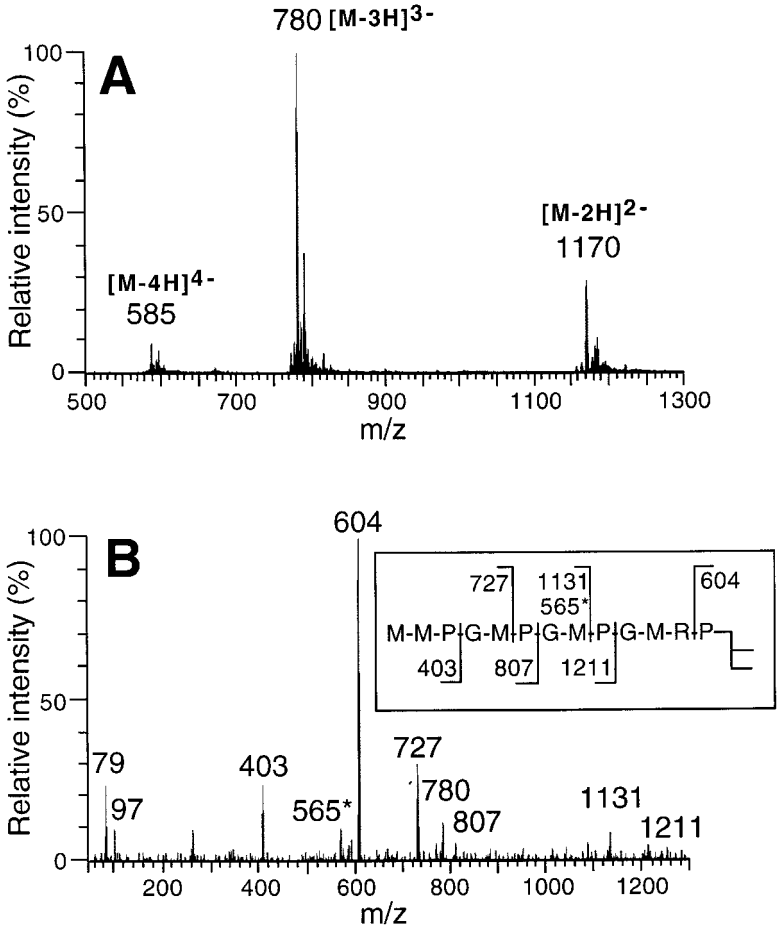

Figure 7. Electrospray mass spectra of compound **3** coupled to dihexadecyl phosphatidylethanolamine (A) and daughter ion spectrum following collision of the triply charged ion at m/z 780 (B). Spectra were recorded in negative mode. Daughter ions at *m/z* 403, 807 and 1211 are thought to involve the formation of a cyclic phosphate [36]. Daughter ions at *m/z* 727, 565 and 1131 are B-type cleavage ions. The ion at *m/z* 604 is essentially formed by direct loss of phosphatidic acid (equivalent to a Z-type cleavage). The daughter ions are principally singly charged, as indicated, except for the ion marked with * at *m/z* 565 which is doubly-charged.

interesting alternative to more traditional neoglycoprotein vaccines since, unlike MHC molecules, CD1 proteins are not polymorphic, reducing the effects of host immunogenetics on the immune response to the neoglycolipid vaccine.

Conclusion

The methods described in this paper allow the preparation of phosphosaccharide-containing neoglycoproteins and neoglycolipids in reasonable yield and high-purity and without degradation of the labile inter-saccharide phosphodiester linkages. These neoglycoconjugates will be useful tools to evaluate whether LPG, or synthetic fragments thereof, are real candidates for an anti-*Leishmania* vaccine, as suggested by earlier studies [13–15].

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