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Investigation towards bivalent chemically defined glycoconjugate immunogens prepared from acid-detoxified lipopolysaccharide of *Vibrio cholerae* O1, serotype Inaba

Cyrille Grandjean · Alain Boutonnier · Bruno Dassy · Jean-Michel Fournier · Laurence A. Mulard

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Abstract A free amino group present on the acid-detoxified lipopolysaccharide (pmLPS) of *V. cholerae* O1 serotype Inaba was investigated for site-specific conjugation. Chemoselective pmLPS biotinylation afforded the corresponding mono-functionalized derivative, which retained antigenicity. Thus, pmLPS was bound to carrier proteins using thioether conjugation chemistry. Induction of an anti-LPS antibody (Ab) response in BALB/c mice was observed for all conjugates. Interestingly, the sera had vibriocidal activity against both Ogawa and Inaba strains opening the way to a possible bivalent vaccine. However, the level of this Ab response was strongly affected by both the nature of the linker and of the carrier. Furthermore, no switch from IgM to IgG, *i.e.* from a T cell-independent to a T cell-dependent immune response was detected, a result tentatively explained by the possible presence of free polysaccharide in the formulation. Taken together, these results encourage further investigation towards the development of potent pmLPS-based neoglycoconjugate immunogens, fully aware of the challenge faced in the development of a cholera vaccine that will provide efficient serogroup coverage.

Keywords Vibrio cholerae O1 · Lipopolysaccharide · Neoglycoconjugate · Cholera vaccine

Abbreviations

Ab	antibody
LPS	lipopolysaccharide
pmLPS	polysaccharide moiety of LPS
CP	capsular polysaccharide
O-SP	O-specific polysaccharide
TT	tetanus toxoid
BSA	bovine serum albumin
MALDI-TOF MS	matrix-assisted laser desorption-ionization time-of-flight mass spectroscopy
SELDI-TOF MS	surface enhanced laser desorption-ionization time-of-flight mass spectroscopy
NHS	*N*-hydroxysuccinimide
EDAC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
NHS-SATA	*N*-succinylimidyl-*S*-acetylthio-acetate
Sulfo-EMCS	(Nε-maleimidocaproyloxy) sulfosuccinimide ester

C. Grandjean · L. A. Mulard
Unité de Chimie Organique, URA CNRS 2128, Institut Pasteur, 25–28, Rue du Docteur Roux, 75724 Paris Cedex 15, France

A. Boutonnier · B. Dassy · J.-M. Fournier
Unité du Choléra et des Vibrions,
Centre National de Référence des Vibrions et du Choléra,
Institut Pasteur, 25–28, Rue du Docteur Roux,
75724 Paris Cedex 15, France

Present address:
C. Grandjean (✉)
Laboratoire des Glucides, UMR 6219 CNRS,
Université de Picardie Jules Verne,
33 Rue Saint Leu, 80039 Amiens, France
e-mail: cyrille.grandjean@u-picardie.fr

Present address:
L. A. Mulard (✉)
Unité de Chimie des Biomolécules URA CNRS 2128,
Institut Pasteur, 25–28, Rue du Docteur Roux,
75724 Paris Cedex 15 France
e-mail: laurence.mulard@pasteur.fr

Introduction

Cholera remains a major public health concern in the developing world. It will remain so unless an adequate level of hygiene is reached in endemic areas. In the meantime, the use of an effective vaccine is highly recommended by the WHO in its program to control enteric diseases [1]. Two inactivated and one live attenuated oral vaccines are currently available [2–4]. However, the high level of protection initially induced by these vaccines declines rapidly, especially in children. Their use is therefore mostly limited to emergency situations, and underlines the need for the development of alternatives [5, 6]. Glycoconjugate vaccines composed of a protein carrier bound to surface carbohydrates of *Vibrio cholerae* serogroups O1 (serotypes Inaba and Ogawa) and O139, the gram-negative bacteria responsible for cholera epidemics [7] might offer such an option due to

- the unique property of polysaccharide–protein conjugate vaccines to induce long-term protection against many bacterial diseases in adults as well as in infants [8, 9],
- the central role played by *V. cholerae* surface polysaccharides in protective immunity against cholera [10].

Indeed, detection of high level of serum vibriocidal anti-polysaccharide Abs strongly correlates with an efficient protection of *V. cholerae* exposed or vaccinated populations [11]. As evidenced by the first *V. cholerae* O139 outbreak which largely affected adults in areas of *V. cholerae* O1 endemicity [12, 13], on field cross-protection between serogroups O1 and O139 is lacking. This phenomenon may be correlated with major differences in the composition and structure of their surface polysaccharides.

A series of *V. cholerae* O139 polysaccharide–protein conjugates have been designed based on either detoxified lipopolysaccharide (LPS) or the capsular polysaccharide (CP), whose repeating unit is identical to that of the *O*-specific polysaccharide part (*O*-SP) of the LPS [14–17]. Immunogenicity analysis in murine models encouraged further investigation of some of these glycoconjugates in clinical trials. In order to access defined *V. cholerae* O1 conjugates, the use of synthetic mono- to hexasaccharides mimicking the non-reducing end of the Ogawa or Inaba *O*-SPs was investigated in detail. Semi-synthetic Ogawa glycoconjugates and in particular hexasaccharide-conjugates induce anti-Ogawa protective Abs in murine models [18, 19]. In contrast, sera induced in mice immunized with the Inaba conjugates, including those incorporating a hexasaccharide hapten, recognize the LPS from both serotypes, but are not protective [20]. Other attempts to develop a *V. cholerae* O1 vaccine have relied on the use of alkali or hydrazine-detoxified LPS [21–25] and the various preparations proved to be immunogenic in mice or rabbits. Nevertheless, Inaba conjugates failed to elicit a sufficient level of protection in humans during a phase 1 clinical evaluation [26]. Thus, the development of glycoconjugate vaccines with broad *V. cholerae* coverage has been hampered greatly by the absence of an efficient formulation against the Inaba serotype.

Herein, the preparation of Inaba LPS-based conjugates is reinvestigated. Indeed, this strategy has led to the most promising Inaba glycoimmunogens to date, but has remained largely undeveloped. Clearly, the LPS detoxification mode [23], the nature of the linker [27], the attachment site, the coupling chemistry [28], and the polysaccharide-to-protein ratio [29] are, amongst the parameters of key impact on immunogenicity. Nevertheless, these have been barely studied in the case of *V. cholerae*. We reasoned that a better understanding and control of these parameters would open the way to improved immunogens. For example, the conjugate used for clinical evaluation involved cyanogen bromide-mediated pre-activation of detoxified LPS [26]. Use of such a potent, non specific nucleophile activator might be expected to lead to ill-defined intermediates whose "protective" antigenic determinant(s), *i.e.* determinants recognized by vibriocidal Abs, could be denatured.

Initial efforts reported herein are intended to take advantage of the presence of a unique D-glucosamine on the LPS core [30], as selective derivatization of the free amino group on this residue would be expected to allow single site conjugation. The amino group reactivity and whether its modification affects the detoxified LPS antigenicity are also investigated. In addition, we report on the immunogenicity in mice of a selection of the newly synthesized detoxified LPS-protein conjugates.

Material and methods

General

TT (MW 157 kDa) (Pasteur Mérieux Connaught) was dialyzed against 0.05 M aqueous NaCl to an actual concentration of 20.6 mg mL^{-1}. PBS, pH 7.3 is a mixture of 0.05 M potassium phosphate buffer and 0.15 M NaCl. Mouse anti-LPS monoclonal Abs (mAbs), IgG I-24-2, F-22-30, and S-20-3 were prepared at the Institut Pasteur by immunizing mice with a lysate from *V. cholerae* O1 serotype Inaba [31–33]. Analytical and semi-preparative RP-HPLC separations were performed using a Perkin-Elmer Series 200 pump and a 785A UV/VIS detector system on a Kromasil C18 (A. I. T. chromato, France) (100 Å, 4.6×250 mm), column at a flow rate of 1 mL min^{-1} (monitoring and analysis), or on a Nucleosil 100-5 C18 (Macherey-Nagel) (100 Å, 5 μm, 10×250 mm) column, at a flow rate of 3 mL min^{-1} (semi-preparative), with detection at 215 nm. Gradient: 0% B for 5 min, 0–40% B

over 40 min or 0–20% B over 60 min, respectively. Solvent system A: 0.05% aqueous TFA; solvent system B: 0.05% TFA in 60% CH_3CN—40% water. Analytical gel filtration was performed on a Superdex 75 column (Amersham Biosciences), at a flow rate of 0.5 mL min^{-1} using PBS 0.1 M, pH 7.3 as eluent.

MALDI-TOF-MS spectra were recorded on a Voyager mass spectrometer. Samples were dissolved in MeOH/H_2O 50:50 and adsorbed on a 2,5-dihydroxybenzoic acid matrix. SELDI-TOF-MS analysis was performed using the ProteinChip® System (Ciphergen Biosystems) calibrated with conalbumin (77,490 Da) and IgG (147,300 Da). NP20 chips and EAM 1 matrices were used. Dialyses were performed with Slide-A-Lyzer® Dialysis Cassettes (Pierce) and centrifugal concentrations were performed on Vivaspin 15R concentrators (Vivascience).

NMR spectra were recorded using a Bruker Advance 400 spectrometer (at 400 MHz for ^1H and 100 MHz for ^{13}C). Chemical shifts of ^1H and ^{13}C spectra were given in ppm. Assignment of signals was confirmed by ^1H homonuclear correlation spectroscopy (COSY), and ^1H–^{13}C heteronuclear single quantum correlation (HSQC), spectroscopy experiments.

Preparation of acid-detoxified LPS of *V. cholerae* O1 serotype Inaba (pmLPS O1 Inaba) **1**

The *V. cholerae* serogroup O1 Inaba strain (CNRVC 950707) was isolated in 1995 from a patient in Mali. Cells were grown in Tryptic soy agar (Difco) in Roux flasks at 37°C for 18 h. Cultures were suspended in distilled water and LPS was obtained by hot phenol/water extraction [34], followed by enzymatic treatment (DNase, RNase and protease), and ultracentrifugation (100,000 g for 3 h). The pellet containing LPS was dialyzed against distilled water and freeze-dried. The LPS preparation contained 1% (w/v) protein and less than 0.2% (w/v) nucleic acid, as determined by electrophoresis [35]. Inaba LPS (10 mg mL^{-1} in 1% (*v*/*v*) aqueous acetic acid was heated at 100°C for 60 min. Precipitated lipid A was removed by low-speed centrifugation (3,000 rpm for 15 min) at 4°C. The supernatant was mixed with an equal volume of chloroform-ethanol (2:1). The reaction mixture was shaken vigorously for 1 min and centrifuged at 10,000 rpm for 60 min at 4°C. The aqueous phase was dialyzed against distilled water to remove ethanol, and then freeze-dried. The residue was dissolved in water and purified by RP-HPLC. The collected fractions were diluted in water and freeze-dried to give **1** in 20–30% yield, depending upon the preparations. Negative MALDI-TOF-MS: *m*/*z* 7057, 6810, 6562, 6315, 6067, 5820, 5573, 5326, 5079, 4831, 4585, 4337, 4090, 3843, 3596, 3348 $(M–H)^-$, (22-mer to 7-mer-pmLPS is composed of a homopolymer whose repeat unit is a 4, 6-dideoxy-4-(3-deoxy-L-*glycero*-tetronamido)-α-D-mannopyranosyl residue linked onto a core polysaccharide). (A small amount of pmLPS with apparent lack of one tetronic acid residue was also detected).

pmLPS O1 Inaba biotinylation

To a solution of 1 (4.77 mg, 0.81 μmol) in 0.1 M potassium phosphate buffer, pH 7.3 (500 μL), was added the biotinylation reagent, sulfo-NHS-lc-lc-biotin (Pierce) (3× 1.53 mg, 3×3 equivalent, dissolved in 50 μL of CH_3CN) in three portions every 2 h. The pH of the reaction mixture was controlled (indicator paper) and maintained at 7–7.5 by addition of 0.5 M aqueous NaOH. Following an additional reaction period of 2 h, the crude reaction mixture was purified by RP-HPLC. The collected fractions were diluted with water and freeze-dried to give mono-biotinylated pmLPS O1 Inaba **2** (1.60 mg, 31% yield), together with recovered starting material 1 (1.13 mg, 23%) as white powders. Negative MALDI-TOF-MS: *m*/*z* 7754, 7508, 7261, 7014, 6767, 6519, 6273, 6025, 5778, 5531, 5283, 5036, 4788, 4542, 4295, 4047, 3800, 3553 $(M–H)^-$, (24-mer to 7-mer of 4,6-dideoxy-4-(3-deoxy-L-*glycero*-tetronamido)-α-D-mannopyranosyl unit) (A small amount of mono-biotinylated-pmLPS with lack of one tetronic acid residue is also detected).

Conjugate preparation

Synthesis of 6-(acetylthio)hexanoic acid succinimidyl ester

To a stirred solution of 6-(acetylthio)hexanoic acid (500 mg, 2.63 mmol, 1 equiv) and NHS (302 mg, 2.63 mmol, 1 equiv) in CH_2Cl_2 (3 mL) was added EDAC (504 mg, 2.63 mmol, 1 equiv), and the mixture was allowed to stir at rt overnight. The crude reaction mixture was diluted with CH_2Cl_2, and this solution was washed with 1 M aq HCl and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to furnish a crude residue, which was purified by rapid chromatography on silica gel (eluent: CH_2Cl_2/EtOAc 96:4). 6-(Acetylthio)hexanoic acid succinimidyl ester (642 mg, 85%) was obtained as a pale yellow oil. R_f= 0.75 (CH_2Cl_2/EtOAc 96:4): ^1H NMR (400 MHz, $CDCl_3$) δ 2.86 (t, 2 H, *J*=7.1 Hz, CH_2–6), 2.78 (br s, 4 H, 2×CH_2 succinimidyl), 2.60 (t, 2 H, *J*=7.4 Hz, CH_2–2), 2.31 (s, 3 H, CH_3), 1.75 (q, 2 H, *J*=7.4 Hz, CH_2–3), 1.63–1.56 (m, 2 H, CH_2–5), 1.52–1.43 (m, 2 H, CH_2–4); ^{13}C NMR (100 MHz, $CDCl_3$) δ 196.2 (COS), 169.6 (2×CO succinimidyl), 168.8 (C-1), 31.1 (C-2), 31.0 (CH_3), 29.4, 29.1, 28.2 (C-6), 25.9 (2×CH_2 succinimidyl), 24.5; CI-MS: *m*/*z* 305 $[M + NH_4]^+$, 288 $[M + H]^+$; Anal. Calcd for $C_{12}H_{17}NO_5S$: C, 50.16; H, 5.96; N, 4.87. Found: C, 50.14; H, 5.96; N, 4.89.

*General procedure for the derivatization of **1** with succinimidyl modifying linkers*

To a solution of 1 (9–23 mg, 1.5–3.8 μmol), 10 mg mL^{-1} in 0.1 M potassium phosphate buffer, pH 7.3, was added sulfo-EMCS or 6-(acetylthio)hexanoic acid succinimidyl ester, respectively, in three portions every 1.5 h, [3×5 equiv dissolved in CH_3CN/H_2O 1:1 (100 μL)]. Following an additional period of 1.5 h, the crude reaction mixture was purified by RP-HPLC. The collected fractions were diluted with water and freeze-dried to give the mono-derivatized derivative. Maleimide activated-pmLPS O1 Inaba **3** (9.53 mg, 23%) was obtained together with recovered starting material **1** (3.36 mg, 15%) as white foams: negative MALDI-TOF-MS: *m/z* 7248, 7001, 6752, 6506, 6260, 6011, 5764, 5518, 5270, 5024, 4777, 4529, 4283, 4035, 3789, 3541 $(M–H)^-$, (22-mer to 7-mer of 4,6-dideoxy-4-(3-deoxy-L-*glycero*-tetronamido)-α-D-mannopyranosyl unit).

Acetylthio activated-pmLPS O1 Inaba **4** (3.7 mg, 28%) was obtained together with recovered starting material 1 (5.5 mg, 42%) as white foams; negative MALDI−TOF-MS: *m/z* 7227, 6978, 6731, 6483, 6238, 5990, 5744, 5497, 5250, 5002 $(M–H)^-$, (21-mer to 12-mer of 4,6-dideoxy-4-(3-deoxy-L-*glycero*-tetronamido)-α-D-mannopyranosyl unit).

*Derivatization of TT with the S-acetylthio linker (TT-SATA, **5**)*

To the stock solution of TT (29.1 mg, 1400 μL, 1.90 μmol) was added NHS-SATA (3×2.19 mg, 3×36 μL of a 60 mg mL^{-1} solution in CH_3CN, 3×50 equiv), in three portions every 45 min. Following an additional reaction period of 45 min, the crude reaction mixture was dialyzed against 3×2 L of 0.1 M potassium phosphate buffer, pH 6.0 at 4°C to eliminate excess reagent; SELDI-TOF-MS 160,178 Da [estimated average (acetylthio)acetyl-to-protein ratio: 31 mol/mol].

*Derivatization of BSA with the S-acetylthio linker (BSA-SATA, **6**)*

BSA (4.68 mg, 0.07 μmol) was treated accordingly with NHS-SATA (3×1.04 mg, 3×50 equiv). SELDI-TOF-MS 71,445 Da [estimated average (acetylthio)acetyl-to-protein ratio: 41 mol/mol]

*Derivatization of TT with the bromoacetyl linker (TT-AcBr, **7**)*

TT (16 mg, 970 μL of the stock solution, 0.11 μmol) was diluted with 0.2 M potassium phosphate buffer saline, pH 7.3 (650 μL). To this solution was added bromoacetic acid succinimidyl ester (3×1.26 mg dissolved in 50 μL of CH_3CN, 3×50 equiv), in three portions every 2 h. Following an additional reaction period of 4 h, the crude reaction mixture was dialyzed against 0.1 M potassium phosphate buffer, pH 6.5 (3×2 L) at 4°C to eliminate excess reagent; SELDI-TOF-MS 160,984 Da (estimated average bromoacetyl-to-protein ratio: 33 mol/mol)

*Derivatization of BSA with the bromoacetyl linker (BSA-AcBr, **8**)*

BSA (4.28 mg, 0.06 μmol) was treated accordingly with bromoacetic acid succinimidyl ester (3×0.75 mg, 3×50 equiv). SELDI-TOF-MS 70,612 Da (estimated average bromoacetyl-to-protein ratio: 30 mol/mol).

*Preparation of conjugates **9** (TT-Mal-pmLPS) and **10** (BSA-Mal-pmLPS)*

Compound **3** (16 equiv) was added to 5 (6.5 mg, 1 equiv, 0.041 μmol), or **6** (1 mg, 1 equiv, 0.015 μmol), in 0.1 M potassium phosphate buffer solution, pH 6. The reaction mixtures were buffered at a 0.5 M concentration by addition of 1 M potassium phosphate buffer, pH 6.0. Then, NH_2OH,HCl (10 μL of a 2 M solution in 1 M potassium phosphate buffer, pH 6), was added to the mixture and the coupling was carried out for 2 h at rt.

*Preparation of conjugates **11** (TT-AcS-pmLPS) and **12** (BSA-AcS-pmLPS)*

Compounds **7** (3 mg, 540 μL), or **8** (1.3 mg, 430 μL), were diluted with 0.1 M $NaHCO_3$, pH 8.3 (400 μL or 430 μL, respectively). The mixtures were degassed and added to **4** at a 1:30 molar ratio under Ar(g). Then 2 M hydroxylamine in 0.1 M $NaHCO_3$, pH 8.3 (15 μL) was added, and the reaction mixture was stirred at rt for 30 h. All conjugates were dialyzed against PBS, pH 7.3 (3×2 L) at 4°C, and further purified by gel permeation chromatography on a sepharose CL-6B column (1 m×160 mm), using PBS, pH 7.3 as eluent at a flow rate of 0.2 mL min^{-1}, with detection by measuring both the optical density at 280 nm and the refractive index. Appropriate fractions were pooled and concentrated by centrifugation. The conjugates were stored at 4°C in the presence of thimerosal (0.1 mg mL^{-1}), and assessed for their total carbohydrate and protein content. PmLPS concentration was measured by a colorimetric method based on the anthrone reaction [36], using pmLPS as a standard. Protein concentration was measured by the Lowry method [37] using BSA as a standard, and confirmed by total acidic hydrolysis (6 M HCl at 110°C for 20 h) using norleucine as an internal standard. Control experiments showed that the measurements were not affected by the presence of either proteins or carbohydrates.

Biological evaluation of the conjugates

Direct ELISA

Flat-bottom microplates (Immuno Microwell; Nunc) were coated with avidin (100 μL at 1 μg mL^{-1}) in PBS for 1 h at 37°C and washed with PBS-Tween. Wells were blocked by incubation with 0.5% gelatin (Prolabo) in PBS for 30 min at 37°C and washed with PBS containing 0.1% Tween-20. 2 was added at various dilutions in PBS-Tween-gelatin (100 μL) to the wells. The plates were incubated for 1 h at 37°C, washed with PBS-Tween and 100 μL/well of monoclonal IgGs F-22-30, I-24-2 or S-20-3, were added. The plates were further incubated for 1 h at 37°C and washed with PBS-Tween. An anti-mouse peroxidase-conjugated IgG (heavy- and light-chain specific) diluted 1:1000 in PBS-Tween containing 0.5% gelatin was added to the wells. The plates were incubated at 37°C for 45 min and washed with PBS-Tween. The enzyme substrate, *o*-phenylenediamine dihydrochloride (100 μL at 0.4 mg mL^{-1}) in 0.1 M sodium citrate (pH 5.2), containing 0.02% hydrogen peroxide, was added to each well and the plate incubated for 10 min at rt. The reaction was stopped by adding 3 M HCl (50 μL per well) and the A490 was read in an EL 800 spectrophotometer (Bio-Tek Instruments).

Inhibition ELISA

Assays were carried out in a two-step ELISA. In step 1, IgG I-24-2 or F-22-30 was incubated with solutions of polysaccharides and in step 2, the resulting mixtures of free and bound Ab were added to microtiter plates coated with Inaba LPS as previously described [32]. Briefly, flat-bottom microplates were blocked by incubation with 0.5% gelatin in PBS for 1 h at 37°C, and rinsed with PBS containing 0.1% Tween-20. Polysaccharide dilutions (100 μL) and mAb (100 μL) in PBS-Tween-gelatin were added to the wells, at a dilution, determined by direct ELISA titration, giving an A490 of 0.5. Mixtures were incubated for 1 h at 37°C, then 100 μL of samples from each well were transferred to a second plate, that had been previously coated by incubation for 2 h at 37 °C with Inaba LPS or pmLPS (5 μg mL^{-1}) in Na_2CO_3/$NaHCO_3$ buffer (0.1 M, pH 9.5), blocked with gelatin and washed with PBS-Tween. This plate was incubated at 37°C for 1 h and washed with PBS-Tween. Detection of the inhibition and reading of the plates were performed as described for direct ELISA.

Serological analysis

Double immunodiffusion was performed in 1% agarose in 0.5 M NaCl for 24 or 48 h at 4°C [38].

Immunizations

Groups of five 6-week-old female BALB/c mice were injected subcutaneously with 10 μg, or 20 μg of Inaba pmLPS alone, or as a conjugate, in saline solutions three times at 2-weeks interval. The mice were bled at days 35, 60 and 90 following the first injection. Controlled groups of mice were immunized similarly with 10 μg of tetanus toxoid (TT) or BSA. Anti-LPS and anti-carrier Ab titers were determined by ELISA. Plates were coated with **2**, LPS, conjugates **9**–**12**, TT or BSA. Serial two-fold dilutions of mouse sera (1/100 to 1/6,400) were analyzed. The secondary Abs used were either peroxidase-conjugated anti-mouse IgG (γ chain specific) or IgM (μ chain specific). The results were calculated for each immunoglobulin class, as a percent of a high-titered reference serum arbitrarily assigned a value of 100 ELISA units by parallel line analysis with a program from the Center for Disease Control and Prevention and expressed as a geometric mean [39]. Following the same method, anti-carrier Ab levels were expressed with respect to a hyperimmune mouse pooled standard serum prepared in the laboratory by repeated immunizations of mice with the carrier.

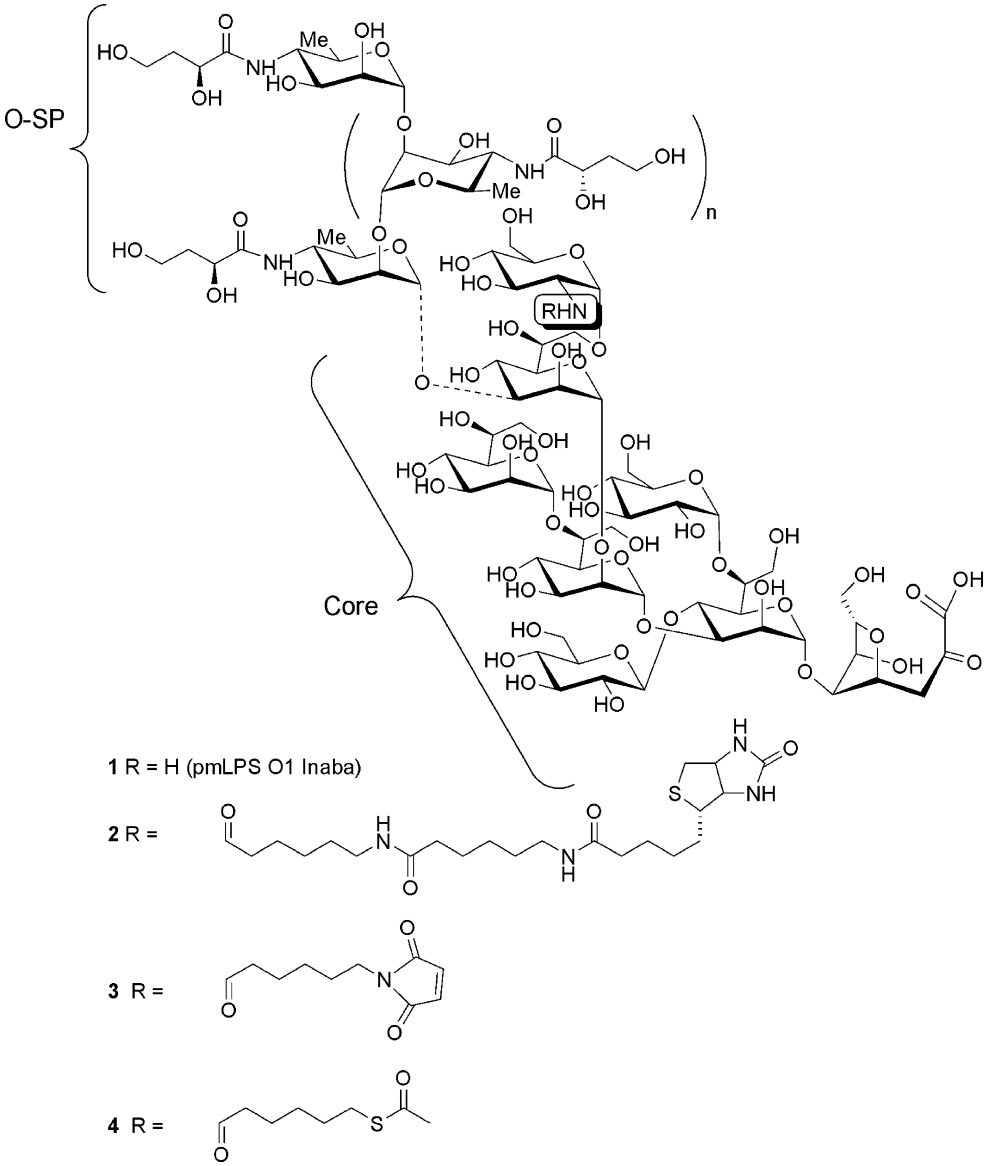
Vibriocidal Ab response

The vibriocidal tests were performed as previously described [31, 40], with two fold dilutions (beginning at 1:10 dilution) using *V. cholerae* O1 strain CNRVC 950707 serotype Inaba and *V. cholerae* O1 strain CNRVC 920172 serotype Ogawa as the target strains, and guinea pig serum as the source of complement. The vibriocidal titer was defined as the reciprocal of the highest serum dilution causing 100% bacterial lysis. Each assay, included a negative control (bacteria and complement only), and a positive control (a hyperimmune rabbit serum obtained from immunizations with the above mentioned *V. cholerae* O1 strains).

Results and discussion

Preparation, purification and characterization of *V. cholerae* O1 Inaba pmLPS

The direct use of LPS for the preparation of conjugates to be used as vaccines is precluded by its intrinsic toxicity. Therefore, LPS obtained from cultured *V. cholerae* O1 serotype Inaba strain was detoxified by mild acid treatment [34] to give the corresponding pmLPS **1** in 20–30% yield following RP-HPLC purification (Scheme 1). This detoxification procedure was preferred to an alkali or hydrazine alternative [23] as it allows the complete removal of the lipid A moiety which is responsible for LPS toxicity.



Scheme 1 Structure of *V. cholerae* O**1** serotype Inaba pmLPS and derivatives **2**, **3** and **4**. The pmLPS structure is proposed according to Vinogradov *et al*. [41]. The point of attachment (dotted line) between the O-SP and the core has not yet been clearly established

Furthermore, the hydrophilic derivative thus obtained is easier to handle and less prone to lipid A contamination since it is devoid of fatty acid chains.

As described earlier [42, 43], the 1% aqueous AcOH treatment causes the release of a D-fructofuranose residue initially linked to a glucose of the core. Acidic hydrolysis also results in the elimination of a phosphate group initially present at C4 of the 3-deoxy-manno-2-octulosonic acid (Kdo) to give an unsaturated ketone intermediate. The latter undergoes further an intramolecular 1,4-addition to give the corresponding cyclic 4,7-anhydro (not shown) or 4,8-anhydro Kdo at the reducing end [44, 45]. The ^{13}C NMR analysis was consistent with previous reports [23, 42, 46]. The MALDI-TOF-MS spectrum of pmLPS **1** contains a series of $[M–H]^-$ pseudomolecular ions ranging from *m/z* 3348 to 7057, with a difference of 247 mass unit between every two neighboring signals (Fig. 1). These observations are consistent with a pmLPS preparation being a homopolymer constituted of 7 to 22 (mainly 13 to 22) 4,6-dideoxy-4-(3-deoxy-L-*glycero*-tetronamido)-α-D-mannopyranosyl residues branched on a core [30]. Furthermore, the mass spectrum reveals that this pmLPS preparation also contains secondary products, such as species lacking one tetronyl side-chain (satellite peaks observed 102 Da below $[M–H]^-$ pseudomolecular ions of the main polymeric mixture] which are clearly present. These contaminants probably arise from non-acylated perosamine incorporation during O-SP elongation.

Assessment of the reactivity of the D-glucosamine NH_2 group; effect of its derivatization on antigenicity

When dealing with synthetic carbohydrate haptens, conjugation is often envisaged from a free amino group present on an aglycone spacer [47, 48]. However, to our knowledge,

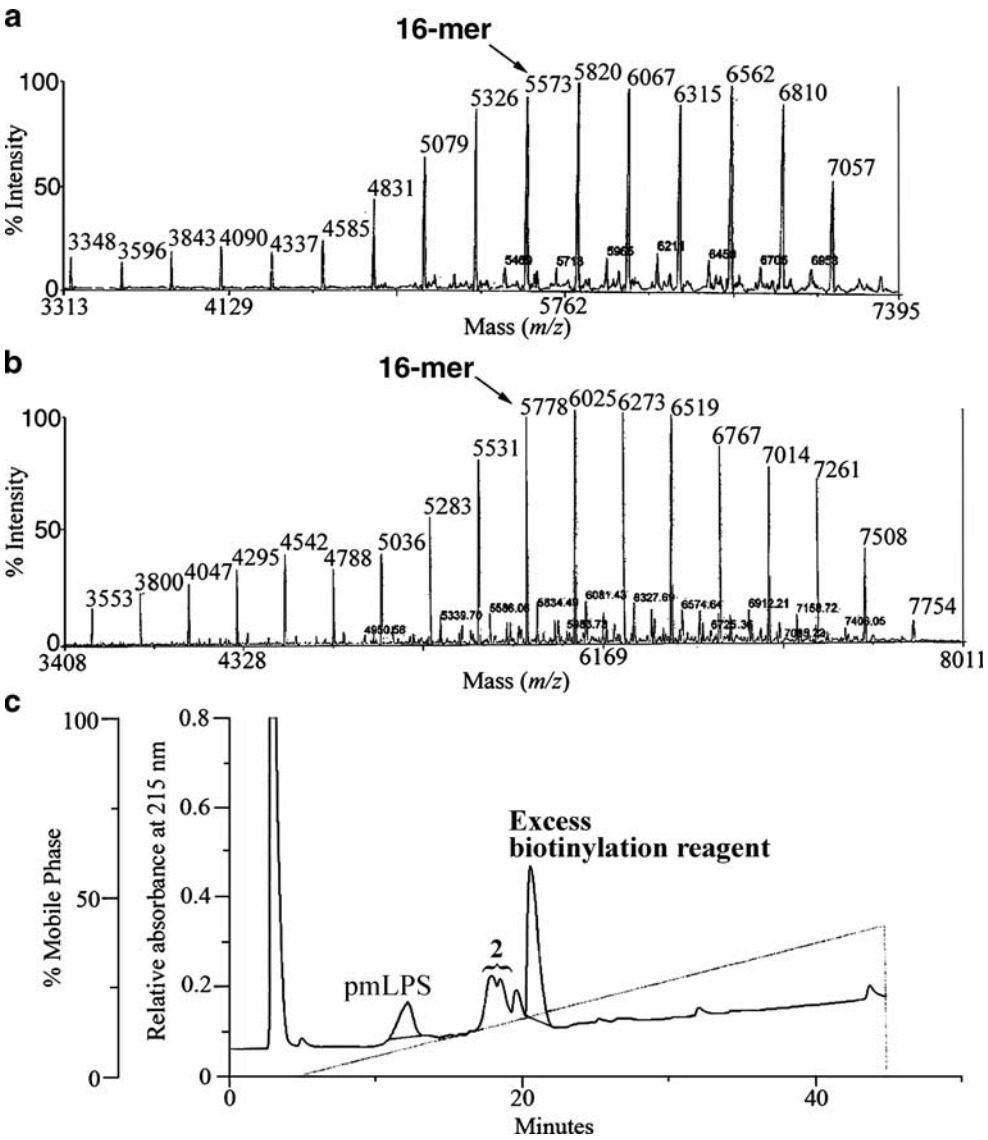


Fig. 1 MALDI-TOF-MS spectra of (a) pmLPS **1**, (b) biotinylated-pmLPS **2**; (c) Analytical RP-HPLC profile of crude biotinylated pmLPS (For elution conditions and detection, see "Material and Methods")

apart from exceptions such as the example of *Streptococcus pneumoniae* type 1 CP [49] and *Shigella sonnei O*-SP [50], amino groups are always acylated when present on bacterial CPs or *O*-SPs of interest as immunogens [51]. Direct [25, 52] or indirect [53] conjugation at such masked amino groups has rarely been contemplated as it would require prior deacylation under harsh conditions and lead with poor reproducibility to reticulated glycoconjugates. However, the use of glycoimmunogens incorporating the core and/or the lipid A carbohydrate moiety of LPS would in principle offer further options for conjugation, thanks to amino groups often present as phosphoethanolamine substituents. The published data indicate that an amino group occurs in the form of a branched D-glucosamine residue of *V. cholerae* O1 core and that this amino group is unique if one neglects the marginal presence of unacylated perosamines. In principle therefore, chemoselective modification at this core residue should be feasible. Furthermore, appropriate choice of the derivation pattern should facilitate the characterization of the intermediates and resulting conjugates. However, long-range nuclear Overhauser effects [41] indicate that the branched D-glucosamine and the Kdo residues are closed to each other and suggests that the accessibility and reactivity of the amino group might be somewhat diminished due to possible steric crowding. To address this point the mono-derivatization of **1** was envisaged using sulfo-NHS-lc-lc-Biotin as the modifying reagent. The long alkyl chain linker in this reagent would be expected to confer hydrophobicity to the pmLPS and facilitate both RP-HPLC monitoring of the conjugation step and purification. Interestingly, biotin-mediated anchoring of such a pmLPS derivative to avidin-precoated microtiter plates might also be expected to open the way to improved ELISA [54–56]. Moreover, the biotinylated-pmLPS/avidin complex could mimic a statically hindered pmLPS–protein conjugate when probing the impact of the site-selective amino-derivatization on antige-

nicity. In particular, this transformation might affect an antigenic determinant shared by both Inaba and Ogawa serotypes and which has previously been shown to be the target of protective Abs. Although its exact structure is unknown, this determinant seems to involve residues from both the core and their *O*-SP [32, 46]. We considered that preserving the integrity of such a determinant upon conjugation would be crucial. Indeed, it was anticipated that a conjugate able to induce a strong Ab response against a "protective" determinant common to serotypes Ogawa and Inaba would open the way to an efficient glycoconjugate vaccine against *V. cholerae* O1 infections.

Thus, 1 was converted, at pH 7.3, into biotinylated 2 in 31% yield, together with 23% of recovered starting material: as expected, these two polymeric mixtures differing by their hydrophobicity were easily separated by RP-HPLC (Fig. 1c). MALDI-TOF-MS analysis revealed an increment of all signals corresponding to 452 Da, confirming the mono-biotinylation of heterogeneous **1** (Fig. 1a and b). Better yields are usually achieved when acylation is performed at pH above 8 [57]. However, in this range of pH, number of side reactions occurred with which made purification and analyses difficult. For example, mass spectra data revealed that all $[M–H]^-$ pseudomolecular ions were accompanied with fragment 46 Da below them, which is consistent with Kdo decarboxylation. Such a transformation has been suggested previously and is favored under basic conditions [58, 59]. The antigenicity of **2** was assessed by direct ELISA experiments using known mAbs IgG3 I-24-2 and IgG1 F-22-30. These mAbs have been reported to recognize the above mentioned antigenic determinant present on the LPS from both Inaba and Ogawa serotypes [46] and shown to protect suckling mice against oral challenge [31, 32]. Whereas binding was observed with both IgGs in this ELISA as expected, no recognition was detected with mAb IgG1 S-20-3 directed against a terminal 2-*O*-methylated perosamine present on the Ogawa O-SP only, and thus establishing its specificity for this serotype (Fig. 2a) [33, 60].

The specificity of the recognition of **2** by the two cross-reactive mAbs was further analyzed by an inhibition ELISA. Biotinylated **2** is as effective as **1** or Inaba LPS in inhibiting the binding of IgG F-22-30 to the latter (Fig. 2b). Identical results were obtained when the experiments were performed with IgG I-24-2 instead of IgG F-22-30 (data not shown). The ELISA data suggests that derivatization at the core glucosamine does not significantly alter the antigenic determinant customarily recognized by these mAbs.

Conjugate preparation

Our next objective was to confirm these results with a series of conjugates composed of the Inaba pmLPS bound to a protein carrier via the amino group. TT, which is clinically acceptable for use in humans, was chosen as the carrier and the readily accessible BSA for comparison. Maleimide-thiol and bromoacyl-thiol conjugation chemistries were selected because of their efficiency [61]. PmLPS functionalization using hydrophobic spacers was envisioned as for the synthesis of 2. Maleimide- and acetylthio-functionalized pmLPS **3** and **4** were isolated in 25% yield together with substantial amounts of recovered **1** (15 and 42%, respectively), using RP-HPLC purification following treatment of pmLPS (**1**) with either sulfo-EMCS or the succinimidyl ester of 6-(acetylthio)caproic acid, respectively. Mass spectroscopic data was consistent with incorporation of a single linker. ELISA experiments conducted with Inaba LPS supported that the integrity of the antigenic determinants had been maintained during the preparation (data not shown).

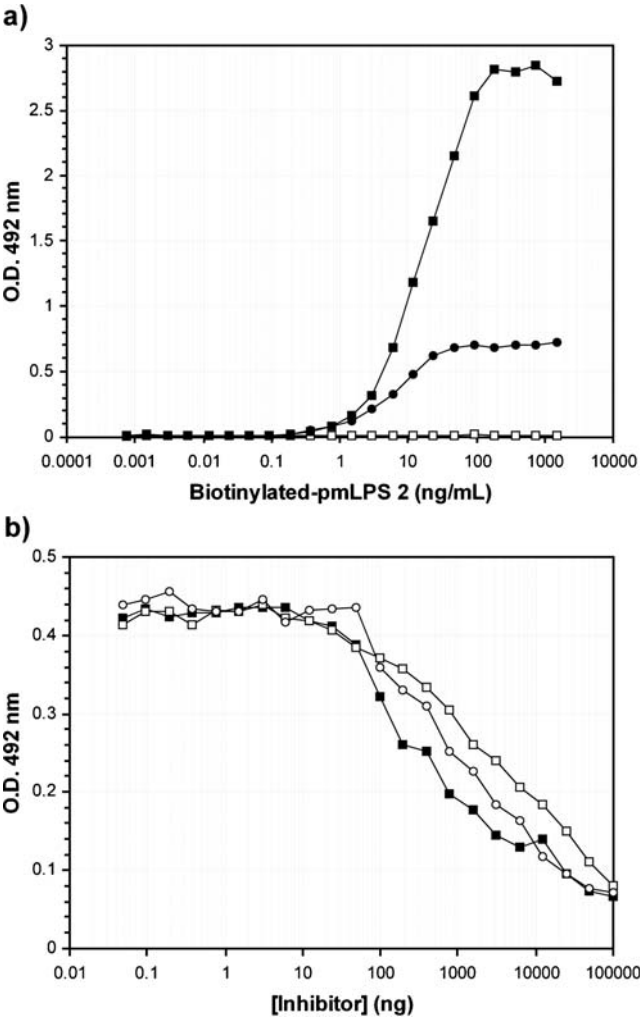
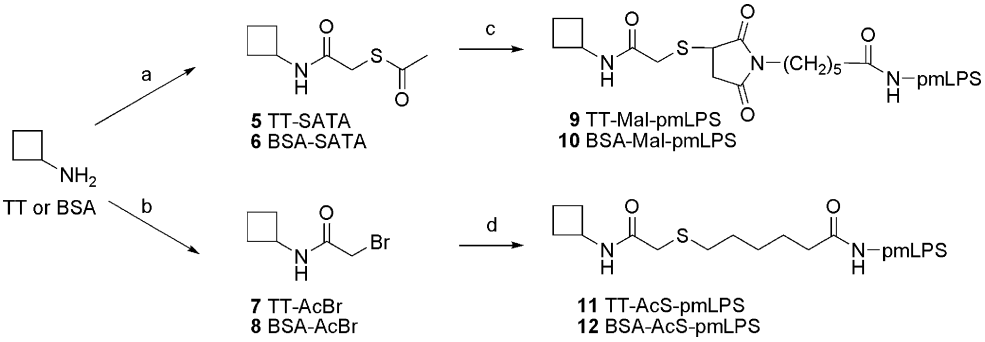


Fig. 2 (a) Direct ELISA binding profiles of biotinylated-pmLPS (**2**) with series of dilution of monoclonal antibodies F-22-30 (*filled square*), I-24-**2** (*filled circle*), and S-20-3 (*hollow square*); (b) ELISA inhibition of the interaction between Inaba LPS and mAb F-22-30 with various concentrations of Inaba LPS (*hollow circle*), **1** (*hollow square*) or biotinylated-pmLPS **2** (filled square)



Scheme 2 Preparation of the pmLPS-TT and BSA conjugates. Conditions and reagents: (a) SATA (3 × 50 equiv), PBS 0.1 M, pH 7.3, rt, 3 h; (b) $BrCH_2COOSu$ (3 × 50 equiv), PBS 0.1 M, pH 7.3, rt, 8 h; (c) **3** (16 equiv); excess NH_2OH, HCl, potassium phosphate buffer 0.5 M, pH 6, rt, 2 h; (d) **4** (30 equiv), excess NH_2OH,HCl, $NaHCO_3$ 0.1 M, pH 8.3, rt, 30 h

Compound **3** was conjugated at pH 6 in the presence of hydroxylamine to thiolated-proteins **5** and **6**, to afford conjugates **9** and **10**, respectively (Scheme 2 and Table 1). Finally, bromoacetylated-proteins **7** and **8**, respectively, were reacted at pH 8.3 with derivative **4** used in large excess in an attempt to minimize the effect of competitive oxidation. In spite of this precaution, polysaccharide-to-protein ratios of resulting conjugates **11** and **12** were lower than those observed for the maleimide-conjugates (Scheme 2 and Table 1). The reduced accessibility of carriers **7** and **8** compared to those of carriers **5** and **6** can not be the route of the above observations since the extent of carrier derivatization as well as the linker length and shape are very similar. The pmLPS heterogeneity, *i.e.* its polymeric distribution, precluded the use of SELDI-TOF mass spectroscopy as has previously been successfully applied to ascertain the sugar/protein ratio of semi-synthetic conjugates [62].

Conjugate antigenicity

The antigenicity of the conjugates was analyzed by the ELISA and double immunodiffusion as illustrated for TT-Mal-pmLPS **9** (Fig. 3). This conjugate was very efficient at inhibiting the interaction between LPS O1 Inaba and mAb F-22-30 (Fig. 3a). On the other hand, a single characteristic precipitate was detected when **9** was tested against IgG3 I-24-2 (Fig. 3b, wells 1 and 3). The immunoprecipitation line is fused with those observed when LPS Inaba or **1** are tested against the same mAb (Fig. 3b, wells 1 and 2 and wells 1 and 4, respectively), indicating immunochemical identity for the three polysaccharides. Similar properties were found for conjugates **10**, **11** and **12**, respectively (not described).

In related work, Gupta *et al*. did not observe a band of precipitation between hyperimmune LPS sera and pmLPS in immunodiffusion assays [23]. Although these two experiments were not strictly identical, the apparent discrepancy might be partly put down to differences in pmLPS preparations. These authors performed the acid-mediated LPS detoxification during a longer period (90 min compared with 60 min). Prolonged exposure to aqueous acidic medium has sometimes in the past been correlated with partial loss of pmLPS antigenicity [43].

Conjugate immunogenicity

BALB/c mice were next immunized subcutaneously three times at 2-week intervals, with 10 μg of carbohydrates, either in the form of free pmLPS or as conjugates **9–12** without any adjuvant. Pre-immune sera and phosphate buffered saline control sera contained no detectable levels of anti-LPS Abs. None of the conjugates elicited any significant anti-pmLPS, or anti-LPS IgG response (Table 2), although high anti-carrier IgG response was detected by ELISA (data not shown). Noteworthy was that mice that received doses of pmLPS alone or as conjugates produced anti-pmLPS IgM Abs after the first immunization. Strik-

Table 1 Characterization of *V. cholerae* O1 Inaba pmLPS-TT and pmLPS-BSA conjugates

Activated-protein (extent of derivatization)[a]	Donjugate	Yield[b] (%)	Composition (μg/mL)[c]		pmLPS/protein[d] (mol/mol)
			CHO	Protein	
TT-SATA (31 mol/mol)	TT-Mal-pmLPS **9**	48	147	260	14
BSA-SATA (41 mol/mol)	BSA-Mal-pmLPS **10**	72	57	144	4
TT-AcBr (33 mol/mol)	TT-AcS-pmLPS **11**	57	170	418	10
BSA-AcBr (30 mol/mol)	BSA-AcS-pmLPS **12**	53	30	140	2

[a] Extents of derivatization were estimated by SELDI-TOF-MS
[b] Yields were calculated on the basis of the weight of protein in the conjugate compared to the starting one determined by the Lowry method [37]
[c] Carbohydrate contents were measured by the anthrone reaction using pmLPS as a standard [36]
[d] Sugar to protein ratio was determined adopting average MWs of 6,500 for pmLPS, 157,000 for TT and 67,000 for BSA

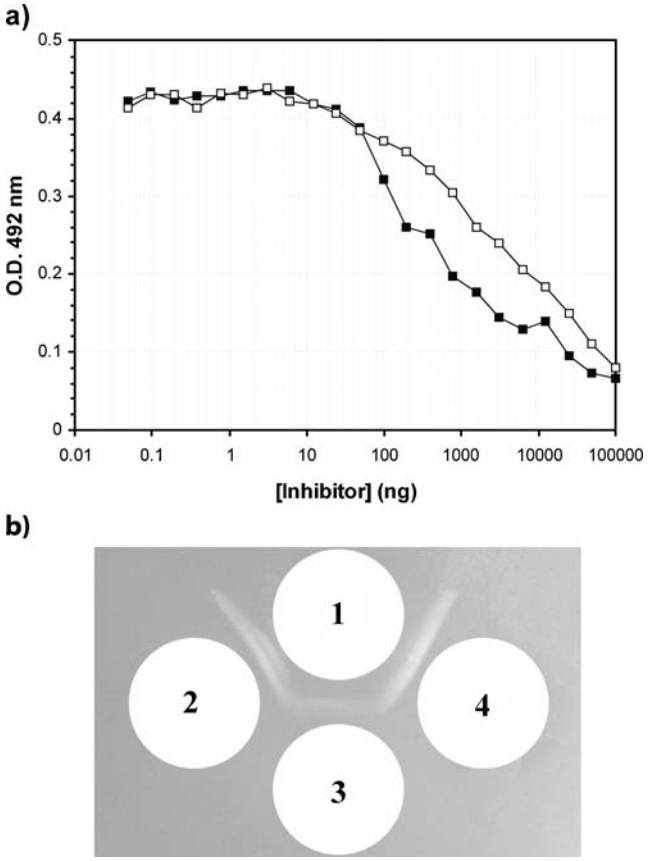


Fig. 3 (a) ELISA inhibition of the interaction between Inaba LPS and mAb F-22-30 with various concentrations of Inaba LPS (*hollow circle*) or TT-Mal-pmLPS (*filled square*); (b) Double immunodiffusion analysis. Wells: 1, mAb I-24-2, 35 μL, (3 mg mL^{-1}); 2, Inaba LPS (1 mg mL^{-1}); 3, TT-Mal-pmLPS, (0.26 mg mL^{-1}); 4, pmLPS Inaba (1 mg mL^{-1})

ingly, this early primary response remains high, even 62 days after the last injection, an observation which might correlate to a persistency of the pmLPS, (free or linked to the protein) in mice. A corresponding anti-LPS IgM response was observed in mice immunized with pmLPS alone or BSA-conjugates. In contrast, this response was somewhat delayed or even absent in mice immunized with TT-conjugates **9** or **11**, respectively (Table 2).

A significant anti-LPS IgM response was, however, induced by conjugate TT-Mal-pmLPS when 20 μg rather than 10 μg of polysaccharide per dose was injected to mice (Table 2). Consistent with the observed antigenicity data, appearance of these anti-Inaba LPS IgM indicates that pmLPS (as prepared and conjugated) effectively expresses antigenic determinants exposed on native Inaba LPS. At least one of the preserved antigenic determinants is shared by Ogawa LPS. Indeed, sera did cross-react with Ogawa LPS (data not shown).

Next whether or not the induced Abs were functional was assessed. Thus, selected pooled sera were analyzed for their vibriocidal activity against both Inaba and Ogawa bacterial strains and compared with hyperimmune rabbit serum as a positive immune control. Interestingly, both pmLPS alone and its conjugates elicited vibriocidal Abs having similar activity for both homologous (Inaba) and heterologous (Ogawa) serotypes (Table 3).

These results are of importance since *V. cholerae* O1 LPS-based vaccines must be able to induce protection against both Inaba and Ogawa serotypes, as both serotypes are equally prevalent. Data presented herein fully agree with observations in the field: vaccines trials run in Asia from 1968 to 1971 showed that killed whole-cell Inaba vaccines protected against cholera caused by both Inaba and Ogawa serotypes, whereas killed whole-cell Ogawa vaccines protected only against the homologous serotype [63–65]. In contrast, Abs elicited in mice by conjugates based on synthetic fragments of the *O*-SP of Inaba LPS cross-reacted with both serotypes, but were not vibriocidal [20]. These conjugates lack those antigenic determinants shared by both Inaba and Ogawa serotypes which have been shown to involve core residues as well as O-SP residues such as those recognized by IgG I-24-2 or IgG F-22-30 [32, 46]. Such epitopes are probably essential for a glycoconjugate immunogen to induce a broad protection against *V. cholerae* O1 disease.

The major concern raised by the present study is the absence of a switch from the T-independent to the T-dependent immune response. That neoepitopes introduced during the preparation of Inaba pmLPS—*e.g.* the acid-treatment of LPS causes the release of a D-fructose from the core while unmasking a cryptic antigenic determinant [66, 67]—or during the conjugation and it is this that diverts the immune response, appears a plausible explanation. If true, such neoepitopes are probably due to the nature of the linker rather than pmLPS or the amide bond formed during derivatization, since the above results demonstrated that sera do not contain IgG able to bind to biotinylated-pmLPS **2**. To check this hypothesis, the IgG response of mice immunized with a given conjugate was re-assessed using the corresponding conjugate formed with the same linker, but with an alternate carrier. This was expected to eliminate any interference caused by anti-carrier Abs. Indeed, using BSA-Mal-pmLPS-coated ELISA plates, high IgG response was measured for all sera of mice that received TT-Mal-pmLPS as shown in Fig. 4.

Since these Abs are not directed against either pmLPS, LPS or BSA, they necessarily bind to an epitope which includes the spacer. Although immunogenicity of maleimide-based spacers has sometimes been mentioned in the literature [58, 68, 69], this result was not fully predictable. Indeed, aliphatic-type maleimide linkers, such as the sulfo-EMCS used in the present study have been shown to mildly or not at all immunogenic, when compared with their cyclic or aromatic counterparts [56, Phalipon et al., unpublished

Table 2 Serum anti-Inaba pmLPS and anti-Inaba LPS antibodies (ELISA) elicited in BALB/c mice following immunization using pmLPS alone or as a conjugate[a]

Immunogen	Dose (μg)	Day	Geometric mean (25–75 centiles)			
			Anti-Inaba pmLPS IgM	Anti-Inaba pmLPS IgG	Anti-Inaba LPS IgM	Anti-Inaba LPS IgG
Inaba pmLPS **1**	10	35	132 (65–339)	5 (4–8)	44 (12–197)	5 (5–7)
		60	98 (53–205)	3 (2–7)	21 (9–68)	1 (1–3)
		90	132 (57–180)	10 (5–19)	24 (12–50)	1 (1–2)
TT-Mal-pmLPS **9**	10	35	171 (160–197)	7 (5–7)	6 (4–6)	3 (1–2)
		60	167 (150–175)	4 (2–5)	6 (4–9)	1 (1–1)
		90	300 (215–381)	7 (4–8)	8 (5–11)	1 (1–1)
BSA-Mal-pmLPS **10**	10	35	152 (115–180)	5.5 (3–8)	5 (4–6)	1 (1–3)
		60	95 (75–109)	4 (3–5)	9 (7–10)	1 (1–1)
		90	101 (97–104)	5 (4–6)	40 (28 – 85)	1 (1–1)
TT-AcS-pmLPS **11**	10	35	111 (29–172)	4 (3–4)	15 (7–17)	1 (1–1)
		60	179 (67–198)	5 (5–5)	15 (15–65)	1 (1–2)
		90	141 (60–164)	3 (2–3)	33 (13–52)	1 (1–1)
BSA-AcS-pmLPS **12**	10	35	64 (57–73)	<1 (1–2)	79 (70–90)	9 (3–19)
		60	112 (82–154)	3 (2–4)	86 (75–86)	2 (2–2)
		90	65 (51–84)	5 (3–7)	165 (161–169)	5 (4–8)
TT-AcS-pmLPS **11**	20	35	161 (70–179)	4 (3–4)	140 (94–262)	2 (1–3)
		60	110 (72–129)	5 (3–7)	115 (81–209)	6 (4–11)
		90	184 (99–210)	6 (4–11)	174 (153–230)	4 (3–8)

[a] Five mice were injected subcutaneously with saline solutions containing the antigen three times at 2-weeks interval. The mice were bled at days 35, 60 and 90

results], and considered appropriate for use in humans [70]. We grafted the maleimide spacer onto pmLPS to avoid the presence of remnant unconnected maleimide moities as well as their corresponding hydrolyzed open maleamic acid form which have also been described as being potentially immunogenic [69]. However, the absence of such an anti-linker immune response, when reproducing the experiment with the acylthioether-type BSA conjugate **12** and sera of mice immunized with conjugate **11** tends to rule out this hypothesis as the sole cause of the absence of anti-LPS IgG in the sera.

An alternate explanation is that the conjugates are contaminated by free pmLPS. In theory, the observed T-dependent response would be decreased and representative of the real amount of covalently linked polysaccharide present in the dose injected into mice [71]. In practice, the presence of free LPS in the immunizing dose may even suppress the development of anti-polysaccharide IgG-producing memory B cells [72–75]. In an attempt to analyze the conjugates for their free polysaccharide contents, Inaba pmLPS and TT-S-pmLPS were injected on a Superdex 75 gel filtration analytical column which has optimal resolution between 3,000 and 70,000 Da. The chromatogram trace of TT-S-pmLPS clearly revealed the presence of a compound eluting at a retention time identical to that observed for pmLPS (Fig. 5b and c). The identity of the peaks was confirmed by co-injecting TT-S-pmLPS with small amounts of Inaba pmLPS, and the results suggest that the formulation does indeed contain free Inaba pmLPS as contaminant (Fig. 5d). At experimental concentrations, the amount of unconjugated polysaccharide could account for between 5 to 25% of the total polysaccharide content of the conjugate preparation. Use of a large excess of Inaba pmLPS together with low conjugation yields may have favored the presence of unconjugated polysaccharide, which apparently was only partially removed during the gel filtration purification.

Suppression of the T-dependent immune response was usually observed for very high doses of free polysaccharide as contaminant, whereas no effect could be determined for pneumococcal conjugates, which typically contain 10% of free polysaccharide [74, 75]. Furthermore, to our knowledge the acceptable amount of free polysaccharide present

Table 3 Vibriocidal activities of sera from BALB/c mice immunized with pmLPS alone or conjugates

Vaccine	Reciprocal vibriocidal titer	
	Inaba	Ogawa
Negative control	<20	<20
Hyperimmune rabit serum	1,280	1,280
Inaba pmLPS day 35 (10 μg/dose)	40	40
Conjugate **12** day 35 (10 μg/dose)	80	80
Conjugate **11** day 35 (20 μg/dose)	80	80
Conjugate **11** day 60 (20 μg/dose)	80	80

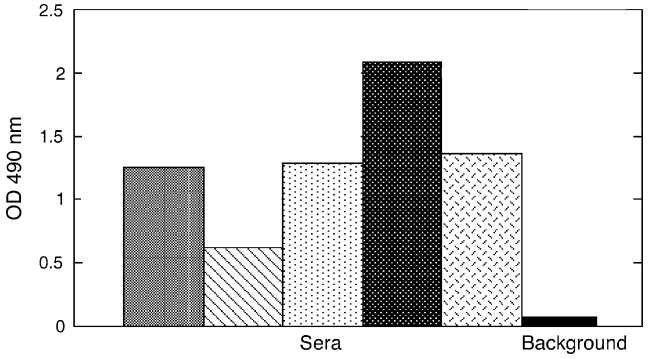


Fig. 4 Anti-spacer IgG Abs response expressed as optical density, at day 60 in the serum at a 200-fold dilution of a group of five mice that received TT-Mal-pmLPS or pmLPS (background)

in polysaccharide–protein conjugate vaccines is 20% of the total PS content. Therefore, the hyporesponsivness which is observed in the present study, if it does originate from free pmLPS, would be unprecedented.

Finally, if the nature or even the formulation of the conjugates is not called into question, the absence of switch to a T-dependent immune response, due to the intrinsic properties of the glycoimmunogen should itself be considered. One possibility is that the characteristics of the B cells that bind serotype Inaba pmLS are different from those of B cells specific *e.g.* for serotype Ogawa and leads to restricted B cell responses and lower IgG Ab titers. Such a hypothesis has been put forward to explain the lesser immunogenicity *Streptococcus pneumoniae* types 4 and 9 V CPs compared to other serotypes [76, 77], but remains to be validated.

Conclusion

Inaba pmLPS-protein conjugates have been prepared for the first time. This study clearly demonstrates the feasibility of using the free amino group of the Inaba pmLPS D-glucosamine core residue to introduce a single modification leading to well-characterized neoglycoconjugates. Conjugates were antigenic and immunogenic, inducing anti-Inaba LPS IgM Abs in BALB/c mice. As anticipated, the induced sera cross-reacted with LPS from serotype Ogawa, the other prevalent *V. cholerae* O1 causative agent of cholera. Interestingly, sera proved vibriocidal for both Inaba and Ogawa serotypes. The absence of significant anti-Inaba LPS IgG Abs in the sera of mice immunized with the conjugates underscores the known difficulty [7, 20, 23] to induce a long lasting immune response against this serotype. Further studies will be necessary to demonstrate whether this hyporesponsiveness is due to immunological

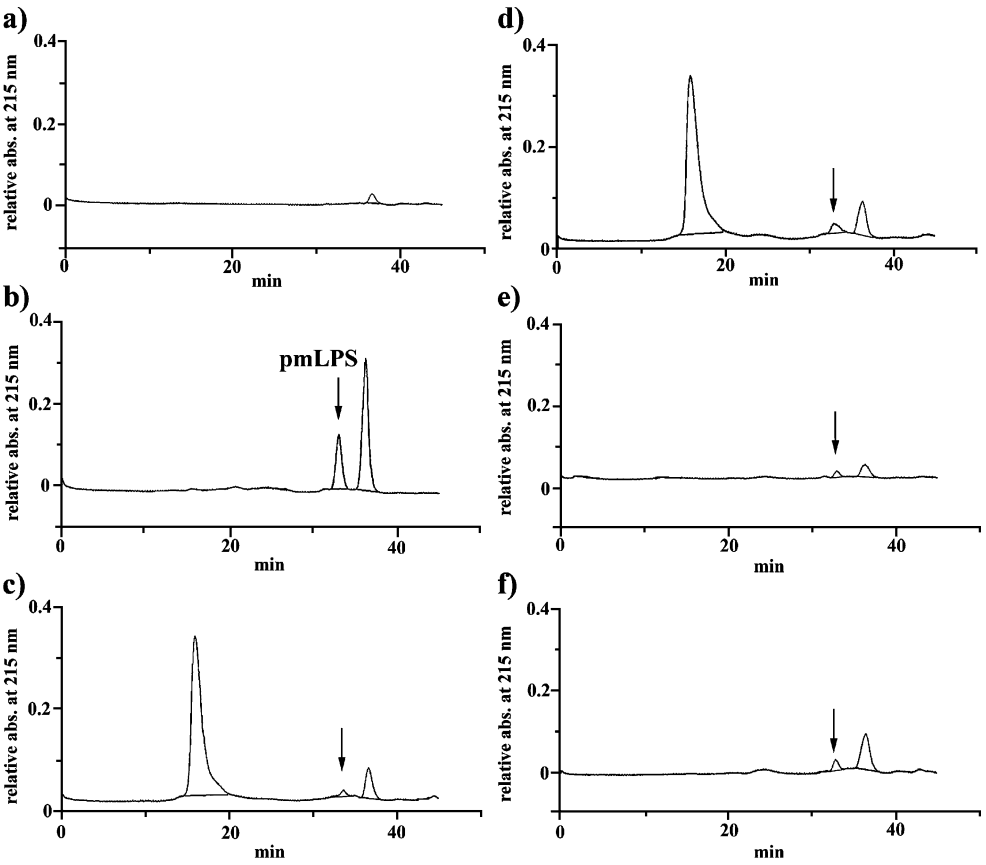


Fig. 5 Gel filtration chromatogram of (a) PBS; (b) Inaba pmLPS (50 μg injected); (c) TT-AcS-pmLPS (dose containing 20 μg polysaccharide injected); (d) TT-AcS-pmLPS (dose containing 20 μg polysaccharide injected) + Inaba pmLPS (5 μg injected); (e) Inaba pmLPS (1 μg injected); Inaba pmLPS (5 μg injected); Conditions: Superdex 75 column, at a flow rate of 0.5 mL min^{-1} using PBS 0.1 M, pH 7.3 as eluent

reasons or to neoglycoconjugate composition. New series of conjugates, involving non immunogenic spacers and efficient conjugation chemistries as well as by limiting the amount of free pmLPS while assuring compatibility with a large range of polysaccharide-to-protein ratios will be next considered.

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