Research

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A model of *Neisseria meningitidis* vaccine based on LPS micelles detoxified by synthetic anti-endotoxin peptides

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Summary We describe a model of vaccine based on detoxified endotoxin (LPS) conserving the supramolecular structure of micelles. Detoxification of LPS from *Neisseria meningitidis* group A, strain A1 (LPS A1), has been achieved by complex formation with a synthetic anti-endotoxin peptide (SAEP 2) binding to the lipid A moiety of LPS A1 with high affinity. Following subcutaneous injection in SW mice, LPS A1/SAEP 2 complex induced high titers of boostable IgG antibodies against the immunotype determinants of LPS A1, cross-reactive with group B LPS in either purified or cell-associated form. These antibodies were able to functionally fix and activate homologous and heterologous species of complement after binding to LPS A1-coated sheep erythrocytes. None of the IgG antibodies induced were specific for lipid A or SAEP 2 and none of the IgG antibodies cross-reacted with heterologous LPS. The purified IgG polyclonal antibodies significantly inhibited serum TNF production in CD1 mice intravenously challenged by homologous but not heterologous LPS. The immunogenic properties of LPS A1/SAEP 2 complex, investigated by the kinetic, magnitude and sub-isotype composition of the polyclonal antibodies induced, were comparable to those of a glycoconjugate obtained by covalent binding of LPS A1, detoxified by SAEP 2, to BSA working as a T-cell dependent carrier protein. The results obtained suggest that LPS behaves in vivo as a T-cell dependent antigen. The strategy of properly delivering to the immune system of mammalians, non-toxic LPS fully expressing its supramolecular antigenic structure, represents a novel approach for development of a new generation of R- and S-LPS/SAEP complex-based vaccines for prophylaxis of specific Gram-negative infections leading to sepsis and endotoxemia.

INTRODUCTION

LPS is the major toxic antigen of the outer membrane of Gram-negative bacteria. When expressed on the cell membrane it is a target for antibodies and once released in the bloodstream of a host, following bacteremia, it produces many biological effects via its lipid A moiety, the conserved region basically consisting of N,O-acyl β-1,6-D-glucosamine 1,4′-bisphosphate.[1] Lipid A is responsible for the toxic effects of LPS, including endotoxic shock,[2] but it may also produce beneficial effects to a mammalian host. In fact, LPS can activate T-lymphocytes (Th-1) of human type by inducing their proliferation and secretion of lymphokines[3] and can downregulate subsets of murine T-lymphocytes ($CD8^+$ $CD4^-$), known as suppressor cells, therefore working as immunologic adjuvant with T-cell

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independent (type 2) antigens, like bacterial polysaccharides,[4] by increasing anti-polysaccharide antibody production.[5]

Lipid A is also responsible for the supramolecular architecture of LPS and, in an aqueous environment, it provides the necessary aggregative forces responsible for an unique structural rearrangement known as the endotoxic conformation.[6] The supramolecular architecture of LPS is today well recognized by studies involving different physicochemical methods of investigation, such as X-ray diffraction, electron microscopy, NMR spectroscopy and molecular mechanics.[7–10] This special feature seems also to play a crucial role in the antigenicity and immunogenicity of LPS.[11,12] In fact, lipid A-deprived oligosaccharides, although containing the antigenic potential, are immunologically silent[13,14] and do not express full antigenic activity, compared to LPS, even when covalently linked to a T-cell dependent carrier protein, unless a lipid A-derived adjuvant is used.[14] Similar observations have been also reported for LPS-specific monoclonal antibodies reacting with synthetic oligosaccharides reproducing the carbohydrate chain of the homologous LPS.[15] Thus, the possibility for LPS expressing full immunogenic activity seems related to the retention of its supramolecular structure, which is also responsible for the expression of toxicity.[8,12] For this reason, LPS has been so far used as antigen only after chemical treatments of the fatty acid residues present in the lipid A moiety,[13] or after complete hydrolysis of the lipid A moiety from the carbohydrate side chain.[14,16,17] In any of these treatments, detoxification of LPS is accompanied by the loss of the supramolecular architecture of lipid A, paralleled by the loss of micellar configuration of LPS, which significantly affects its immunogenic potential.

In this study, we have tested the hypothesis demanding the supramolecular architecture of LPS for fully expressing its immunogenicity, by delivering LPS A1 to the immune system of a mammalian in the form of micelles, following its detoxification by specific binding of a synthetic anti-endotoxin peptide (SAEP 2) to the lipid A binding site.[8] By this strategy, R- and S-LPS retain their micellar structure, as shown by electron microscopy and molecular sieving chromatography.[8] In contrast, R- and S-LPS in complex with SAEP, significantly loose their toxicity in various assays and different animal models, where detoxification occurs systemically and locally, especially in key target organs of LPS.[8,18] SAEP 2 features a polymyxin B-like amino acid sequence whose characteristics have been also found in some amino acid sequences within the primary structure of several proteins known to be natural targets of LPS.[19,20] On this basis, the likely mechanism of action of SAEP in vivo is related to the antagonistic effect on cell receptor proteins (like CD14) by binding competition for lipid A, which leads to the stability of the complex formed.[18,21]

The choice of LPS from *N. meningitidis* strains, specifically LPS A1, has been made for various reasons:

1. Current unavailability of a vaccine for prevention of bacterial meningitis due to the group B meningococcus.

2. The carbohydrate structure of LPS A1 contains the immunotypes L8, L10 and L11, also present in the LPS structure from the most pathogenic groups of meningococci including group B and C.[14] In contrast, its structure does not contain immunodeterminants like lacto-N-neotetraose, which are identical to those present on human erythrocytes and lymphocytes, therefore avoiding the risk of inducing an antibody response cross reactive with human targets.[14]

3. The immune response of mice and rabbits to the oligosaccharide chain of LPS A1 deprived of lipid A is not comparable to that induced by (toxic) LPS A1 even when the lipid A-free oligosaccharide is presented to the mammalian immune system in the form of glycoconjugate via a T-cell dependent carrier protein.[14]

4. The immune response of rabbits to (toxic) LPS A1 has been shown to be bactericidal by complement dependency for homologous and heterologous strains of *N. meningitidis*.[14]

5. High serum levels of LPS are well documented in human meningococcal meningitis, responsible for the significant incidence of unfavourable prognosis,[22] which stimulates investigation on the role of anti LPS A1 polyclonal antibodies for prevention of meningococcemia as well as of the effects associated with the consequent endotoxemia.

MATERIALS AND METHODS

Preparation of antigens

Strains of *N. meningitidis* A1, BB431, 44/77 and purified *N. meningitidis* LPS A1 were prepared and characterized as previously described by Gu and Tsai.[14] The cyclic peptide SAEP 2 was synthesized on solid phase, oxidized and characterized according to the methods described in a previous paper.[8] The covalent conjugate BSA-LPS A1 was prepared as follows. LPS A1 (5 mg/ml) in PBS, containing two reactive moles of amino group per mole of oligosaccharide-lipid A monomer, was transformed to mono-succinimidyl ester by incubation (60 min at room temperature) with *bis*-succinimidyl ester of adipic acid (0.7 mg/ml) in dimethylsulfoxide (DMSO), basically as

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reported for amino-activated bacterial capsular oligosaccharides.[23] The LPS A1 derivative had more than 98% of the amino groups contained in its structure transformed in highly reactive ester groups, when detected by trinitro-benzene sulphonic acid (TNBS) reaction.[23] The ester-derivative of LPS A1 was then mixed with a 0.1 M sodium bicarbonate solution pH = 8.0 containing BSA (Sigma Chemical Co., St Louis, MO, USA) at 0.9 mg/ml. The stoichiometry of the reagents in reaction was equivalent to a molar ratio between amino groups of BSA:monoester groups of LPS A1 = 2. The solution was stirred for 4 h at room temperature and the BSA-LPS A1 conjugate was then recovered by precipitation with ethanol (60% v/v final concentration), solubilized in 0.1 M sodium bicarbonate pH = 8.0 and finally purified on gel chromatography (Sepharose 6B, Pharmacia, Uppsala, Sweden), sterile-filtered by 0.22 μm membrane and freeze-dried. The conjugate was assayed for chemical identity by amino acid analysis and lipid A content, according to the methods described.[8] Stoichiometry of the conjugate was consistent with a ratio BSA:LPS A1 = 1 (w/w).

Preparation of vaccines

Complexes of SAEP 2 with either LPS A1 or BSA-LPS A1 were prepared as reported.[18] Briefly, sterile solutions (0.22 μm membrane-filtered) of LPS A1 or BSA-LPS A1 and SAEP 2 were mixed at the ratio LPS/SAEP = 1:250 (w/w) and incubated 30 min at 37°C, added of sodium merthiolate 0.01% (w/v) as preservative and stored at 4°C. The immunizing dose of LPS A1 was 5 μg in the volume of 0.2 ml, in all formulations of vaccine tested. The suitable stoichiometry used in the preparation of the vaccines was previously found in in vivo experiments of lipid A detoxification with mice and rabbits.[8,18]

Safety of LPS A1/SAEP 2 complexes

LAL activity

Lipid A-induced LAL clotting for LPS A1 and the competitive inhibition by SAEP 2 in complex with LPS A1 was tested as previously reported,[8] using the Pyrogent Monolal reagent (BioWhittaker, Walkersville, MD, USA). The ratio between the highest concentration of LPS A1, alone or in complex with SAEP 2, showing consistent clotting activity gave the measure of the efficiency of detoxification for SAEP 2.[8]

Serum TNF production following subcutaneous injection in mice

Groups of five CD1 mice each, were injected s.c. with one dose of each vaccine. 90 min later, animals were bled and serum TNF titered for each mouse by bioassay, as previously reported.[8,18]

Hemorrhagic dermonecrosis (local Shwartzman reaction) in rabbits

Groups of three New Zealand white rabbits each, were injected intradermally in the shaved back with one dose of each vaccine according to Demitri et al.[18] 72–96 h later, animals were controlled for the presence of hemorrhagic necrosis at the site of injection.

General safety test in mice and guinea pigs

This test has been done according to the procedures reported in the Code of Federal Regulations,[24] by injection of each detoxified vaccine formulation in SW mice and guinea pigs.

Immunogenicity of LPS A1/SAEP 2 complexes

Kinetic, quantitative and qualitative analysis of the antibodies

Groups of 6 SW mice each, aged 6–8 weeks, were immunized s.c. with one dose of each antigen, at intervals of 3 weeks. Bleedings occurred 2 weeks after each injection was administered. Sera were obtained and serological analysis for isotype antibodies was performed by ELISA on the sera pool, basically under the conditions previously reported[14] using O-phenylendiamine as substrate for goat anti-mouse IgG and IgM antibody conjugated to peroxidase (Sigma). Titer of each sera pool was determined by end-point ELISA and defined as the highest serum dilution showing an optical density value, at 492 nm, twice that given by the control reaction. The kinetics of antibody production were followed by assaying the pool of sera for each group of mice at the standard dilution 1:200 (v/v). Sub-isotypes of the IgG polyclonal antibody populations induced were determined by inhibition-ELISA, using goat anti-mouse IgG_1, IgG_2, IgG_{2a}, IgG_{2b} and IgG_3 antibody (Sigma) diluted 1:50 (v/v). Each goat anti-mouse IgG antibody was preincubated with each pool of mice sera for 30 min at 37°C, before ELISA assay was performed in the conditions above reported.

Specificity of the antibodies

The specificity of the antibodies induced in the sera pool of mice immunized with the complex LPS A1/SAEP2 was assessed by inhibition ELISA, using a variety of purified heterologous LPS (Sigma), lipid A purified from *Bordetella pertussis*[8] and SAEP 2, at the highest concentration used

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in the ELISA assay (50 μg/ml). Each antigen was pre-incubated with the sera pool (30 min at 37°C) at serum dilutions in the range 1:200–1:1600 (v/v), before the ELISA assay was performed under the conditions reported above.

Specificity for LPS expressed on the cell membrane of homologous and heterologous meningococcal bacteria was assessed by inhibition ELISA using heat-killed strains of group A (strain A1) and group B (strains BB 431 and 44/76), all expressing the immunotype L8.[14] Before use, the bacterial suspensions (concentration range 5.5–9.5 $\log^{-1}$ cells/ml) were extensively washed in PBS until their supernatants were consistently negative in the LAL clot test (sensitivity of the assay according to the manufacturer was 0.125 EU/ml, equivalent for LPS A1 at 200–400 pg/ml). Each bacterial suspension containing killed cells of the stains, was pre-incubated (30 min at 37°C) with the sera pool diluted 1:200 (v/v), before ELISA was performed using the conditions reported above.

Biological functionality of the antibodies

Complement fixation

Efficiency and specificity of anti-LPS A1 IgG mouse antibody to fix and activate the complement pathway of homologous (mouse) and heterologous (guinea pig) species were assessed either by direct or inhibited passive immunohemolysis (PIH) using LPS-coated sheep erythrocytes (SRBC), according to Kuhn et al.[25] Mouse and guinea pig complement (Sigma) were diluted in the assays at 1:15 and 1:30 (v/v), respectively. Specificity was investigated by *Escherichia coli* LPS B5-coated SRBC, using an anti-LPS B5 mouse antiserum[19] as control, in addition to the inhibition of PIH by pre-incubation (15 min at 37°C) of homologous LPS A1 at the concentration of 50, 5, 0.5 μg/ml with the mouse antisera at various dilutions.

Inhibition of LPS A1-induced serum TNF in mice by affinity-purified IgG polyclonal antibodies induced by LPS A1/SAEP 2 complex

Groups of 5 CD1 mice each, were injected intravenously with IgG antibodies (250 μg/mouse) purified to homogeneity, 30 min before a challenge of LPS A1 (250 ng/mouse) was given by the same route. 90 min after challenge, animals were bled and serum TNF titered by bioassay as reported.[8,18] Controls included mice challenged with *E. coli* LPS B5 (50 ng/mouse), after i.v. administration of the purified IgG antibody (50 μg/mouse). Purification of the mouse IgG polyclonal antibodies induced by LPS A1/SAEP 2 complex was performed by ammonium sulphate precipitation (final concentration equal to 50% saturation) followed by affinity chromatography with goat anti-mouse IgG antibody covalently linked to agarose (Sigma) in 0.01 M sodium phosphate buffer pH = 7.2 with additionally 0.5 M sodium chloride. Elution of the bound mouse IgG antibody occurred by 0.1 M glycine pH = 2.4 in 0.15 M sodium chloride. The purified IgG antibody was freeze-dried and then characterized for purity by silver stained SDS-PAGE analysis in non-reducing conditions, for protein content as well as for occasional LPS contamination by LAL clot assay. Anti LPS A1 titer of the purified mouse IgG polyclonal antibody was assessed by ELISA, as reported above.

RESULTS

Safety of LPS A1/SAEP 2 complexes

LAL activity

LPS A1 gave consistent clotting at the end-point titers of 200–400 pg/ml while the BSA-LPS A1 conjugate showed activity in the range 400–800 pg/ml. Efficiency of SAEP 2 in the competitive inhibition of LPS A1-induced LAL activity was higher than 99%, comparable to the one previously reported for the meningococcal group B LPS.[8] On weight basis, the stoichiometric ratio between SAEP 2 and LPS A1 giving consistent inhibition of LAL activity at the end-point titer was 10 and 100 for LPS A1 and BSA-LPS A1, respectively.

Serum TNF production following subcutaneous injection in mice

Serum TNF titers are shown in Figure 1. The dose–response efficiency of SAEP 2 on LPS A1-induced TNF production was clearly related to the stoichiometry between SAEP 2 and LPS A1. At the ratio SAEP 2:LPS A1 = 250 (w/w), serum TNF induced by the complex was consistently within 10% of the value found for the control LPS A1 (9.3 ± 3.6 ng/ml), a result in agreement with the previously reported inhibition of local and systemic TNF when S-LPS and SAEP 2 were injected by intravenous route.[18] The BSA-LPS A1 conjugate was about 4 times less active than control LPS A1 in inducing production of serum TNF. As in the case of LPS A1, SAEP 2 inhibited the levels of TNF induced by the conjugate in a dose-dependent fashion. At the weight ratio SAEP 2/BSA-LPS A1 = 250, the conjugate complex induced less than 1% of the serum TNF induced by LPS A1 alone. When BSA was just mixed with LPS A1 as a control, no inhibitory activity on LPS A1-induced serum TNF production was detected, consistent with our experimental observation that BSA does not have any binding activity for the lipid A moiety of LPS (data not shown).

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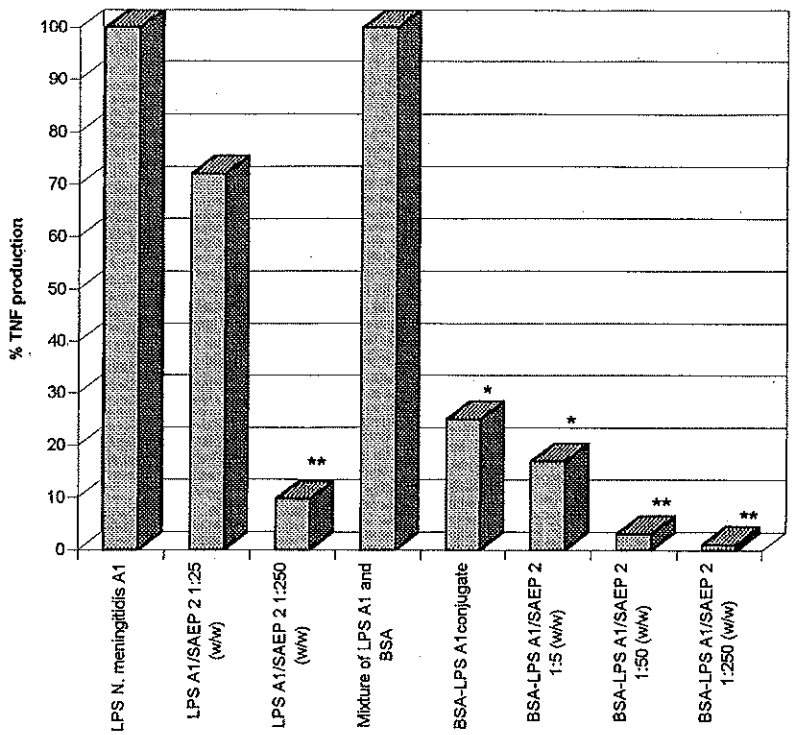

Fig. 1 Detoxification of LPS A1 and BSA-LPS A1 conjugate antigens by complex formation with SAEP2, as estimated by serum TNF production. Formulations of SAEP2 with the antigens were prepared at various stoichiometries for comparison of the inhibitory effects on the production of LPS-induced serum TNF in SW mice. Mice received all antigens s.c. Animals were bled 90 min later and serum TNF titered. $^{*}P < 0.05$, $^{**}P < 0.01$ by Student's *t*-test.

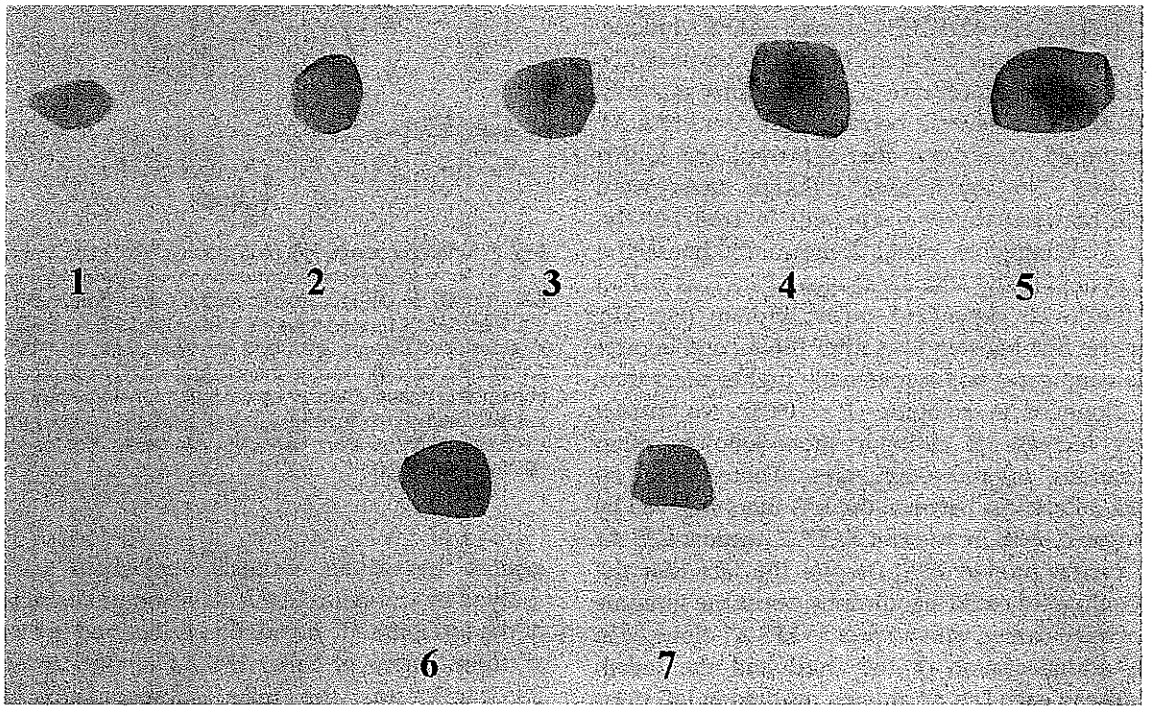

Fig. 2 Micelle-based vaccine of *N. meningitidis* LPS A1: detoxification by complex formation with SAEP2 or Polymyxin B as quantified by local hemorrhagic necrosis in the rabbit. **1**, Saline. **2**, **3**, **4**, and **5**, LPS A1 at 2.5, 5, 10, 25 μg respectively. **6**, LPS A1 at 10 μg in complex with SAEP 2 (1:250 w/w). **7**, LPS A1 at 10 μg in complex with Polymyxin B (1:100 w/w). Complexes were injected i.d. in the dorsal region of rabbits and the sites of injection were observed 72–96 h later.

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Hemorrhagic dermonecrosis (local Shwartzman reaction) in rabbits

Injection of one dose of either complex with SAEP 2 (LPS A1 or BSA-LPS A1) did not result in any detectable skin lesion in contrast to control LPS A1 (Fig. 2), a result consistent with earlier findings with R-LPS.[18] In some cases, the complex between polymyxin B and LPS A1, used as control at the ratio polymyxin B/LPS A1 = 100 (w/w), resulted in a significant reduction of the hemorrhagic lesion but not in its complete inhibition. This observation is explainable with a sub-optimal stoichiometry used in vivo for polymyxin B in complex with LPS A1, due to the convenient dose of this drug before polymyxin B-related toxic effects in the animals could be detected.[8,18]

General safety test in mice and guinea pigs

Animals did not show any sign of abnormal behaviour and gained weight normally during the period of observation.

Immunogenicity of LPS A1/SAEP 2 complexes

Kinetic, quantitative and qualitative analysis of the antibodies

The kinetic pattern of the IgG antibodies specific for LPS A1, induced by the various vaccines, is shown in Figure 3A. The complex LPS A1/SAEP 2, as well as LPS A1 alone, boosted the immune system of mice consistently, although a significant increase in antibody titer was only obtained after the second booster dose. Anti-LPS A1 titers continued to increase after the third and fourth booster dose. No significantly different titers were detectable between the two antigens. In contrast, the BSA-LPS A1 conjugates could significantly boost the immune system of the animals against LPS A1, as well as against the T-cell dependent carrier protein (Fig. 3B), following the first booster dose. After the second booster dose, the conjugates had significantly higher titers than LPS A1 alone or in complex ($P < 0.01$). No further significant increase of antibody titers followed other administrations of the conjugates. There were no significant differences between the IgG titers induced by the conjugates, with or without SAEP 2, against LPS A1 and the carrier protein BSA. Despite the fact that the protein conjugates could significantly boost the immune system of mice earlier, with respect to LPS A1 alone or in complex, administration of the third and the fourth booster dose of each LPS A1 antigen resulted in IgG titers that were no longer significantly different from those induced by the protein conjugates. At completion of the immunization period, titers of the IgG antibody induced

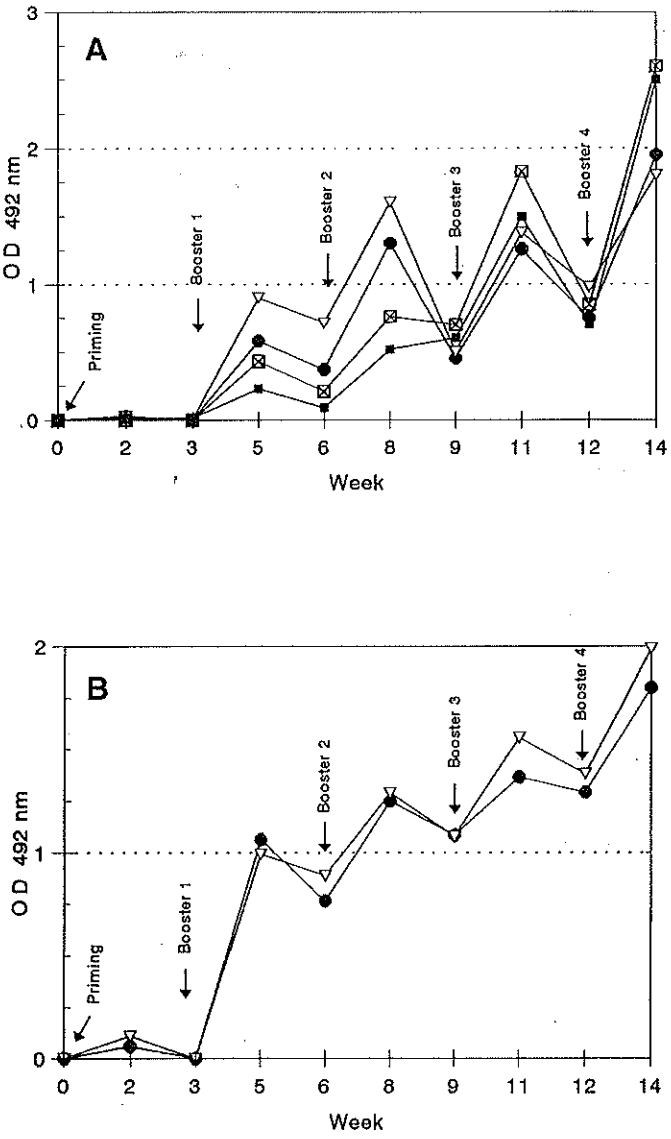

Fig. 3 Kinetic of serum IgG production, in SW mice, induced by LPS A1 in various vaccine formulations. (**A**) Serum IgG specific for LPS A1. (**B**) Serum IgG specific for the carrier protein BSA. Filled squares, LPS A1; open squares with cross, LPS A1/SAEP2; inverted open triangle, BSA-LPS A1; filled circle, BSA-LPS A1/SAEP2. Arrows indicate the week at which mice received the immunizing doses. Titers of serum IgG, represented by the OD value of each symbol, were detected by ELISA in the sera pool of each animal group. Each sera pool was assayed at the standard dilution 1:200 (v/v).

against the various antigens were about 1:15 000 as assayed by end-point ELISA. Sub-isotype characterization of the IgG antibody population is reported in Figure 4. In all formulations of either LPS A1 or the carrier protein BSA, IgG_2 was the main sub-population induced, followed by IgG_1 and IgG_3. Within the IgG_2 sub-isotype, equal amounts of IgG_{2a} and IgG_{2b} were detected in all immune sera (data not shown). No significant titers of

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LPS A1-specific IgM antibodies could be detected in any of the immune sera and no evident relation between expression of immunogenicity and expression of toxicity for LPS A1 could be seen among the various vaccine formulations.

Specificity of the antibodies

Inhibition-ELISA performed with the various inhibitors listed in Figure 5, including homologous and heterologous

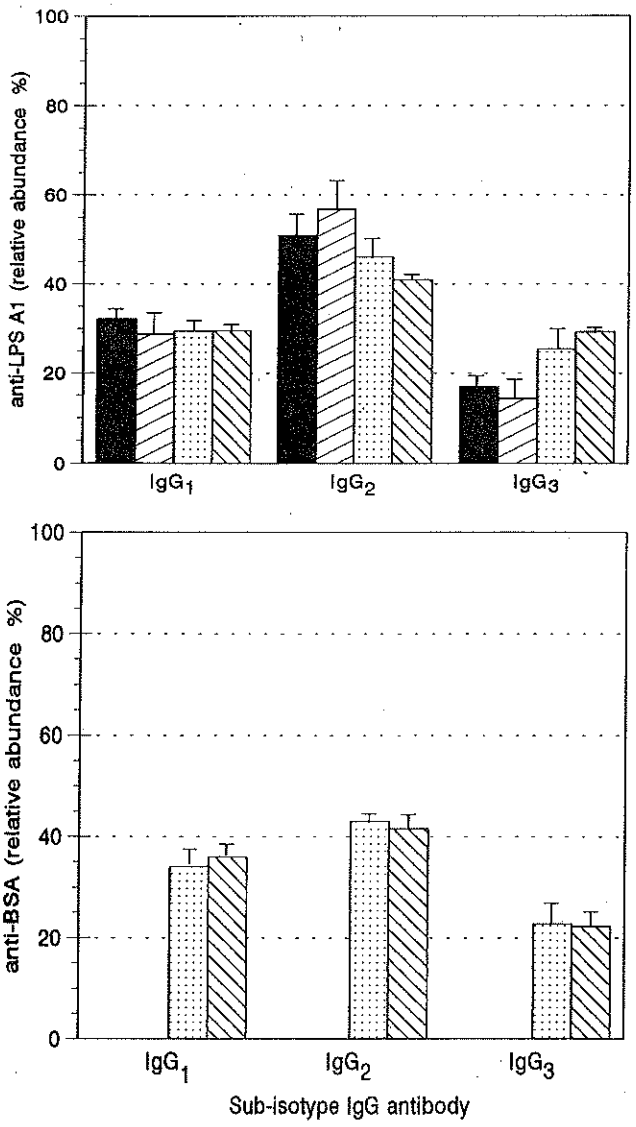

Fig. 4 Sub-isotyping of serum IgG induced in SW mice by LPS A1 in various vaccine formulations. From left to right: LPS A1, LPS A1/SAEP2, BSA-LPS A1, and BSA-LPS A1/SAEP2. Sera pools of each animal group immunized were assayed for the relative abundance of each sub-isotype antibody present within the IgG polyclonal population specific for LPS A1 and the carrier protein BSA. Sera pools were assayed by inhibition-ELISA, at the standard serum dilution 1:200 (v/v), using goat anti-mouse IgG_1, IgG_2 and IgG_3.

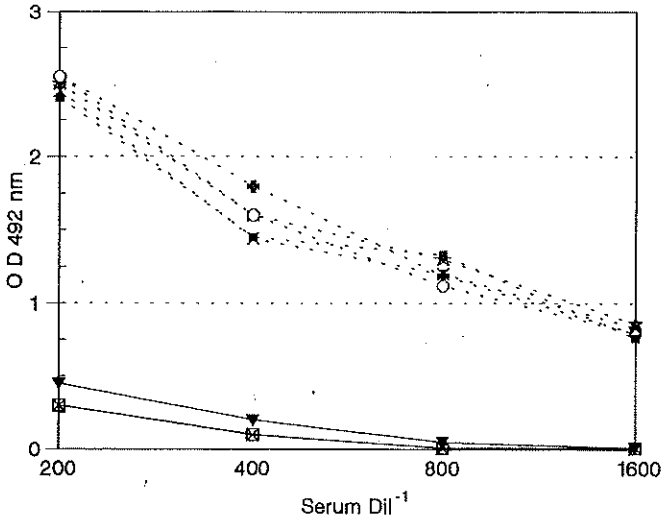

Fig. 5 Immunochemical specificity of anti-LPS serum IgG induced in SW mice by the complex LPS A1/SAEP 2 for purified homologous and heterologous antigens. Open square with cross, *N. meningitidis* LPS A1; inverted filled triangle, *N. meningitidis* LPS BB431; open circle, SAEP2; filled square, *B. pertussis* LPS, lipid A (purified from *B. pertussis*); star, *E. coli* LPS B5, *Klebsiella pneumoniae* LPS, *Pseudomonas aeruginosa* LPS; ×, *Haemophilus influenzae* LPS, non-typeable *H. influenzae* LPS; cross, *Salmonella typhosa* LPS, *Salmonella enteritidis* LPS, *Salmonella dysenteriae* LPS, *Shigella flexneri* 2a LPS. The sera pool containing anti-LPS A1 IgG antibody was assayed by inhibition-ELISA, using the purified antigens listed. Each antigen was pre-incubated with each dilution of the sera pool, diluted in the range 1:200–1:1600 (v/v) (see Materials and methods), before ELISA was performed.

LPS, showed that the IgG population induced by LPS A1/SAEP 2 complex was specific for the carbohydrate chain of LPS A1 since no inhibitory activity was detected for purified lipid A or SAEP 2. Most importantly, inhibition by bacterial cells demonstrated specificity for LPS expressed on the cell membrane of group A (strain A1) and group B (strains BB 431 and 44/76) meningococci (Fig. 6), a finding expected on the basis of the shared L8 immunodeterminant.[14]

Biological functionality of the antibodies

Complement fixation

In the presence of either homologous (mouse) or heterologous (guinea pig) source of complement, anti LPS A1 serum antibodies showed comparable erythrocyte-lytic capability on LPS A1-coated SRBC (Table 1). The reaction was specific for the LPS A1 antigen as shown by either the absence of activity of mouse anti-LPS A1 serum IgG on *E. coli* LPS B5-coated SRBC or inhibition of PIH by homologous LPS in a dose-dependent fashion.

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Inhibition of LPS A1-induced serum TNF in mice by affinity-purified mouse IgG polyclonal antibodies induced by LPS A1/SAEP 2 complex

Affinity-purified polyclonal IgG antibodies had an LPS A1-specific titer of 1: 15 000 when assayed by end-point ELISA, comparable to the titer range detected in the immune-sera. Silver stained SDS-PAGE analysis in non reducing conditions, revealed one single band in the expected range of molecular weight, by loading 2 μg of protein. LAL assay showed no occasional LPS contamination at least at the level of less than 50 pg/250 μg protein ($< 2 \times 10^{-5}$%). Prophylactic i.v. administration of the purified polyclonal antibody resulted in the significant inhibition of serum TNF induced by the i.v. challenge of homologous but not heterologous LPS (Table 2).

DISCUSSION

Delivery of LPS antigens to the mammalian system requires a careful control of the toxicity associated with the lipid A moiety. Since lipid A is responsible for the biological effects of LPS as well as for its supramolecular architecture, in this study we have presented a model of micelle-based vaccine obtained by detoxification of LPS A1 through complex formation with SAEP 2, a synthetic peptide specifically tailored to fit into the binding site of lipid A.[8] The physical chemical characteristics of peptides binding to and detoxifying the lipid A binding site of LPS have been well understood. They are consistent with an optimal size encompassing about 10 amino acids and

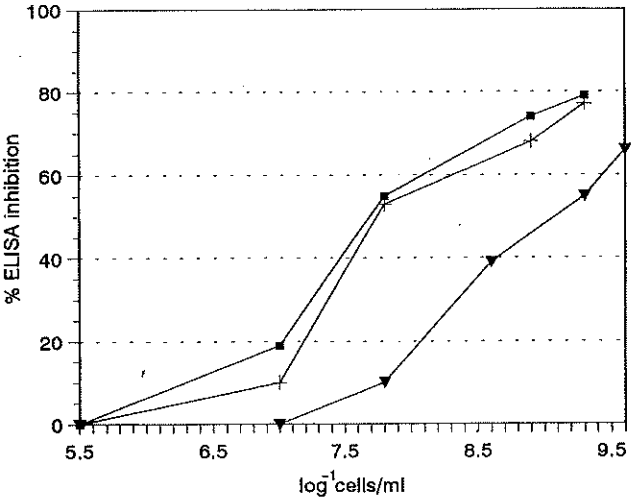

Fig. 6 Immunochemical specificity of anti-LPS A1 serum IgG antibody, induced in SW mice by the complex LPS A1/SAEP2, for cells of *N. meningitidis* group A and B. filled squares, A1 cells (group A); cross, 44/76 cells (group B); inverted filled triangle, BB431 cells (group B). The sera pool containing anti-LPS A1 IgG antibody was assayed by inhibition-ELISA, using the bacterial suspension in the conditions reported in Materials and methods. Each bacterial suspension containing killed cells of the strain A1 (Group A), BB431 and 44/76 (Group B) was pre-incubated with the sera pool diluted at the standard dilution 1:200 (v/v), before ELISA was performed.

Table 1 Functional specificity of anti-LPS A1 serum IgG in fixing and activating the complement system of either mouse or guinea pig species, as detected by either direct or inhibited passive immunohemolysis (PIH)[25]

Mouse antiserum	SRBC-saline	SRBC-LPS A1	SRBC-LPS B5
Anti LPS A1 serum IgG	< 10	200	< 10
Anti LPS B5 serum	< 10	< 10	400
Anti LPS A1 serum IgG + 50 μg/ml LPS A1	< 10	< 10	
Anti LPS A1 serum IgG + 5 μg/ml LPS A1	< 10	< 10	
Anti LPS A1 serum IgG + 0.5 μg/ml LPS A1	< 10	200	

The assay was performed using SRBC coated with either purified homologous *N. meningitidis* LPS A1 or heterologous *E. coli* LPS B5. Lytic activity of the serum IgG in the presence of guinea pig complement (as an example) and its inhibition by homologous LPS A1 is shown at the reciprocal of the indicated serum dilution.

Table 2 Effect of purified anti-LPS A1 polyclonal IgG antibodies on the inhibition of serum TNF production in CD1 mice challenged i.v. with either homologous *N. meningitidis* LPS A1 or heterologous *E. coli* LPS B5

Mice (total)	IgG anti LPS A1 (mg/mouse)	Challenge by LPS A1 TNF (pg/ml)	Challenge by LPS B5 TNF (pg/ml)
10	Saline	6110 ± 2062	2371 ± 1471
10	0.25	1579 ± 591 (inhibition 74%, $P < 0.01$)	n.d.
10	0.05	n.d.	2608 ± 1864

Data represent mean of two independent experiments with 5 mice/group. Purified IgG anti-LPS A1 were injected i.v. 30 min before an i.v. challenge of either *N. meningitidis* LPS A1 (250 ng/mouse) or *E. coli* LPS B5 (50 ng/mouse). TNF was titered by bioassay as described in Materials and methods. Data are mean ± SD; n.d. = not done.

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expressing cationicity and amphipathicity in association with a secondary re-arrangement of the molecule which increases affinity of binding. These features were first elucidated by studies of binding thermodynamic using synthetic peptide analogues of polymyxin B[8] and confirmed by studies of molecular mechanics based on NMR spectra of SAEP 2[26] and of a peptide analogue in complex with LPS.[27]

Detoxification of LPS A1 has been significantly achieved after complex formation with SAEP 2 in a variety of in vitro and in vivo assays. This finding parallels earlier data obtained with various R and S-LPS, where lipid A-induced LAL activity, pyrogenicity and hemorrhagic dermonecrosis in rabbit, as well as local and systemic TNF release in mice, were consistently inhibited by SAEP 2 at comparable values.[8,18,19] Unlike previous studies, in this work we have purposely administered LPS A1 s.c., mimicking in the animals the preferred immunization route of humans. The s.c. injection of LPS A1, however, resulted in the induction of serum TNF, after 90 min, at levels comparable to those reported for the intravenous and intraperitoneal injections of LPS.[8,18] Conjugation of LPS A1 to BSA resulted in an antigen with reduced toxicity, when compared to LPS A1 alone, a finding likely explainable with the role played by the carrier protein to sterically hinder the binding of LPS to a variety of humoral and cellular receptors.[28] Complex formation of LPS A1 with SAEP 2 drastically reduced the residual toxicity of the conjugate as well, showing that the specific binding of SAEP 2 to lipid A was not affected by the conjugation process. The observation that LPS A1/SAEP 2 complex is inactive in the local Shwartzman reaction, in contrast to LPS A1, is of particular interest in view of the immunization route chosen, as well as in light of the molecular mechanism elucidated for this lipid A-specific biological reaction, which involves the production and release of the proinflammatory cytokines IFN-γ, TNFα and IL-12.[29,30] Once released, these key mediators are responsible for the devastating effects produced in the tissues of various organs through the induction of cell apoptosis due to DNA fragmentation.[31]

The immunogenic properties of the antigens in mice, were consistent with the induction of IgG isotype antibodies specific for the carbohydrate structure of LPS A1, irrespective of the fact that LPS A1/SAEP 2 complex was covalently bound to a T-cell dependent carrier protein or not. The carbohydrate structure of LPS A1, lacking the lacto-N-neotetraose determinant, expresses an immunotype similar to those of several important group B strains, involving 7–8 monosaccharide residues.[14] None of the induced IgG antibodies had specificity for lipid A, although in the inhibition studies we have used a lipid A that was purified from *B. pertussis* LPS.[8] However, on the basis of the comparable structure reported for lipid A of LPS originating from pathogenic *N. meningitidis*,[32] it seems reasonable to conclude that no specificity for the lipid A moiety of LPS A1 was present in the sera of the immunized mice. Nevertheless, this issue deserves further investigation with the purified homologous lipid A. Also, no antibodies were induced against SAEP 2, an observation consistent with the well known lack of immunogenicity in mammals to small molecules like oligopeptides and oligosaccharides. This is a very desirable property for a detoxifying carrier like SAEP 2, since no antibodies to undesired (neo)determinants could be induced by the complex LPS A1/SAEP 2. Covalent conjugation of LPS A1 to the carrier protein affected the kinetics of the antibodies induced but not their overall qualitative and quantitative features. In particular, analysis of the sub-isotype IgG population revealed no statistically significant differences among the antibodies induced by LPS A1 alone or by LPS A1/SAEP 2 complex with or without conjugation to the T-cell dependent carrier protein. As in the case of glycoconjugate antigens of different composition,[23] in this study the carrier protein showed the familiar immunogenic behaviour of a T cell-dependent antigen, so that high IgG titers were induced to BSA and to the carried LPS A1 since the first booster dose of the conjugate complex was given to the animals. Very interestingly, the main sub-isotype populations of antibodies induced by the glycoconjugate against the carrier protein and LPS A1 were IgG_1 and IgG_2, quantitatively comparable to those induced by LPS A1/SAEP 2 complex and LPS A1 alone. Only the amount of IgG antibodies induced by the first booster dose of each antigen could be used as a parameter discriminating the immunological activity of BSA-LPS A1/SAEP 2 conjugate complex from that of LPS A1/SAEP 2 complex and LPS A1 alone. Following the third booster dose, in fact, all antigens were immunochemically indistinguishable, raising IgG antibodies at comparable levels. Altogether, these findings strongly suggest that LPS A1 expresses a T-cell mediated activity on the immune system of mammals, which is quite comparable to that expressed by LPS conjugated to a carrier protein, in terms of quality and quantity of inducible IgG antibodies. The kinetics of antibody production, however, are slightly different, an observation likely related to different kinetics of clearance for different LPS antigens by the immune system. In this study, we have not investigated at the cellular level the kind of T-cell immune response induced by the various antigens. However, based on the IgG sub-isotypes induced by LPS A1 in various formulations and on the general knowledge that the IgG_{2a} sub-isotype is mainly associated with the stimulation of the Th1 subset of $CD4^+$ T-lymphocytes while the IgG_1 sub-isotype is mainly associated with the stimulation of the Th2 subset of $CD4^+$ T-lymphocytes,[33] it seems reasonable to speculate that LPS A1 triggers in mice an immunological response which involves both

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kinds of T-cell mediated immunity. Indeed, a very recent study in mice has shown that *E. coli* LPS B5 induces, in addition to the polyclonal activation of B cells and macrophages, a strong stimulation of T cells where both $CD4^+$ and $CD8^+$ T cells are involved.[34] Thus, when LPS A1 is presented to the immune system of a mammal with its complete supramolecular structure, via the lipid A moiety which is structurally intact although significantly silent from the toxic point of view because the blockade of its binding site by SAEP, the antigenic features are fully expressed immunogenically. Moreover, comparison of the biological properties of LPS A1 as related to the expression of toxicity and immunogenicity, appear to be clearly dissociated, in agreement with recent observations reported for the immunomodulatory activity of meningococcal LPSs expressing different degrees of toxicity by association with outer membrane proteins.[35]

The complement-dependent bactericidal characteristics of the IgG isotype antibody induced in rabbits by purified (toxic) LPS A1 have been already reported,[14] in parallel to earlier findings that meningococcal bactericidal polyclonal antibodies are present in human sera and can be absorbed out with homologous LPS.[36] Therefore, the biological functionality of the IgG antibody induced was investigated in relation to the three ideal properties searched for in a candidate anti-meningococcal vaccine, that is: (i) cross-reactive binding to LPS expressed on the surface of bacterial cells; (ii) efficiency in fixing and activating complement; and (iii) inhibition of the effects due to endotoxemia. All these desirable features were expressed by the IgG antibody population induced by LPS A1/SAEP 2 complex in either conjugated or unconjugated formulation with the T-cell dependent carrier BSA. As shown, the induced IgG antibodies were able to recognize LPS present on the surface of meningococci belonging to group A and B on the basis of the shared immunodeterminant L8;[14] to fix and activate complement of homologous and heterologous species; and to significantly inhibit the systemic production of TNF following an i.v. challenge of homologous LPS. In mice, however, complement-dependent bactericidal activity of anti-LPS A1 antibodies could not be demonstrated in some studies[14,37] and several concerns related to the conditions generally used in the bactericidal assay have been raised, which might include the source of complement.[37] In the present study, we have shown that the IgG antibody induced in mice against LPS A1 by the various formulations, are functionally fixing and activating complement from homologous and heterologous species. Thus, while other factors should be considered to explain the bactericidal characteristics of murine LPS-specific antibodies, their protective mechanism has to be further investigated even considering previous studies showing the capability of anti-LPS meningococcal antibodies to promote opsonization in the absence of complement-dependency[38] and the fact that *N. meningitidis* is strictly a human pathogen and there are no widely applicable or generally accepted animal models for disease.

The mechanism by which IgG antibodies specific for the carbohydrate chain of LPS A1 can reduce the effects of endotoxemia, as the significant inhibition of homologous LPS-induced serum TNF, is likely related to the physiological property of antibodies which contribute to the removal of antigens from the bloodstream, by complex formation. In this case, the IgG/LPS A1 complex may accelerate the clearance of LPS, partly avoiding the consequences deriving from the massive distribution of LPS from the bloodstream to target organs which results in the production of local and systemic TNF.[18] Indeed, the importance of antibodies and complement in the clearing process of LPS has been recently well-documented in antibody-deficient mice.[39] Also, similar findings have been reported on the inhibition of LPS-induced TNF production in rabbits by human immunoglobulin preparations, such inhibition resulting in the parallel suppression of LPS-induced fever and mRNA encoding for TNFα.[40] In the latter study, however, a clear demonstration on the binding specificity of the immunoglobulins for the S-LPS used in the assays (originating from *E. coli* B6) was not provided.

In conclusion, since the protective biological mechanisms of meningococcal LPS-specific antibodies may include different paths,[41] the features shown by the polyclonal population of IgG antibodies induced in this study seem to be a necessary condition for a candidate meningococcal LPS-based vaccine. Whether these features will be also considered as a sufficient condition in the prophylaxis of meningococcemia and related endotoxemia will need future human studies in order to unequivocally prove efficacy of the candidate vaccine in a host where the antibody population, source of complement, sensitivity to LPS and susceptibility to the disease can be well monitored. To this end, it may be of interest to note that the antibody response in humans to meningococcal LPS in a multicomponent experimental vaccine for group B meningococcus, containing detergent-extracted vesicles of LPS and outer membrane proteins from strain 44/76, involved mainly the IgG isotype.[42] In parallel, the IgG isotype was the most significant antibody population in the humoral response to *Salmonella typhi* LPS in humans affected by typhoid fever in endemic areas, with IgG_1 and IgG_2 being the most relevant sub-isotypes induced.[43] Comparable data have been recently reported for *Shigella* infections occurring in human subjects exposed to natural infection.[44]

It can be anticipated that human trials with this model of vaccine will not be affected by safety concerns on the basis of unwanted immunological cross reactions with

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antigens expressed in the host, as in the case of protein-capsular polysaccharide group B conjugates,[45] because LPS A1, unlike other LPS from *Neisseria* and *Haemophilus* species,[46] does not contain determinants potentially capable to induce such immunological cross-reactions.[14] Finally, the implications of these findings include fundamental practical aspects for development of a novel new generation of R- and S-LPS-based vaccines, in cases where more than one LPS is needed to cover a broad spectrum of pathogenic Gram-negative bacteria. In this case, one may consider a simple mixture of LPS/SAEP complexes as a multivalent vaccine formulation.

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