Improvement of Immunogenicity of Meningococcal Lipooligosaccharide by Coformulation with Lipidated Transferrin-Binding Protein B in Liposomes: Implications for Vaccine Development

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Among various meningococcal antigens, lipooligosaccharide (LOS) and recombinant lipidated transferrin-binding protein B (rlip-TbpB) are considered to be putative vaccine candidates against group B Neisseria meningitidis. In the present work, we report the development of a new liposome-based vaccine formulation containing both rlip-TbpB and L8 LOS. The endotoxic activity of the liposomal LOS was evaluated in vitro using the Limulus Amebocyte Lysate assay and compared to the endotoxic activity of free LOS. Above a 250:1 lipid/LOS molar ratio, liposomes were shown to effectively detoxify the LOS as the endotoxic activity of the LOS was reduced by more than 99%. Immunogenicity studies in rabbits showed that the presence of rlip-TbpB dramatically increased the immunogenicity of the LOS. While the formulation raised a strong anti-TbpB response, it elicited a higher anti-LOS IgG level than the liposomal LOS alone. Sera from rabbits immunized with rlip-TbpB/liposomal LOS displayed increased ability to recognize LOS on live bacteria expressing the L8 immunotype and increased anti-LOS–specific bactericidal activity compared to sera from rabbits immunized with liposomal LOS alone. Measurement of interleukin-8 (IL-8) produced by HEK293 cells transfected with Toll-like receptor (TLR) after stimulation with rlip-TbpB showed that the protein is a TLR2 agonist, which is in accordance with the structure of its lipid. Furthermore, an in vivo study demonstrated that the lipid moiety is not only required for its adjuvant effect but also has to be linked to the protein. Overall, the rlip-TbpB/LOS liposomal formulation was demonstrated to induce an effective anti-LOS response due to the adjuvant effect of rlip-TbpB on LOS.

Neisseria meningitidis is one of the most important causes of bacterial meningitis and septicemia worldwide in both endemic and epidemic forms. The bacteria are classified into serogroups based on the structure of their capsular polysaccharides. Thirteen different serogroups have been identified (69), but only five (A, B, C, W135, and Y) are responsible for the majority of infections although epidemic meningitis due to meningococcal serogroup X is emerging in the meningitis belt of Africa (46). Two effective quadrivalent polysaccharide–protein conjugate vaccines have been developed and licensed against serogroups A, C, W135, and Y (30, 53). In contrast to the other capsular polysaccharides, the group B polysaccharide is a poor immunogen because of structural similarities with polysialic acid chains present in human cells (22). These properties of the group B polysaccharide have impeded the development of a polysaccharide-based vaccine against group B Neisseria meningitidis (MenB) and led to the development of alternative vaccine approaches based on subcapsular antigens.

A large number of new vaccine candidates have been identified from genomics, proteomics, and immunological approaches. Among them, transferrin-binding protein B (TbpB) has been demonstrated to be a potential vaccine candidate. TbpB is an outer membrane lipoprotein presumably attached to the outer membrane through an N-linked terminal lipid anchor (41). TbpB, along with TbpA, is expressed by Neisseria meningitidis to overcome the lack of bioavailable iron within the host by acquiring its iron directly from human transferrin. Based on the antigenic and genomic features of TbpB, N. meningitidis isolates can be classified into two major families: isotype I (tbpB gene of 1.8 kb and TbpB protein with a mass of approximately 68 kDa) and isotype II (tbpB gene of 2.1 kb and TbpB protein with a mass of approximately 80 to 90 kDa). The two TbpB isotypes are not equally represented among N. meningitidis clonal complexes as isotype I is mainly restricted to the ST11 complex while isotype II is widely represented in all other major complexes responsible for meningococcal infections (56). Despite its heterogeneity, TbpB constitutes an attractive vaccine candidate since it is expressed by all clinical isolates and is not subject to phase variation. Recombinant TbpB (rTbpB) has been found to elicit serum bactericidal antibodies in preclinical studies (17) and was safe and immunogenic in phase I human clinical trials (B. Danve, personal communication).

Lipooligosaccharide (LOS) is another potential vaccine candidate since anti-LOS antibodies have been found in convalescent and normal human sera displaying bactericidal or opsonic activity (21, 33, 52, 54, 61). While 12 LOS immunotypes have been identified, at least 70% of invasive MenB strains produce L3 and L7 LOS, which contain the lacto-N-neotetraose (LnNT) structure in sialylated and nonsialylated forms, respectively (34, 74), whereas most carrier isolates are L8 (34). The LnNT structure is, however, present in glycosphingolipids of human red blood cells (44),
which raises safety concerns. As a result, most studies with LOS-based vaccines have been conducted with truncated LOS structures, among them L8 structures (75).

Various preclinical studies have been conducted on LOS conjugate vaccines and shown the efficacy of the conjugates to elicit anti-LOS-specific bactericidal antibodies (15, 26). However, the nature of the immune response seems to be strongly dependent on the LOS structure, the spacer molecule, and the coupling chemistry (15, 16, 47), which has impeded the development of a broadly effective LOS conjugate vaccine.

The relevance of LOS as a vaccine antigen has been shown in the context of clinical trials performed with outer membrane vesicle (OMV) vaccines. OMV vaccines have been developed to present antigens in their native conformations as they are similar to membrane blebs released by meningococci during natural growth. Various tailor-made OMV vaccines derived from outbreak strains have been successfully used to control serogroup B epidemics in Norway, Cuba, and New Zealand (for a review, see reference 65). OMV vaccines contain a cocktail of immunogenic antigens and have been considered an appropriate means to deliver LOS and outer membrane proteins (OMPs). While the most dominant immunogen in OMV vaccines is the highly variable outer membrane protein PorA (66), intranasal immunization in humans with native OMVs (NOMVs) that contained large amounts of fully active LOS was safe and found to induce anti-LOS antibodies with bactericidal activity (19), indicating that an effective anti-LOS response can be obtained in humans with such a vaccine. Tailor-made OMV vaccines for parenteral administration have been prepared using deoxycholate treatment to reduce LOS content and, hence, local and systemic reactogenicity. However, the concentrations of detergent typically used to extract LOS from OMVs to decrease endotoxic activity also decreased the immunogenicity of LOS as the level of anti-LOS antibodies raised by OMV vaccines generally correlated with the amount of LOS present in the vaccine (72). This has led some groups to develop either modified detergent-extracted OMVs or NOMVs from meningococcal mutant strains expressing genetically modified penta-acylated LOS (lpxl1 mutant strain) in order to generate LOS-enriched OMV vaccines with attenuated LOS toxicity (68, 72, 75). A modified detergent-extracted OMV vaccine was developed by Weynants et al. (72) from the lpxl1 mutant of strain H44/76 (L3 immunotype), which was genetically engineered to express a truncated LOS lacking either the terminal sialic acid (L7 LOS) or the terminal galactose of LnNT in order to prevent potential antigenic cross-reactivity with human antigens. In addition, PorA was deleted to avoid PorA immunodominance. In mice, the PorA-negative mutant OMV vaccine with the highest LOS content (~15%) elicited the highest anti-LOS serum antibody titers with broad bactericidal activity. In addition to decreasing the amount of LOS in OMV vaccines, detergent extraction also removes phospholipids and immunogenic lipoproteins and modifies the environment as well as the native conformation of outer membrane proteins. Thus, Zollinger et al. have developed a multivalent vaccine based on NOMVs from three epidemic strains genetically modified to ensure safety and increased breadth of protection (75). The NOMVs have three sets of antigens: LOS, PorA, and conserved outer membrane proteins (factor H-binding protein or \textit{N. meningitidis} adhesin A). The strain was modified to express penta-acylated LOS (lpxl1 mutation) with a shorter lactose alpha chain (L8 LOS) and various inner core structures, to express a second PorA, and to increase expression of at least one conserved protein. Preclinical and phase 1 clinical studies showed a broad bactericidal antibody response and indicated that antibodies to LOS are major contributors to the bactericidal activity (35, 75).

With the aim of developing an LOS-based vaccine, we considered liposomes as an attractive alternative approach to conjugates and OMVs. Liposomes have been used for many years in pharmaceutical sciences as biocompatible carriers for various drugs and antigens (60). Incorporation of meningococcal LOS into liposomes has been reported to be effective in modulating the biological activity of LOS and to significantly decrease its reactogenicity without altering its immunogenicity (50). The most effective liposomes in this regard are those comprising cationic lipids such as 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (ethyl-DOPC) (43). In addition, liposomes are very effective systems for antigen delivery as well as protein presentation (42). This has driven the development of a liposome formulation that allows presentation of meningococcal outer membrane proteins in their native conformation and that contains chemically decylated LOS as a second antigen. In a recently reported phase I clinical study, the liposome formulation was shown to be well tolerated and effective in presenting the deacylated LOS as an antigen (76).

In the present study, we report the development of a well-defined formulation based on cationic liposomes with the aim of associating two meningococcal antigens, L8 LOS and recombining lipidated TbpB (lipT-TbpB), while providing a means to decrease the toxicity of the LOS and increase the immunogenicity of the antigens. Interestingly, immunogenicity results have shown that lipT-TbpB is a potent adjuvant of anti-LOS responses. The lipoprotein was demonstrated to be a Toll-like receptor 2 (TLR2) agonist \textit{in vitro}, which is in accordance with the structure of its lipid moiety.

**MATERIALS AND METHODS**

\textit{N. meningitidis} strains. \textit{N. meningitidis} strain A1 (also called 2E, immunotype L8) is one of the strains recommended by the World Health Organization for the production of group A capsular polysaccharide. It was a gift of E. C. Gotschlich (Rockefeller University) to Institut Mérieux in May 1969. It was used in the present study to purify L8 LOS. \textit{N. meningitidis} strain B RH873 (also called M978) and 8680 both express the L8 immunotype and belong, respectively, to the ST41/ST44 and ST32 complexes. They were kindly provided by L. Saarinen and D. Caugant (NIPH, Norway), respectively.

Purification and characterization of L8 LOS. L8 LOS from \textit{N. meningitidis} strain A1 was purified from broth-grown cells by the hot phenol-water extraction procedure (71). The LOS structure was determined by oligosaccharide (OS) carbotyping using high performance anion exchange chromatography with pulsed amperometry detection (HPAEC-PAD) (48). Briefly, OSs were isolated from whole cells by mild acid hydrolysis with 1% acetic acid at 100°C for 1 h. After cell debris was removed by centrifugation, acid was removed under a stream of nitrogen, and the samples were redissolved in water and passed through a centrifugal filter unit (10-kDa molecular-mass cutoff; Ultrafree Biomax, Millipore) to remove proteins and other macromolecules. The OSs were separated on a Dionex system using a CarboPac PA1 (4 mm by 250 mm) analytical column (Dionex, Sunnyvale, CA) with a guard column (4 mm by 50 mm) previously equilibrated in 80 mM sodium acetate (NaOAc) in 75 mM NaOH at a flow rate of 1 ml/min. The OSs were separated isocratically using 80 mM NaOAc in 75 mM NaOH for 15 min followed by a linear gradient to 400 mM NaOAc in 75 mM NaOH over 50 min. The localization of phosphoethanolamine (PEA) substituents was determined by nu-
clear magnetic resonance (NMR) spectroscopy as previously described (49).

Overexpression and purification of recombinant lipated TbpB and histidine-tagged nonlipidated TbpB. Recombinant lipidated TbpB (rip-TbpB) of MbnE strains M982 and B16B6 and histidine-tagged nonlipidated TbpB (His-rTbpB) of strain M982 were overexpressed in *Escherichia coli* and purified as described previously (37, 38, 39) with the exception that 2% Elugent (vol/vol) (Calbiochem, San Diego, CA) was used instead of 0.005% Zwittergent during the last step of rip-TbpB purification.

Liquid-phase digestion and MALDI-TOF MS analyses of strain M982 rip-TbpB. First, M982 rip-TbpB was reduced and alkylated in 3 M urea at pH 8.5. The sample was then mixed with trypsin (Promega, Co., Madison, WI) at a 50:1 ratio (wt/wt) in 0.3 M urea–50 mM ammonium bicarbonate, pH 8.5, and incubated at 37°C overnight. The reaction was stopped by the addition of trifluoroacetic acid (TFA) to a final concentration of 0.2%. The trypptic peptides were purified by reverse-phase extraction with C4 ZipTip (Millipore, Molsheim, France) prior to matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). Mass analyses were performed with a MALDI-TOF mass spectrometer (AutoFlex II; Bruker Daltonique SA, Wissembourg, France) set on the positive reflectron mode. The same spectrometer was used for tandem mass spectrometry (MS/MS) analysis. Saturated α-cyano-4-hydroxy-cinnamic acid in acetonitrile–0.1% trifluoroacetic acid (1:2, vol/wt) was used as a matrix. Peptides were identified from MS data using the Biotools software (Bruker Daltonique SA), and peptide sequences were identified using the Swiss Institute of Bioinformatics (SIB) BLAST database.

Incorporation of LOS into liposomes. Small cationic liposomes were prepared by the Bangham method (5) from ethyl-DOPC and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (Avanti Polar Lipids, Inc., Alabaster, AL) in a 3:2 molar ratio, and LOS was incorporated using a detergent-removal method. Briefly, 0.3 mol of ethyl-DOPC and 0.2 mol of DOPE in chloroform were evaporated to complete dryness, resuspended in 30 ml of a 10 mM Tris buffer, pH 7.0, and bath sonicated until the formation of a homogeneous suspension of small unilamellar liposomes. The liposomes were then disrupted by adding 3.33 ml of a 1 M solution of octyl-β-D-glucopyranoside (OG) Sigma-Aldrich, St. Quentin Fallavier, France) in 10 mM Tris buffer, pH 7.0, so as to obtain mixed ethyl-DOPC/DOPE/OG micelles that were aseptically filtered through a 0.45-μm-pore-size Millex HV membrane (Millipore, Molsheim, France). Liposomes comprising increasing amounts of LOS, ranging from a 25:1 to 400:1 lipid/LOS molar ratio were then prepared by adding various amounts of a sterile solution of purified LOS at 1 mg/ml in 10 mM Tris–100 mM OG, pH 7.0, to the mixed ethyl-DOPC/DOPE/OG micelles followed by OG removal by dialysis against 10 mM Tris, pH 7.0, under aseptic conditions. Thiometers and NaCl were then added from sterile concentrated stock solutions so as to obtain LOS-containing cationic liposomes in 10 mM Tris, 150 mM NaCl, and 0.001% thiomersal, pH 7.0 (formulation 1).

In the second method, the protein was mixed with the cationic vesicles. Briefly, Elugent contained in rip-TbpB buffer was removed by incubation with activated Biobeads as described above, and the protein was then mixed with preformed LOS liposomes at a protein-to-LOS ratio of 1:1 (wt/wt). Thiometers and NaCl were then added from sterile concentrated stock solutions so as to obtain LOS-containing liposomes in 10 mM Tris, 150 mM NaCl, and 0.001% thiomersal, pH 7.0 (formulation 2).

The protein–containing liposome suspensions were stored at 5°C. The size of the liposomes was determined by dynamic light scattering on a Malvern Zetasizer nano-ZS (Malvern Instruments, Worcestershire, United Kingdom). Phospholipids were quantified by reverse-phase high-performance liquid chromatography (RP-HPLC) using a Zorbax C18 Extend column (5-μm particle size; 3 by 150 mm) (Agilent, Santa Clara, CA) using isocratic elution with acetonitrile-water-TFA at 95/5/0.1 at a flow rate of 0.5 ml/min and UV detection at 200 nm. The total content of LOS in liposomes was determined after extraction of the phospholipids in chloroform-methanol at 2:1 (vol/vol) through quantification of 3-keto-deoxyoctulosonic acid (KDO) by HPAEC-PAD according to Kiang et al. (36). The decrease in LOS toxicity was evaluated by the *Limulus* Amebocyte Lysate (LAL) Endosafe KTA kit (Charles River, Wilmington, MA) and by measuring in *vitro* interleukin-6 (IL-6) release from human peripheral blood mononuclear cells (PBMC). SDS-PAGE with densitometric analysis of the bands was used to assess the integrity of rTbpB and to estimate the protein content in the liposomal LOS formulation. Association of rip-TbpB to the liposomes was evaluated after purification of the liposomes by ultrafiltration on a discontinuous sucrose gradient (28).

**Immunization of rabbits.** Seven-week-old female New Zealand KBL rabbits (Charles River Laboratories, St. Germain-sur-l’Arbresle, France), 2.2 to 2.5 kg, were first housed for 2 weeks before immunization. Animals were housed and handled according to European regulations. All protocols were approved by the Sanofi Pasteur animal care committee.

Animals received a dose of 0.5 ml in two 0.25-ml intramuscular (i.m.) injections at two different anatomical sites on two occasions 3 weeks apart. Sera were sampled at the time and 2 weeks after the second immunization. Unless specified in the tables and graphs, animals received 40 μg of L8 LOS and/or 40 μg of rip-TbpB.

**Anti-TbpB ELISA.** Serum IgG antibodies directed against rTbpB were titrated by a manual enzyme-linked immunosorbent assay (ELISA) according to the following procedure. Dynex 96-well microplates (Dynex Technologies VWR International, Fontenay, France) were coated overnight at 4°C with 200 ng/well of rip-TbpB in 0.05 M sodium carbonate–bicarbonate buffer, pH 9.6 (Sigma-Aldrich, Saint Quentin Fallavier, France). The plates were then blocked for 1 h at 37°C with 150 μl/well of PBS, pH 7.1, 0.05% Tween 20, and 1% powdered skim milk (wt/vol) (Difco, West Chester, PA), (PBS-Tween-milk). All subsequent incubations were carried out in a final volume of 100 μl, followed by four washings with PBS (pH 7.1)—0.05% Tween 20.

Serial 2-fold dilutions of serum samples, performed in PBS-Tween-milk, starting from 1/100 or 1/1,000, were added to the wells and incubated for 90 min at 37°C. After the plates were washed, anti-rabbit IgG peroxidase conjugate (Sigma-Aldrich, St. Louis, MO) diluted in PBS-Tween-milk at 1/40,000 was added, and the plates were incubated for 90 min at 37°C. The plates were further washed and incubated in the dark for 30 min at room temperature with 100 μl/well of a ready-to-use 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution (Tebu Bio, Le Perray en Yveline, France). The reaction was stopped with 100 μl/well of 1 M HCl.

Optical density (OD) was measured at 450 nm to 650 nm with a plate reader (Versa Max Molecular Device, Saint Gregory, France). The blank (mean value for negative controls) was subtracted from the data. The IgG antibody titers were calculated using Codunit software for an OD value range of 0.2 to 3.0 from a standard titration curve obtained from the titration of rabbit hyperimmune serum on each plate. The IgG titer of this

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standard curve, expressed in arbitrary ELISA units (EU), had previously been determined in several experiments and was defined as the log$_{10}$ arithmetic mean of the reciprocal dilution giving an OD of 1.0 in each of the experiments.

The threshold of antibody detection was 1.3 (log$_{10}$ EU). For each titer inferior to this threshold, an arbitrary value of 1.0 was assigned.

Anti-LOS ELISA. An ELISA of LOS-specific antibodies in the serum samples was performed by a robotic application (Staccato robot; Caliper Life Science Villepinte, France) according to the following protocol. Dynex 96-well microplates were coated for 2 h at 37°C and overnight at 4°C with 1 μg of L8 LOS, in PBS and 10 mM MgCl$_2$, pH 7.1. The plates were then blocked for 1 h at 37°C with 150 μl of PBS–0.05% Tween 20–1% (wt/vol) powdered skim milk. All subsequent incubations were carried out in a final volume of 100 μl, followed by three washings with PBS–0.05% Tween 20.

Serial 2-fold dilutions of the samples performed in PBS–Tween milk (starting from 1/40) were added to the wells and incubated for 90 min at 37°C. After three washings, an anti-rabbit IgG peroxidase conjugate diluted 1/10,000 in PBS–Tween milk was added, and the plates were incubated for 90 min at 37°C. The plates were further washed three times and incubated in the dark for 20 min at room temperature with 100 μl per well of a ready-to-use TMB substrate solution. The reactions were stopped with 100 μl of 1 M HCl.

Flow cytometry analysis of LOS expression. The ability of polyclonal antiserum elicited by the recombinant proteins and LOS to bind to the surface of live MenB strains was determined using flow cytometric detection of indirect fluorescence. Strains RH873 and 8680 were grown overnight at 37°C with 10% CO$_2$ on brain heart infusion (BHI) agar (Difco, Detroit, MI). The fluorescent staining of bacteria was analyzed after 2.5 h, which corresponds to early exponential growth phase. The bactericidal activity of purified rabbit IgG and mouse serum was evaluated using pooled baby rabbit serum as a source of complement, as described earlier, with slight modifications (57). Briefly, 50 μl of 2-fold serial dilutions of IgG solutions or serum was added to 96-well microtiter plates (Nunc) and incubated with 25 μl of a meningococcus suspension adjusted to 2 × 10$^4$ CFU/ml and 25 μl of baby rabbit complement. After 1 h of incubation at 37°C, 20 μl of the mixture from each well was plated onto Mueller-Hinton agar (MHA) plates. The plates were incubated overnight at 37°C in 10% CO$_2$. The bactericidal titer of each serum or IgG preparation was expressed as the inverse of the last dilution of serum at which ≥50% killing was observed compared to the control complement. For complement-sensitive strains, the complement was adsorbed on formaldehyde-fixed bacteria (10$^7$ CFU [3 ml of complement]) before use.

HEK293/TLR experiments. HEK293 cells expressing human TLRs were used to assess the role and nature of TLR signaling in the immunostimulatory effect of rlip-TbpB. Cell lines expressing various TLRs were used: HEK293 TLR1/TLR2 (293-htlr1/2; Invivogen, San Diego, CA), HEK293 TLR2/TLR6 (293-htlr2/6; Invivogen), and HEK293 TLR4/MD2 (obtained by transfection in Sanofi Pasteur). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) Gibco-BRL/Invitrogen, Villebon sur Yvette, France) supplemented with 10% fetal calf serum (FCS) and 1% penicillin-streptomycin. After the first two to three passages, basicidalin (Invivogen) was used as a selection antibiotic (1 μl/ml).

The cells were cultured 24 h before stimulation in 96-well round-bottom plates (50 000 cells/ml in 200 μl of DMEM without basicidalin). Various stimulating agents were tested at different concentrations, as follows: LOS alone at 0.1, 1, 10, 100, and 1,000 ng/ml; the TLR2/TLR1 agonist Pam3CAS (Bachem, Weil am Rhein, Germany) at 10, 100, and 500 ng/ml; the TLR2/6 agonist Pam2CSK4 (Invivogen, San Diego, CA) at 10, 100, and 500 ng/ml; M982 rlip-TbpB in PBS–0.2% Elugent (vol/vol) at 0.001, 0.01, 0.025, 0.05, 0.1, and 1 μg/ml; M982 His-rTbpB in PBS–10% glycerol at 0.01, 0.025, 0.05, 0.1, and 1 μg/ml; M982 His-rTbpB in PBS–10% glycerol (wt/vol)–2% Elugent (vol/vol) at 0.001, 0.01, 0.025, 0.05, 0.1, and 1 μg/ml; M982 His-rTbpB in PBS–10% glycerol (wt/vol)–2% Elugent (vol/vol) at 0.001, 0.01, 0.025, 0.05, 0.1, and 1 μg/ml; and medium. After stimulation for 24 h, IL-8 was measured in the supernatants using an ELISA (BD OptEIA set human IL-8; BD Bioscience Pharmingen, San Diego, CA).

Statistical analysis. Comparisons between groups were performed using a t test for antibody titers and a Mann-Whitney rank-sum test for serum bactericidal titers.

RESULTS

L8 LOS of strain A1 is a di-PEA-substituted LOS. L8 was chosen as an LOS prototype to assess the liposome formulation since it is devoid of the LnNT structure. To avoid any residual capsule B, it was purified from serogroup A strain A1 (25) in which it has been shown that expression of the L8 immunotype is stable due to a frameshift mutation in the lgtA gene (73). Carbotyping by HPAEC-PAD provided more detailed information on its OS structure since the analysis clearly demonstrated the presence of two glycoforms: a mono-PEA-substituted OS and a di-PEA-substituted OS. To de-
was measured by LAL assay and compared with the endotoxic activity of free L8 LOS (Fig. 2). The results showed an increase in the detoxification rate with an increase in the lipid/LOS ratio. From a 250:1 lipid/LOS ratio, the LAL activity of the liposomal L8 LOS could be reduced to a plateau value of 80 EU/g, corresponding to a greater than 99.0% reduction of its original value (13,070 EU/g). The 250:1 ratio was then selected for further experiments. The potency of the cationic liposomes to significantly decrease the toxicity of LOS was also confirmed by measuring in vitro IL-6 release from human PBMCs. In the latter experiments, liposomal L8 LOS was shown to be 1,000 times less potent than free LOS in inducing IL-6 secretion (data not shown).

Liposomal LOS is a well-defined formulation. To adjust the LOS dose used for animal testing, the concentration of liposomal L8 LOS was measured and found to range from 115 to 118 EU/ml. Depending on the batch produced, the liposome size ranged from 55 to 68 nm with a polydispersity ranging from 0.44 to 0.45. When rlip-TbpB was coformulated with liposomal L8 LOS, the concentration of the sample was adjusted to contain 40 µg of protein and 40 µg of LOS per dose injected. Association of the protein with liposomes was evaluated after ultracentrifugation of the samples on sucrose cushion. Protein concentration was estimated by quantitative densitometry of SDS-PAGE gel, and the values obtained were normalized after quantification of lipids in the liposome sample recovered from ultracentrifugation. In all cases, protein association to liposomal L8 LOS ranged from 90 to 100%.

Strain M982 rlip-TbpB is mainly a triacylated lipoprotein. To characterize the lipidation of rlip-TbpB, the purified protein was digested with trypsin, and the tryptic peptides were analyzed by MALDI-TOF MS. The mass spectrum obtained revealed the presence of several posttranslational lipid modifications (Table 1 and Fig. 3). The peaks at m/z 2,837.65, 3,075.89, and 3,103.87 were further analyzed and identified by MS/MS. Following fragmentation, the peptide sequence detected was identical for the four lipopeptides and was identified as the N-terminal sequence of TbpB (data not shown). These results confirmed the lipidation of rlip-TbpB at its N terminus.

The lipid-modified peptides differed in the numbers and lengths of their acyl chains and indicated that the protein is di- and triacylated. To adjust the LOS dose used for animal testing, the concentration of liposomal L8 LOS was measured and found to range from 115 to 118 µg/ml. Depending on the batch produced, the liposome size ranged from 55 to 68 nm with a polydispersity ranging from 0.44 to 0.45. When rlip-TbpB was coformulated with liposomal L8 LOS, the concentration of the sample was adjusted to contain 40 µg of protein and 40 µg of LOS per dose injected. Association of the protein with liposomes was evaluated after ultracentrifugation of the samples on sucrose cushion. Protein concentration was estimated by quantitative densitometry of SDS-PAGE gel, and the values obtained were normalized after quantification of lipids in the liposome sample recovered from ultracentrifugation. In all cases, protein association to liposomal L8 LOS ranged from 90 to 100%.

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triacylated. The major peak was detected at m/z 3,075.87, which could be assigned to the 23-residue-long N-terminal sequence of TbpB modified by three C16 acyl chains, suggesting that the major modifying lipid of recombinant TbpB is N-palmitoyl-S-(1-2-di-palmitoyl)glyceryl-cysteinyl (Pam3-Cys). The mass measured was 2 Da less than the calculated mass for all lipopeptides containing diacylglyceryl cysteine (Table 1). Thus, we could deduce that the diacylglyceryl moiety contains an unsaturated bond.

In summary, the MS/MS results of the tryptic digests of rlip-TbpB confirmed that the protein is lipidated through its N-terminal cysteine and is a di- and triacylated protein, where the triacylated protein is the major form.

**rlip-TbpB is an adjuvant for anti-LOS responses in rabbits.**

In the first experiment, 40 μg of rlip-TbpB was combined with 40 μg of liposomal L8 LOS. L8 LOS and rlip-TbpB (isotype II from strain M982) were coformulated at the time of preparation of liposomal L8 LOS (formulation 1), or rlip-TbpB was mixed to the preformed liposomal LOS (formulation 2). Serum IgG levels were measured by ELISA after each immunization. First, the results confirmed the strong immunogenicity of rlip-TbpB. In addition, a potent adjuvant effect of rlip-TbpB was evidenced on anti-L8 LOS IgG titers, whatever the formulation used, compared to the response induced by liposomal L8 LOS alone (Fig. 4). The positive adjuvant effect was seen on the IgG response after the first and second immunizations. On the other hand, no adjuvant effect of LOS was observed on anti-TbpB IgG titers under the conditions of the present test, which used high doses of rlip-TbpB. Similar studies carried out in mice confirmed the adjuvant effect of rlip-TbpB on L8 LOS in another species (data not shown).

A dose-ranging study was then performed, using three different doses of rlip-TbpB, still with 40 μg of liposomal L8 LOS. As in the previous experiment, 40 μg of rlip-TbpB increased the anti-L8 LOS IgG titers by more than 10-fold compared to liposomal L8 LOS alone. Similarly, 10 μg and 2.5 μg of rlip-TbpB increased anti-L8 LOS IgG titers but to a lesser degree. In all three groups, the difference in relation to the group that received liposomal L8 LOS alone was statistically significant (Table 2).

Interestingly, an additional experiment showed the same statistically significant adjuvant effect of the second isotype of TbpB (isotype I from strain B16B6) on anti-L8 LOS IgG titers (Table 3).

Liposomal L8 LOS in combination with rlip-TbpB is able to induce specific antibodies that recognize the surface of MenB strains and are bactericidal. In an attempt to determine if functional antibodies could be generated against L8 LOS, rabbit serum raised against liposomal L8 LOS with or without rlip-TbpB was tested against two strains, 8680 and RH873, for which L8 LOS expression was detected by cytometry analysis using the monoclonal antibody (MAb) L8. Strains 8680 and RH873 (Fig. 5) were
TABLE 2 Dose effect of rlip-TbpB on anti-L8 LOS IgG responses in individual rabbits

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<th>Liposome formulation</th>
<th>Anti-L8 LOS titer*</th>
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</tr>
<tr>
<td>Liposomal L8 LOS without adjuvant*</td>
<td>3.30</td>
<td>2.55</td>
<td>2.90</td>
</tr>
<tr>
<td></td>
<td>3.55</td>
<td>2.83</td>
<td>2.88</td>
</tr>
<tr>
<td>Liposomal L8 LOS with M982 rlip-TbpB at (µg):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>3.53</td>
<td>3.59</td>
<td>3.29</td>
</tr>
<tr>
<td></td>
<td>3.60</td>
<td>3.59</td>
<td>3.29</td>
</tr>
<tr>
<td></td>
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<td>3.94</td>
</tr>
<tr>
<td>40</td>
<td>4.16</td>
<td>3.92</td>
<td>3.94</td>
</tr>
</tbody>
</table>

* ELISA titers were determined in individual rabbit serum samples after two injections of 40 µg of liposomal L8 LOS and various doses of rlip-TbpB at a 3-week interval. Blood sampling was performed 2 weeks after the second injection. The titer is expressed as the log₁₀ titer.
b GMT, geometric mean titer.
c Statistical analysis was performed using a t test. Groups were compared to the liposomal L8 LOS reference group (*) containing no M982 rlip-TbpB.

grown on BHI medium without addition of the iron-chelating agent (Desferal) in order to detect LOS and not TbpB expression. The data obtained on strain RH873 (Fig. 5A) clearly indicated that TbpB is not detected under the growth conditions used. As limited detection of L8 LOS was observed on bacteria using serum raised against liposomal L8 LOS, rlip-TbpB is needed in the formulation to increase L8 LOS recognition at the bacterial surface. In addition, the data obtained on strain 8680 (Fig. 5B) indicated that rlip-TbpB from both strain M982 (isotype II) and strain B16B6 (isotype I) are effective in improving L8 LOS recognition at the bacterial surface by serum raised against the rlip-TbpB/liposomal L8 LOS formulation. The bactericidal activity of the rabbit serum was determined against strain RH873. The increase in L8 LOS recognition due to the adjuvant effect of rlip-TbpB on LOS immunogenicity resulted in an increase in serum bactericidal activity (SBA) on strain RH873. The major portion of the bactericidal activity against RH873 could be attributed to anti-L8 LOS antibodies since the level of bactericidal activity was weak when rlip-TbpB was injected alone (Table 4). The low level of anti-TbpB-specific bactericidal antibodies can be explained by the fact that RH873 expresses a heterologous variant of TbpB compared to the one used in the vaccine formulation (58). There was a positive correlation between anti-L8 LOS IgG titers and the bactericidal titers of the sera (Fig. 4).

**rlip-TbpB is a TLR2 agonist in vitro, which requires its lipid moiety.** Because TbpB is lipidated, we investigated whether or not it could activate cells through TLR2, as do other lipoproteins. Experiments were carried out on HEK293 cells expressing TLR1/2 or TLR2/6 heterodimers or TLR4/MD2 complex. Lipidated and nonlipidated TbpB proteins were used to address the role of lipoprotein activation. Synthetic TLR2 agonists (Pam2 and Pam3) or LOS was used as positive controls for activation of TLR2-positive (TLR2⁺) or TLR4⁺ cells, respectively. As shown in Fig. 6, rlip-TbpB can trigger IL-8 production in TLR1/2⁺ or TLR2/6⁺ cells, as do synthetic Pam2CSK4 and Pam3CAG, while no significant secretion

TABLE 3 Rabbit anti-L8 LOS and anti-rlip-TbpB (B16B6) IgG responses

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Anti-L8 LOS titer*</th>
<th>Anti-TbpB (B16B6) titer*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Predose</td>
<td>Postdose 2</td>
</tr>
<tr>
<td>Liposomal L8 LOS*</td>
<td>1.00</td>
<td>3.07</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>2.75</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>2.58</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>2.94</td>
</tr>
<tr>
<td>GMTb</td>
<td>1.00</td>
<td>2.83</td>
</tr>
<tr>
<td>B16B6 rlip-TbpB/liposomal L8 LOS*</td>
<td>1.00</td>
<td>4.27</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>3.98</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>3.87</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>3.96</td>
</tr>
<tr>
<td>GMTb</td>
<td>1.00</td>
<td>4.02</td>
</tr>
</tbody>
</table>

* ELISA titers were determined in individual rabbit serum sera after two injections of 40 µg of liposomal L8 LOS with or without 40 µg of B16B6 rlip-TbpB (isotype I) at a 3-week interval. Blood sampling was performed 2 weeks after the second injection. The titers are expressed as log₁₀ titer.
b GMT, geometric mean titer.
c Statistical analysis was performed using a t test, and comparison was made to the liposomal L8 LOS reference group (*) containing no B16B6 rlip-TbpB. For anti-L8 LOS titers, P = 0.001; for anti-TbpB titers (strain B16B6), P = 0.001.

FIG 5 Flow cytometry analysis of the binding of rabbit sera to live cells of N. meningitidis strain RH873 (A) or strain 8680 (B1 and B2). Binding of rabbit sera raised to empty liposomes was used as a negative control (black line on each graph). (A) Binding of rabbit sera to M982 rlip-TbpB (light gray), to liposomal L8 LOS (dark gray), or to M982 rlip-TbpB/liposomal L8 LOS (black). (B1) Binding of rabbit sera to M982 rlip-TbpB mixed with empty liposomes (light gray) or to M982 rlip-TbpB/liposomal L8 LOS (black). (B2) Binding of rabbit sera to B16B6 rlip-TbpB mixed with empty liposomes (light gray) or to B16B6 rlip-TbpB/liposomal L8 LOS (black).
TABLE 4 Bactericidal activity of individual rabbit sera against N. meningitidis strain RH873

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Predose</th>
<th>Postdose 2</th>
<th>P&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>PBS</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td></td>
</tr>
<tr>
<td>GMT</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td></td>
</tr>
<tr>
<td>Empty liposomes</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td></td>
</tr>
<tr>
<td>Liposomal L8 LOS*</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Liposomal L8 LOS (formulation 1)</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Liposomal L8 LOS (formulation 2)</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>GMT</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Mix of liposomal L8 LOS and M982 rlip-TbpB (formulation 2)</td>
<td>&lt;4</td>
<td>32</td>
<td>&lt;4</td>
<td>64</td>
</tr>
<tr>
<td>GMT</td>
<td>&lt;4</td>
<td>128</td>
<td>152</td>
<td>0.029</td>
</tr>
<tr>
<td>M982 rlip-TbpB/liposomal L8 LOS</td>
<td>&lt;4</td>
<td>128</td>
<td>76</td>
<td>0.029</td>
</tr>
<tr>
<td>M982 rlip-TbpB</td>
<td>&lt;4</td>
<td>128</td>
<td>76</td>
<td>0.029</td>
</tr>
<tr>
<td>GMT</td>
<td>&lt;4</td>
<td>76</td>
<td>152</td>
<td>0.029</td>
</tr>
</tbody>
</table>

<sup>a</sup> Each rabbit received two i.m. injections of one of the formulations at a 3-week interval. Protein and LOS doses were both 40 μg. Blood sampling was performed 2 weeks after the second injection. Bacteria were grown with chelating agent in order to express TbpB.

<sup>b</sup> Bactericidal activity is expressed as the reciprocal value of the serum dilution yielding 50% killing of bacteria. Titers corresponding to a greater than 4-fold increase over the corresponding preimmune titers are highlighted in bold.

<sup>c</sup> For GMT calculations and comparisons, values of <4 were considered as 2. Statistical analysis was performed using a Mann-Whitney rank-sum test. Groups were compared to the liposomal L8 LOS reference group (*) containing no rlip-TbpB.

was observed after stimulation by nonlipidated TbpB or LOS. On the other hand, rlip-TbpB or synthetic Pam2CSK4 and Pam3CAG agonists did not induce significant IL-8 levels in TLR4/MD2 cells while LOS did, as expected. These data demonstrate that rlip-TbpB is a TLR2 agonist through its lipid moiety.

The lipid moiety of rlip-TbpB is required for in vivo LOS adjuvanticity while a simple synthetic TLR2 agonist is not active. We then investigated whether or not the lipid moiety was also required for adjuvanticity in vivo and if a synthetic TLR2 agonist could be as effective as rlip-TbpB in this respect.

As shown in Table 5, we confirmed the adjuvant effect of rlip-TbpB on SBA responses against strain RH873 due to anti-L8 LOS-specific antibodies, although at lower levels than in previous experiments (close to statistical significance). However, no difference was seen between the groups having received liposomal L8 LOS alone, in combination with nonlipidated TbpB, or with a synthetic TLR2 agonist (Pam2CSK4). Moreover, the combination of nonlipidated TbpB with the synthetic TLR2 agonist was similarly ineffective, showing the importance of having the lipid moiety directly linked to the protein antigen.

**DISCUSSION**

LOSs of *N. meningitidis* have been considered potent vaccine candidates for the last 2 decades (16, 26, 70, 72, 75). They must be detoxified before being used as vaccine antigens. The lipid A moiety is responsible for the toxicity of LOS, and the removal of only one acyl chain is sufficient to decrease the toxicity of the molecule. Genetically constructed penta-acylated LOS (lpxl1 LOS) has been extensively used as a nontoxic alternative to LOS and has been shown to retain immunogenicity and adjuvant activity in mice (67). In order to mimic natural presentation of antigens at the surface of *N. meningitidis*, OMV vaccines, in which LOS alone is considered the antigen candidate or is part of a cocktail of antigens, have been developed from strains expressing lpxl1 LOS (68, 75). However, Steeghs et al. (63) reported that the lpxl1 LOS does not activate the human TLR4/MD2 complex and may even act as an antagonist of wild-type LOS in humans (62). For these reasons, use of wild-type LOS as a human vaccine antigen should be more advantageous, provided that it is effectively detoxified. Although previous studies suggested that incorporation of wild-type LOS into liposomes was effective in decreasing its toxicity (2, 7, 18, 50), none of these studies used cationic ethyl-DOPC/DOPE liposomes. In some preliminary experiments, we found this particular liposomal composition to be much more effective to this end than standard neutral liposomal formulations (29). In the present study, we have shown that incorporation of wild-type L8 LOS into cationic ethyl-DOPC/DOPE liposomes decreased the toxicity of LOS by more than 99%.

In addition, liposomes can easily incorporate lipidated proteins allowing us to coformulate LOS with rlip-TbpB in liposomes. We thus combined the two antigens in the same liposomes, with the aim of delivering them together to the same antigen-presenting cells and to study how this would affect immune responses. Although previous studies reported the incorporation of wild-type LOS into liposomes, with LOS acting as an adjuvant on antiprotein responses (3, 4), none of these studies ever demonstrated that the meningococcal protein could act as an adjuvant on the anti-LOS response. Surprisingly, we found that rlip-TbpB was a potent adjuvant for anti-LOS responses when it was coformulated with LOS in liposomes, but we did not observe an adjuvant effect of LOS on the anti-TbpB response. We attributed the lack of adjuvant effect of LOS to the fact that rlip-TbpB on its own is highly immunogenic in rabbits at the dose used in our formulation. Indeed, when we used lower doses of rlip-TbpB (i.e., 0.6 μg) in subsequent studies, we were able to demonstrate an adjuvant effect of LOS on the lower doses of the protein (data not shown).

Conversely, we speculated that the potent adjuvant effect of rlip-TbpB on anti-LOS responses could result from the ability of the bacterial lipoprotein to stimulate TLR2, although at this stage we cannot rule out the possibility that the lipidated antigen may act through a TLR2-independent pathway. In any case, we first confirmed that rlip-TbpB was a TLR2 agonist in vitro and that the lipid moiety was required for such activity. It is of note that other lipidated bacterial proteins, such as the OspA antigen of *Borrelia*...
burgdorferi, have also been demonstrated to be potent systemic and mucosal adjuvants (20). The adjuvant effect of lipidated TbpB observed in vivo on anti-LOS responses was also dependent on the lipid moiety; however, we observed that a synthetic TLR2 agonist (Pam2CSK4) was not active in this regard. This suggests that the protein moiety of rlip-TbpB also plays a role, by providing T-helper (Th) epitopes or another additive signal. This hypothesis is in line with a recent paper (40), showing that a lipidated protein (recombinant lipidated DEN3 construct) had a better stimulating effect in vitro on mouse spleen cells than a synthetic peptidic agonist (Pam3), inducing, in particular, a different gene expression profile. A simple mixture of a synthetic TLR2 agonist (Pam2CSK4) with the nonlipidated protein is not active as an adjuvant for LOS, suggesting that a covalent link between the protein and the lipid moiety is required. This may anchor the protein more strongly in the liposome, augmenting the chance of codelivering the protein and the LOS to the same cells. Thus, the rlip-TbpB/LOS liposomal formulation can be considered a “virtual conjugate.”

It is indeed required that the same polysaccharide-specific B cell recognizes the polysaccharide antigen (in our case LOS) and presents the peptides derived from the protein carrier to specific Th cells, in order to turn a T-independent (Ti) antigen into a T-dependent (Td) antigen. A liposomal formulation, such as the one used here, could thus constitute a kind of immunostimulatory LOS “conjugate” vaccine, delivering LOS, the T-helper component, and the TLR adjuvant in the same vehicle. Such liposomes can trigger responses through both TLR4 and TLR2, acting in synergy in cells coexpressing these receptors, for example, dendritic cells (DCs). PorB from N. meningitidis, which is a nonlipidated TLR2 agonist, has also been shown to improve the protective responses induced in mice after immunization with Francisella tularensis LOS (12, 13). However, this effect was observed only on IgM levels, and the formulations used in our study seem to be more effective in this respect.

The presence of an additional signal emanating from a pathway other than TLR4 may be even more critical to induce significant anti-LOS responses in humans. In fact, the pattern of TLRs expressed by B cells varies between species; in particular, naive or memory human B cells, unlike mouse B cells (24, 31), do not express TLR4 and are not activated by LOS (32, 59; also our personal observations). The status of rabbit B cells in this respect is unknown, but their poor in vivo responses to LOS alone suggest that they more closely resemble human B cells and would not be stimulated directly by LOS due to the absence of TLR4 expression.

In normal nonactivated human B cells, TLR2 expression is known to be very low, while TLR4 expression is known to be absent (23, 32). However, it has been shown that naive human B cells can be directly activated through TLR2 to produce cytokines and chemokines (1). In any case, various B cell subsets can be stimulated by TLR2 agonists: partially differentiated B cells (23), tonsillar B cells (45), or B cells preactivated by costimulatory signals such as provided by Staphylococcus aureus protein A (6) or...
T-cell help (59). Of particular note with regard to our present work the study from Ruprecht and Lanzavecchia (59) demonstrated that a combination of T-cell help, B-cell receptor (BCR) stimulation, and some TLR stimulation was able to efficiently promote B-cell activation, leading to proliferation, isotypic switch, and differentiation to Ig-secreting cells; importantly, among TLR agonists, TLR2 but not TLR4 agonists were able to do so. In fact, other signals are needed to stimulate B-cell activation and differentiation into IgG(M)-secreting plasmocytes (8, 9, 10, 59), in addition to B-cell receptor stimulation. Thus, as a single stimulus alone (BCR stimulation, TLR stimulation, or T-cell help) is not able to trigger LOS-specific B-cell responses, synergistic combinations of these three signals are required, which may indeed be the case for the rlip-TbpB/L8 LOS liposomal formulation used in our study.

In particular, it can thus be postulated that a TLR2 agonist would compensate for the absence of TLR4 stimulation by the LOS on B cells, assuming that (naive) marginal-zone B cells react in the same manner as “classical” B2 cells. Moreover, in various experiments in rabbits performed in-house, complete Freund’s adjuvant followed by incomplete Freund’s adjuvant induced high IgG and bactericidal antibody titers to LOS, while a TLR4 agonist was unable to do so. Freund’s adjuvant contains, among other active components, TLR9 and TLR2 agonists, therefore supporting our hypothesis.

In any case, a second TLR agonist could synergize with the signals induced through TLR4, e.g., on dendritic cells, as demonstrated by several examples in the literature with protein antigens (for a review, see reference 27). Given the intrinsic capacity of LOS to stimulate TLR4, adding a second TLR agonist could be beneficial at the DC and/or B-cell level: at the DC level, it could synergize with LOS on DCs and enhance their activation; at the B-cell level, it could substitute for an absent TLR4-induced signal in species in which B cells do not express that receptor. All in all, the stimulation of both DCs and B cells could potentially induce a higher response. In agreement with this hypothesis, it has been shown that multiple TLRs must be involved following immunization in order to induce optimal responses against a detoxified LOS-group B meningococcal OMP complex (11).

The rlip-TbpB/L8 LOS liposomal formulation provided a strong homologous anti-L8 LOS bactericidal response. In our study, serum bactericidal activity was evaluated using rabbit complement, as rabbit complement has been widely used to assess MenB vaccine candidates at the preclinical stage, among them antigens that are now included in the MenB vaccine recently submitted for licensing in Europe (51). Our structural analysis of L8 LOS produced by \textit{N. meningitidis} serogroup A (MenA) strain A1 showed that the LOS contains a di-PEA-substituted HepII. In contrast, L8 LOS from the MenB strains used for the bactericidal assay and cytometry analyses (strains RH873 and 8680) was shown to contain only mono-PEA-HepII substituted at position 3 (data not shown). Nevertheless, the anti-L8 LOS response raised by the formulation was able to strongly recognize the two group B strains and kill strain RH873, suggesting that the second PEA substitution at position 6 has no impact on the specificity of the anti-L8 LOS response and does not induce a specific response to PEA disubstituted L8 LOS.

The L3 and L7 immunotypes are prevalent in invasive MenB strains, while the L8 immunotype is found predominantly in carriers (34, 74) and can be coexpressed with L3 and L7 by some MenB strains. Schmiel et al. (61) recently reported a study on the specificity of bactericidal antibodies in normal, convalescent, and postvaccination human sera using a bactericidal antibody depletion assay. They observed that human sera with bactericidal activity toward L3,7 strains also have bactericidal activity against L8 strains, suggesting the presence of a subset of cross-reactive antibodies specific to the core. However, the possibility that these human sera contained bactericidal antibodies specific both to L3,7 and L8 LOS cannot be ruled out. In any case, L8 LOS could potentially be effective in providing protection against meningococcal infections, provided that cross-reactivity of anti-L8 LOS is demonstrated on strains expressing the L3,7 immunotypes. For further evaluation of the formulation as a potential vaccine, the coverage of the rlip-TbpB/liposomal LOS formulation, using either L8 LOS or L6 LOS as an alternative to L8, is currently being investigated.

In conclusion, we have developed a new vaccine formulation based on liposomes, in which rlip-TbpB and L8 LOS antigens were associated. Incorporation into liposomes resulted in detoxification of LOS and the presence of rlip-TbpB dramatically increased the immunogenicity of LOS. Furthermore, we showed that the lipoprotein is a TLR2 agonist and that the lipid moiety is not only required for its adjuvanticity but must also be linked to the protein for such activity.

**TABLE 5** Bactericidal activity of rabbit serum against \textit{N. meningitidis} strain RH873$^a$

<table>
<thead>
<tr>
<th>Liposome and adjuvant</th>
<th>SBA titer$^b$</th>
<th>GMT</th>
<th>$P^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty liposomes + Pam2CSK4</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Liposomal L8 LOS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None*</td>
<td>8</td>
<td>16</td>
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</tr>
<tr>
<td>M982 rlip-TbpB</td>
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<td>256</td>
<td></td>
</tr>
<tr>
<td>M982 His-rTbpB</td>
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<td>16</td>
<td>0.857</td>
</tr>
<tr>
<td>Pam2CSK4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pam2CSK4 + M982 His-rTbpB</td>
<td>4</td>
<td>6</td>
<td>0.857</td>
</tr>
</tbody>
</table>

$^a$ Each rabbit received two i.m. injections of 40 µg of liposomal L8 LOS containing either no adjuvant, 40 µg of M982 rlip-TbpB, 40 µg of nonlipidated M982 rTbpB (His-rTbpB), 25 µg of the synthetic TLR2 agonist (Pam2CSK4) or both 40 µg of His-rTbpB and 25 µg of Pam2CSK4.

$^b$ Bactericidal titers are expressed as the reciprocal value of the serum dilution yielding 50% killing of bacteria. Titers corresponding to a greater than 4-fold increase over the corresponding preimmune titers are highlighted in bold.

$^c$ For GMT calculations and comparisons, values of $<4$ were considered as 2. Statistical analysis was performed using a Mann-Whitney rank-sum test. Groups were compared to the liposomal L8 LOS reference group containing no rlip-TbpB (*).
ACKNOWLEDGMENTS

We thank Alexandra Buffet for characterization of the bacterial strains, Laurent Bellamy for purification of the proteins, Olivier Pirot for providing purified LPS LOS, Sandrine Vialle and Frédéric Blanc for characterization of LOS, Fabien Martial and Jean-François Cotte for characterization of liposomal LOS, Catherine Berry and Frank Raynal for animal testing and serology assays, and Christophe Chabanel and Nadine Petiot for SBA experiments. We also acknowledge the personnel of the animal facility and in particular Christophe Charnay for excellent animal care. We are grateful to Graham Smith for editorial assistance and Nicolas Burdin for constant support.

REFERENCES


