INFECTION AND IMMUNITY, May 1993, p. 1873–1880
0019-9567/93/051873-08$02.00/0
Copyright © 1993, American Society for Microbiology
Vol. 61, No. 5

Preparation, Characterization, and Immunogenicity of Meningococcal Lipooligosaccharide-Derived Oligosaccharide-Protein Conjugates

XIN-XING GU AND CHAO-MING TSAI*

Center for Biologics Evaluation and Research, Food and Drug Administration, 8800 Rockville Pike, Bethesda, Maryland 20892

Received 17 December 1992/Accepted 9 February 1993

A method was developed for coupling carboxylic acid-containing oligosaccharides (OS) to proteins. An OS was isolated from *Neisseria meningitidis* group A strain A1 lipooligosaccharide (LOS). This LOS has no human glycolipid-like lacto-*N*-neotetraose structure and contains multiple immunotypes, including L8, found in group B and C strains. The carboxylic acid at 2-keto-3-deoxyoctulosonic acid of the OS was linked through adipic acid dihydrazide to tetanus toxoid. The molar ratio of the OS to tetanus toxoid in three conjugates ranged from 11:1 to 19:1. The antigenicity of the OS was conserved in these conjugates, as measured by an enzyme-linked immunosorbent assay (ELISA) and an inhibition ELISA with polyclonal and monoclonal antibodies to A1 LOS. These conjugates induced immunoglobulin G antibodies to A1 LOS in mice and rabbits. The immunogenicity of the conjugates in rabbits was enhanced by use of monophosphoryl lipid A plus trehalose dimycolate as an adjuvant. The resulting rabbit antisera cross-reacted with most of 12 prototype LOSs and with LOSs from two group B disease strains, 44/76 and BB431, in an ELISA and in Western blotting (immunoblotting), which revealed a 3.6-kDa reactive band in these LOSs. The rabbit antisera showed bactericidal activity against homologous strain A1 and heterologous strains 44/76 and BB431. These results indicate that conjugates derived from A1 LOS can induce antibodies against many LOS immunotypes from different organism serogroups, including group B. OS-protein conjugates derived from meningococcal LOSs may therefore be candidate vaccines to prevent meningitis caused by meningococci.

Neisseria meningitidis is a capsulated gram-negative bacterium that causes meningitis and septic shock in humans. In industrialized countries, the annual incidence is 1 to 5 in 100,000, while in nonindustrialized countries, it is estimated that 330,000 persons suffer from meningococcal disease, with 35,000 deaths per year (22, 45). More than half of the cases occur in children below the age of 5 years, with the highest incidence occurring in the first 2 years. About 90% of the cases are caused by serogroups A, B, and C; the remainder are caused by serogroups Y and W135 (18, 22, 41).

Two major problems in the development of vaccines against this pathogen are as follows: (i) the current A, C, Y, and W135 capsular polysaccharide (PS) vaccines are less immunogenic in young children, especially infants (37, 42); and (ii) group B capsular PS, a polymer of α(2→8)-linked sialic acid which is also present in some human gangliosides and a number of fetal glycoproteins (14, 16), is poorly immunogenic in humans (60). The immunogenicity of the capsular PSs can be improved by coupling them to proteins (3, 4, 31), but this procedure may not be desirable for group B capsular PS (31, 33) because antibodies to the PS might cross-react with human tissue antigens. Therefore, lipooligosaccharide (LOS) and outer membrane protein antigens are currently being studied as potential vaccine candidates (17, 32, 43, 56).

LOS or lipopolysaccharide (LPS) plays a key role in the induction of septic shock by gram-negative bacteria in humans. The studies of Brandtzaeg et al. (5) indicated a direct correlation between the severity of systemic meningococcal disease and circulating levels of LOS. So far, 13 different immunotypes of meningococcal LOS are known (1, 25, 39, 59). On the basis of the oligosaccharide (OS) structures of immunotypes L1, L2, L3, 7, 9, L5, and L6 (12, 28, 29, 40), Mandrell et al. (38) and Tsai and Civin (54) found that at least 8 of the 13 LOS immunotypes have a terminal lacto-*N*-neotetraose (LNnT) structure, which is also present in paragloboside, a glycolipid found in a variety of human cells. In this study, we use group A strain A1 LOS as a vaccine candidate; it has an immunotype similar to those of several important group B disease strains, 44/76, BB431, and M978, but does not have the LNnT moiety (24, 25). This LOS could induce bactericidal monoclonal antibodies (MAbs) in mice (24), and bactericidal polyclonal antibodies in human sera could be absorbed out with the LOS (21b).

A method has been developed for coupling LOS-derived OS to tetanus toxoid (TT) by use of a spacer, adipic acid dihydrazide (ADH). The antigenicity and immunogenicity of OS-TT conjugates were evaluated. The reactivities of rabbit antisera elicited by the conjugates were analyzed with 12 LOS immunotypes and LOSs from a few group B disease isolates by an enzyme-linked immunosorbent assay (ELISA) and by Western blotting (immunoblotting). The bactericidal activities of the rabbit antisera were also tested with the homologous strain, A1, and two group B strains, 44/76 and BB431.

MATERIALS AND METHODS

Isolation of OS. LOS was isolated from both cells and culture broth of group A meningococcal disease strain A1 (25) by the gel filtration method as described previously (23). Strain A1 was cultured on brain heart infusion (BHI) agar at

* Corresponding author.

A. Oligosaccharide-COOH (OS) + NH_2NH-CO-$(CH_2)_4$-CO-$NHNH_2$ (ADH)

$\xrightarrow{\text{EDC/Sulfo-NHS}}$ NH_2NH-CO-$(CH_2)_4$-CONH-NHCO-Oligosaccharide (AH-OS)

B. Protein-COOH + AH-OS $\xrightarrow{\text{EDC}}$

Protein-[CONH-NHCO-$(CH_2)_4$-CONH-NHCO-Oligosaccharide]$_n$

FIG. 1. Reaction scheme for coupling of OS to protein. (A) A hydrazide group is introduced by coupling of ADH to the carboxylic acid group of the terminal KDO moiety of the OS to form AH-OS. (B) AH-OS is coupled to carboxylic acid groups on the protein. Both reactions yield conjugates with stable peptide bond linkages. Coupling of OS to OS or proteins to proteins because of the presence of free amino groups is avoided by the use of excess amounts of ADH (A) or AH-OS (B).

37°C in 5% CO_2 for 8 h and then transferred to 200 ml of tryptic soy broth in a 500-ml bottle. The bottle was shaken at 140 rpm in an incubator shaker at 37°C overnight, and the culture broth was further transferred to six 2.8-liter Fernbach flasks each containing 1.4 liters of tryptic soy broth. The flasks were shaken at 140 rpm in the shaker at 37°C overnight and then at 110 rpm and 32°C for another 24 h. The strain released a large amount of outer membrane vesicles (OMV) into the medium during its growth (about 40 μg of LOS per ml). The culture broth was centrifuged at 15,000 × *g* for 30 min to separate cells and OMV. The OMV in the supernatant were concentrated by use of an Amicon hollow-fiber cartridge with a 100,000-molecular-weight cutoff and then pelleted by ultracentrifugation at 150,000 × *g* for 3 h. The OMV suspension (~10% [vol/vol] in water) was treated with 2% sodium deoxycholate, and the dissociated LOS was purified on a Sephacryl S-300 column (5.0 by 90 cm; Pharmacia LKB Biotechnology, Uppsala, Sweden). Crude LOS from cells was also obtained by phenol extraction and further purified on the S-300 column as described above. About 400 mg of LOS was obtained from both cells and OMV in a run.

Two grams of purified LOS from several runs was dissolved in distilled water to 10 mg/ml and then hydrolyzed in 1% acetic acid at 100°C for 2 to 3 h (57). The lipid A portion and unhydrolyzed LOS were removed by centrifugation at 150,000 × *g* for 5 h. The pellet contained about 10% unhydrolyzed LOS and was resuspended, hydrolyzed, and centrifuged again. Both supernatants were combined and lyophilized. For removal of the free 2-keto-3-deoxyoctulosonic acid (KDO), the lyophilized material was dissolved in a small volume of distilled water (300 mg/ml) and centrifuged at 2,000 × *g* for 10 min. The supernatant (about 1 ml for each run) was applied to a Bio-Gel P-4 column (1.6 by 90 cm; Pharmacia) equilibrated with 25 mM ammonium acetate and monitored with a differential refractometer (R-400; Waters, Milford, Mass.). The eluate was assayed for sugar content (13), and the major OS fractions from several runs were pooled and lyophilized twice to remove the salt. These OSs were used for the preparation of conjugates. The yield of OS from A1 LOS was about 35% by weight.

Purification of TT. TT was obtained from Sclavo S.P.A. (Siena, Italy). TT was concentrated and passed through a Sephacryl S-300 column (2.6 by 90 cm; Pharmacia) equilibrated with 0.9% NaCl containing 0.02% sodium azide. The major middle peak (corresponding to a molecular mass of about 150 kDa), which had the highest reactivity with a reference anti-TT serum in an ELISA, was collected and concentrated to 10 mg/ml (Omega membrane; 30,000-molecular-weight cutoff; Pharmacia). The protein concentration was measured by the micro-bicinchoninic acid method (Pierce, Rockford, Ill.) (50), and bovine serum albumin (BSA) was used as a standard.

Derivatization of OS. ADH (Aldrich Chemical Co., Milwaukee, Wis.) was coupled to OS by carbodiimide-mediated condensation with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysulfosuccinimide (sulfo-NHS) (Pierce) (51) as shown in Fig. 1A. OS (70 mg) was dissolved in 10 ml of 431 mM ADH. The molar ratio of ADH to OS was about 90:1. Solid sulfo-NHS was added to a concentration of 8 mM. The pH was adjusted to 4.8 with 2 N HCl, and EDC was added as a solid to a concentration of 0.1 M. The reaction mixture was stirred at pH 4.8 for 3 h in a pH Stat (Radiometer, Copenhagen, Denmark). The solution was adjusted to pH 7.0 and applied to a Bio-Gel P-4 column (1.6 by 90 cm) as described before. The eluate was assayed for sugar content (15) and for adipic hydrazide (AH) content of derivatized OS (AH-OS) (34) in microtiter plates with the modification that AH groups were measured at A_{490}. Peaks containing both sugar and AH were pooled and lyophilized twice to remove the salt. For quantitation, OS or ADH was used as a standard and each sample was measured in triplicate.

Conjugation of AH-OS to TT. The coupling reaction was performed at pH 5.6 with EDC as shown in Fig. 1B. AH-OS (20 mg) was dissolved in 2 ml of distilled water, and the solution was mixed with 1 ml of TT (10 mg/ml). The molar ratio of AH-OS to TT was about 200:1. The pH was adjusted to 5.6 with 0.2 N HCl, and solid EDC was added to a concentration of 0.1 M. The reaction mixture with or without 8 mM sulfo-NHS was stirred at pH 5.6 for 1 to 3 h. The OS–TT conjugation solution was adjusted to pH 7.0, centrifuged at 1,000 × *g* for 10 min, and purified on a Sephacryl S-300 column (1.6 by 90 cm) equilibrated with 0.9% NaCl containing 0.02% sodium azide. The eluate was monitored for protein at A_{280} and assayed for sugar content (15). Peaks that contained both protein and sugar were pooled and concentrated by ultrafiltration (30,000-molecular-weight cutoff). The conjugates (OS-TT1, OS-TT2, and OS-TT3) were analyzed for total sugar content and protein content (50).

HPLC analysis. A1 OS and AH-OS were analyzed by high-performance liquid chromatography (HPLC). A CarboPac-PA1 anion-exchange column and pulsed amperometric detection (Dionex Corp., Sunnyvale, Calif.) (26) were used with an eluant of 1 mM NaOH–125 mM sodium acetate, a flow rate of 1 ml/min, and 300-nA output for the detector.

Antigenicity determination. The antigenicities of the OS-TT conjugates were tested by an ELISA and an inhibi-

tion ELISA (25) with rabbit immune serum to A1 whole cells and a mouse MAb to A1 LOS.

For the ELISA, a 100-μl sample of OS-TT conjugate at 3 μg/ml (sugar content) or LOS at 10 μg/ml in phosphate-buffered saline (PBS; pH 7.4; containing 10 mM $MgCl_2$) was used as a coating antigen in a microplate (Immuno I plate; Dynatech Laboratories, Inc., Alexandria, Va.) overnight. The plate was blocked with 150 μl of 1% BSA for 1 h, and 100 μl of diluted rabbit serum (1/1,000) was added for a 3-h incubation. Then, alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) was added for a 3-h incubation. All steps were performed at room temperature, and 0.9% NaCl containing 0.05% Tween 20 (pH 7.4) was used in five washings between steps. After the enzyme substrate was added, the reactions were read with an automated microplate reader (EL309; Bio-Tek Instruments) at A_{405}.

For the inhibition ELISA, A1 LOS was used as a coating antigen and MAb 4387A5 (25) was used as a binding antibody. OS-TT3 conjugate, A1 LOS, or A1 OS in serial dilutions was incubated with the purified MAb in 1% BSA in PBS at 4°C overnight. The incubated solutions were transferred to a microplate coated with A1 LOS, and the subsequent steps were performed as for the ELISA, except that alkaline phosphatase-conjugated rabbit anti-mouse IgG was used. The 50% inhibition value was determined as the concentration (millimolar) of the conjugate, LOS, or OS required for 50% inhibition.

Immunogenicity determination. The immunogenicities of the OS-TT conjugates were tested in both mice and rabbits. Five-week-old BALB/c mice (female), 10 per group, were immunized subcutaneously with 5 μg of the conjugates (OS content), LOS, or OS in 0.1 ml of 0.9% NaCl. The mice were injected three times at 2-week intervals and bled 7 to 10 days after each injection. LOS-specific antibody levels were expressed in ELISA units, with A1 LOS as a coating antigen and two mouse MAbs against A1 LOS as reference standards assigned a value of 1,000 units each for IgG and IgM.

New Zealand White rabbits (female, 2 to 3 kg), two or three rabbits per group, were immunized subcutaneously with 50 μg of the conjugates (OS content), LOS, or OS in 0.5 ml of 0.9% NaCl. For another two conjugate groups, every dose of the conjugates contained either $Al(OH)_3$ (250 μg) or Ribi-700 adjuvant (containing 125 μg of monophosphoryl lipid A and 125 μg of synthetic trehalose dicorynomycolate; Ribi ImmunoChem Research, Inc., Hamilton, Mont.). As a control, one group received only 0.5 ml of Ribi-700 adjuvant. The rabbits were injected twice at 3-week intervals and bled 2 weeks after the first injection and 1 week after the second injection. LOS-specific antibody levels were expressed in ELISA units, with A1 LOS as a coating antigen and a rabbit immune serum to A1 whole cells as a reference standard assigned values of 1,500 units for IgG and 150 units for IgM.

For TT antibody determinations in both mouse and rabbit sera, a similar ELISA was performed, except that TT (3 μg/ml) was used as a coating antigen and horse anti-TT serum (20 IU/ml) was used as a reference standard assigned a value of 40 ELISA units for IgG.

Binding reactivity determination. The reactivities of the rabbit antisera elicited by the A1 OS-TT conjugates, A1

TABLE 1. Yield and ratio for AH-OS

Lot	Reaction OS (mg)[a]	AH-OS (mg)[a]	Yield (%)	Ratio of AH to OS (mol of AH/mol of OS)[b]
1	47	40	85	0.95
2	69	58	84	1.06
3	90	75	83	0.84
4	70	58	83	0.84
5	75	65	87	0.99

[a] Based on the amount of OS measured by the phenol-sulfuric acid method (13).

[b] Based on molecular weights of 173 for AH and 1,400 for OS (25). The amount of AH was measured by a modified TNBS (trinitrobenzenesulfonic acid) method (34).

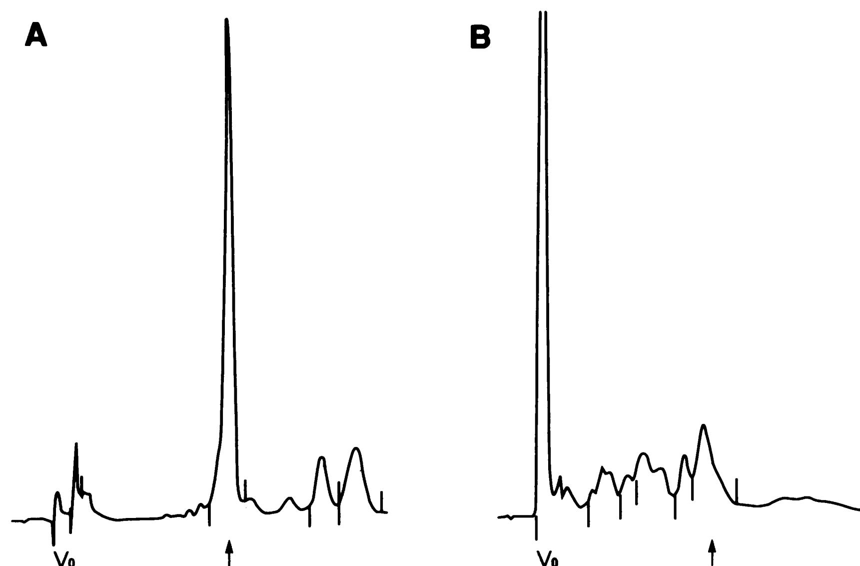

FIG. 2. Analysis of strain A1 OS and AH-OS by HPLC on a CarboPac-PA1 anion-exchange column (Dionex). OS (5 μg; A) or AH-OS (10 μg; B) was eluted with 4 mM NaOH–125 mM sodium acetate. The retention times at the voided volume (V_0) and at the arrow in both panels are 2.5 and 11.5 min, and the total running time for each panel is 20.0 min. The major peak of OS at 11.5 min (A) was shifted to the voided volume after derivatization of OS with ADH (B).

LOS, and A1 whole cells were analyzed by an ELISA with different LOSs as coating antigens (10 μg/ml) purified from 12 prototype strains (25) and strains A1, BB431 (19), and 44/76 (27). An appropriate dilution was chosen for each rabbit serum (1/100 to 1/500), and the binding reactivities of the different LOSs for each serum were determined at A_{405}.

SDS-PAGE and Western blot assay. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the Western blot assay were performed as described previously (25), except that 3-amino-9-ethylcarbazole was used as a substrate for the Western blot assay.

Bactericidal activity assay. The bactericidal activity assay was based on a modification of a microbactericidal activity assay (20, 30). Rabbit pre- and postimmune sera (after two injections) were inactivated at 56°C for 30 min. Threefold dilutions of the sera were made in Dulbecco's PBS containing 0.1% gelatin (DPBSG) so that 50 μl of a serum dilution was present in each well of sterile 96-well plates. Meningococcal strains were grown on BHI agar (23) at 37°C in 5% CO_2 for 4.5 h. The bacteria were diluted in DPBSG, and 30 μl of a suspension containing 50 to 80 CFU was added to each well. Pooled baby rabbit serum was added (15 μl per well) as a source of complement. The plates were incubated at 37°C for 30 min. Fifty microliters of the mixture was removed from each well and spread on BHI plates (100 by 15 mm). The plates were incubated at 37°C in 5% CO_2 overnight, and the colonies were counted. Controls included complement, inactive complement, and bacteria. The highest serum dilution causing ≥75% killing was considered the bactericidal titer of the serum (expressed as the reciprocal). The assay was repeated for each serum dilution.

Statistical analysis. Antibody levels were expressed as the geometric mean ELISA units of n independent observations ± the standard deviation or range ($n < 4$). Significance was tested with the two-sided t test, and P values of <0.05 were considered significant.

RESULTS

Chemical characterization of AH-OS and OS-TT conjugates. A1 OS was first derivatized with ADH by use of EDC and sulfo-NHS. The resulting AH-OS was purified and analyzed for sugar and AH contents. The ratio and the yield for five lots of AH-OS are shown in Table 1. The molar ratio of AH to OS was about 1, and the yield of AH-OS was 83 to 87%, on the basis of sugar content. In the absence of sulfo-NHS, the yield of AH-OS decreased by 10 to 20%. Figure 2A shows the HPLC profile of the OS with a major peak at 11.5 min. After derivatization, the major peak shifted to the voided volume (Fig. 2B), a result indicating that most of the OS was derivatized.

AH-OS was further coupled to TT by use of EDC with or without sulfo-NHS. Three purified OS-TT conjugates were analyzed for their sugar and protein contents. The molar ratio of OS to TT for three lots of the conjugate preparations ranged from 11:1 to 19:1 (Table 2). In the absence of sulfo-NHS, the OS-TT3 conjugate had a higher OS/TT ratio and a higher yield. Sulfo-NHS caused cross-linkage and precipitation of the conjugates, which resulted in a low yield of soluble conjugates. The optimum pH for coupling of OS to protein varied when BSA, CRM_{197} (a nontoxic diphtheria toxin), and meningococcal outer membrane proteins were used. TT and BSA were found to be the best carriers among the proteins tested, with an optimum pH of 5.6.

TABLE 2. Composition and antigenicities of OS-TT conjugates

Conjugate	OS (μg/mg of protein)	Ratio of OS to TT (mol of OS/mol of TT)[a]	A_{405}[b]
OS-TT1	104	11	0.9
OS-TT2	122	13	1.2
OS-TT3	181	19	1.1

[a] Based on molecular weights of 1,400 for OS and 150,000 for TT (25).

[b] The antigenicity of OS-TT conjugates was expressed as ELISA reactivity at A_{405} when the OS-TT conjugates were used as coating antigens and rabbit serum (1/1,000) elicited by strain A1 whole cells was used as a binding antibody.

TABLE 3. Murine IgG antibody response to *N. meningitidis* A1 LOS elicited by OS-TT conjugates, OS, or LOS

Immunogen[a]	Geometric mean ± SD (range) IgG ELISA unit(s)[b] after injection:		
	1	2	3
OS	0.6 (0.4–1.1)	0.5 (0.3–0.9)*	0.8 (0.5–1.4)*
OS-TT1	0.7 (0.4–1.3)*	4.2 (1.4–13)**	9.0 (1.4–56)**
OS-TT2	0.6 (0.3–1.4)*	4.5 (1.8–11)**	11 (1.8–69)**
OS-TT3	1.1 (0.6–1.9)	3.0 (1.2–7.6)**	10 (2.0–51)**
LOS	0.4 (0.3–0.6)†	1.4 (0.4–4.3)††	14 (3.1–61)††

[a] Ten mice for each group were subcutaneously immunized three times, at 2-week intervals, with 5 μg of OS, LOS, or conjugates. Blood samples were collected 1 week after the first or second injection and 10 days after the third injection.

[b] Based on a mouse MAb against A1 LOS. Preimmune sera contained 0.5 (0.3 to 0.8) unit. For * versus ** and † versus ‡, $P < 0.01$.

Antigenicities of OS-TT conjugates. The antigenicities of the OS-TT conjugates were tested with both polyclonal antibodies and MAbs. All conjugates reacted well with rabbit immune serum to A1 whole cells, and the ELISA reactivities were not correlated with the molar ratio of OS to TT (Table 2). In an inhibition ELISA with a mouse MAb to A1 LOS, the 50% inhibition values were 0.0028 mM (10 μg/ml) for LOS, 1 mM (1.4 mg/ml) for OS, and 0.057 mM for the OS-TT3 conjugate (540 μg/ml, containing 80 μg of conjugated OS per ml). Although OS was antigenically active, its reactivity was reduced after it was hydrolyzed from LOS. However, the antigenicity of OS could be improved after conjugation to TT.

Immunogenicities of OS-TT conjugates. The immunogenicities of the OS-TT conjugates were tested in both mice and rabbits. First, three OS-TT conjugates were tested in BALB/c mice. There was no increase in IgG levels after three injections of OS (Table 3). In contrast, all OS-TT conjugates were immunogenic. There was a booster effect on the IgG levels elicited by OS-TT conjugates between the first injection and the second or third injection ($P < 0.01$). The

TABLE 4. Murine IgG antibody response to TT elicited by OS-TT conjugates

Immunogen[a]	Geometric mean ± SD (range) IgG ELISA units[b] after injection:		
	1	2	3
OS-TT1	<1*	27 (12–59)**	56 (27–117)†
OS-TT2	<1	26 (11–64)**	63 (34–117)†
OS-TT3	<1	32 (15–71)**	56 (23–137)

[a] See Table 3, footnote *a*.

[b] Based on a reference horse serum. For * versus **, $P < 0.01$; for ** versus †, $P < 0.05$.

TABLE 5. Rabbit IgG antibody response to *N. meningitidis* A1 LOS elicited by OS-TT conjugates, OS, or LOS

Immunogen[a]	No. of rabbits	Geometric mean (range) IgG ELISA unit(s)[b] on day:		
		0	14	28
OS	2	0.7 (0.4–1.2)	0.7*	0.7
OS-TT	3	0.4	11**	67 (32–97)††
OS-TT + Al (OH)$_3$	2	0.4	11	56 (32–97)***
OS-TT + Ribi-700[c]	3	0.4	25 (22–32)†	607 (292–875)†††
LOS	2	0.4	11	1,237 (875–1,750)†††

[a] Rabbits were subcutaneously immunized on days 0 and 21 with 50 μg of OS, LOS, or OS-TT conjugate (three conjugates mixed), with or without adjuvants. Blood samples were collected on days 0, 14, and 28.
[b] Based on a reference rabbit serum against A1 cells. For * versus ** or †, ** versus ††, † versus †††, and †† versus †††, $P < 0.01$; for *** versus †††, $P < 0.05$.
[c] Sera from two rabbits injected with two injections of Ribi-700 adjuvant alone contained 0.7 (0.4 to 1.2) unit on day 28.

TABLE 6. Rabbit IgG antibody response to TT elicited by OS-TT conjugates

Immunogen[a]	No. of rabbits	Geometric mean (range) IgG ELISA units[b] on day:		
		0	14	28
OS-TT	3	<1	12 (6–77)*	657 (456–1,367)**
OS-TT + Al (OH)$_3$	2	<1	6 (6)	456 (456)**
OS-TT + Ribi-700	3	<1	12 (6–17)	2,843 (1,367–4,101)**
TT	1	<1	51	1,367

[a] See Table 5, footnote *a*.
[b] Based on a reference horse serum. For * versus **, $P < 0.01$.

antibody levels elicited by LOS were also increased after immunization. No significant difference in IgG levels was observed between the conjugates and LOS after three injections. Some IgM was present in preimmune sera, and no significant increase in IgM levels was observed after any immunogen injections, except that LOS induced a more marked IgM response after three injections (data not shown). Antibodies to TT were present at less than 1 unit after the first injection (Table 4). After the second injection, all mice showed the booster response ($P < 0.01$). The third injection further increased TT antibody levels in OS-TT1- and OS-TT2-immunized mice ($P < 0.05$).

The three OS-TT conjugates were combined and further

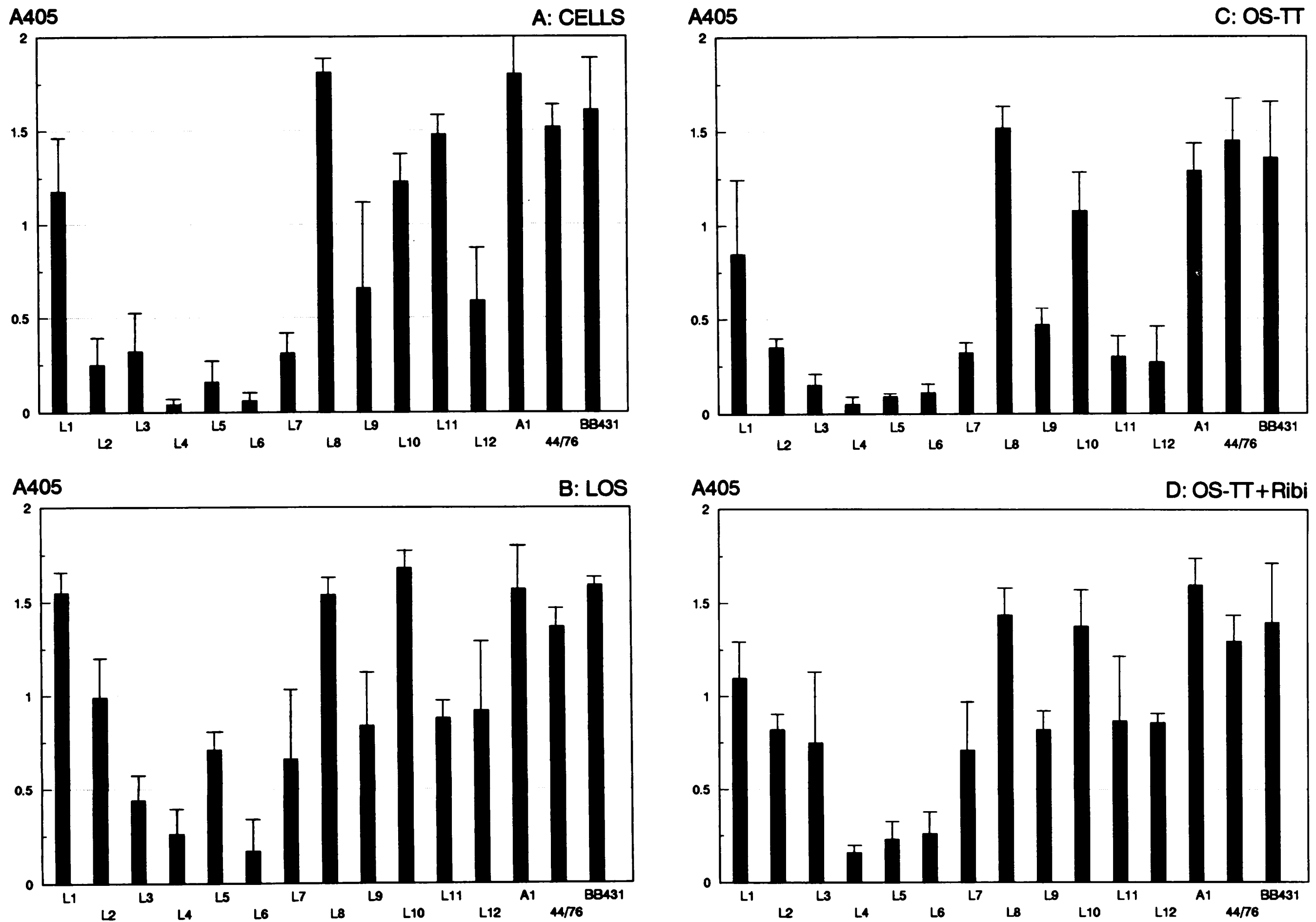

FIG. 3. Specificity of rabbit antisera elicited by strain A1 whole cells (A), LOS (B), OS-TT conjugate (C), and conjugate–Ribi-700 adjuvant (D), as analyzed by an ELISA with LOSs purified from 12 prototype strains (126E, 35E, 6275, 891, M981, M992, 6155, M978, 120M, 7880, 7889, and 7897) and strains A1, 44/76, and BB431 as coating antigens (*x* axis). The binding reactivities of the different LOSs to each rabbit serum are shown as A_{405} values (*y* axis). Each bar represents the mean ± the standard deviation for two or three rabbit sera.

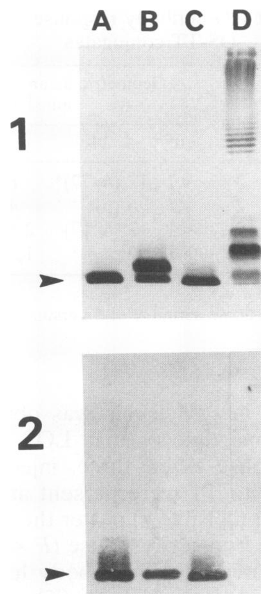

FIG. 4. SDS-PAGE and Western blotting of purified meningococcal LOSs from strains A1, 44/76, and BB431 (lanes A through C, respectively) and *Escherichia coli* O111 LPS (lane D). (Panel 1) Silver-stained gel; 0.5 μg of LOS or 5 μg of LPS in each lane. (Panel 2) Western blot of the LOSs and LPS from panel 1 with a rabbit antiserum (88061) elicited by two injections of OS-TT conjugate. Arrowheads on the left indicate the locations of the 3.6-kDa band of strain A1 LOS.

tested in rabbits. No increase in IgG levels was elicited by OS (Table 5). In contrast, a significant increase in IgG levels was elicited by the conjugate after the first injection, and a booster response was elicited after the second injection ($P < 0.01$). Similar results were obtained when the conjugate was mixed with $Al(OH)_3$. Mixing the conjugate with Ribi-700 adjuvant enhanced the immunogenicity of the conjugate: there was a ca. ninefold increase in IgG levels after two injections ($P < 0.01$). The IgG levels induced by LOS were also increased after immunization. There was no significant difference between the antibody levels elicited by conjugate–Ribi-700 adjuvant and LOS after two injections. For IgM, the results were similar to those obtained in mice (data not shown). For antibodies to TT, less than 1 unit was detected in preimmune sera (Table 6). All rabbits showed an antibody response to TT after the first injection and showed a booster effect after the second injection ($P < 0.01$).

TABLE 7. Bactericidal activities of OS-TT conjugate antisera against group A and B *N. meningitidis*

Immunogen[a]	Rabbit antiserum	Bactericidal titers[b] for strain (group)[c]:		
		A1 (A)	BB431 (B)	44/76 (B)
OS	89603	0	0	0
	88412	0	0	0
OS-TT	88042	9	3	0
	86234	3	3	0
	88061	9	9	0
OS-TT + Al $(OH)_3$	88082	9	9	3
	88035	9	3	0
OS-TT + Ribi-700	86240	27	27	3
	36096	9	9	3
	93024	27	9	3
LOS	86200	27	27	27
	88402	81	27	81

[a] See Table 5, footnote *a*. Pre- and postimmune sera obtained after two injections were used.

[b] Expressed as the fold increase above the value for preimmune sera and given as the reciprocal of the serum dilution causing ≥75% killing of each strain.

[c] Strain A1 is the homologous strain for OS-TT conjugates, and BB431 and 44/76 are group B strains. All strains share the L8 epitope in their LOSs.

Binding reactivities of rabbit antisera elicited by the conjugates. The reactivities of the rabbit immune sera elicited by the conjugates were analyzed by an ELISA with different LOS immunotypes and comparison with the immune sera induced by A1 LOS or A1 whole cells (Fig. 3). All antisera showed strong binding reactivities to L1, L8, L10, A1, 44/76, and BB431 LOSs. The whole-cell antisera also showed strong reactivity to L11 (Fig. 3A). The conjugate antisera showed less cross-reactivity than the LOS antisera to L2, L3, L5, L7, L9, L11, and L12 (Fig. 3B and C). The above-described cross-reactions were enhanced in the conjugate–Ribi-700 adjuvant antisera, except for L5 (Fig. 3D). In Western blot analysis, the conjugate antisera bound strongly to a 3.6-kDa LOS from homologous strain A1 and two group B strains, 44/76 and BB431, as shown in Fig. 4. They also bound to the 3.6-kDa component in other reactive prototype LOSs.

Bactericidal activities of rabbit antisera elicited by the conjugates. The bactericidal activities of the rabbit conjugate antisera were determined by use of homologous strain A1 and two heterologous strains (Table 7). The conjugate antisera showed bactericidal activities against strains A1 and BB431. The antisera obtained with Ribi-700 adjuvant had higher bactericidal activities, and bactericidal activities also appeared against strain 44/76. There was some correlation between LOS IgG antibody levels and the bactericidal titers of these antisera (Tables 5 and 7).

DISCUSSION

There are many coupling methods for LPS-derived PS-protein conjugates (6, 8, 10, 52) or for LOS-derived OS-protein conjugates (32, 36, 56); they are based on two main approaches: random (multiple-ended) activation and selective (single-ended) activation of the PS or the OS before conjugation to proteins. For LOS-derived OS, the selective mild modification method is required to produce an effective immunogenic OS-protein conjugate (56). We present a simple and efficient method for coupling OS through the carboxylic acid of KDO to proteins without further modification of the carbohydrate antigens. This method resulted in an effective immunogenic OS-TT conjugate in rabbits and mice. The immunogenicity of the conjugate was less marked than that of the native LOS but could be enhanced with Ribi-700 adjuvant (Table 5), a result consistent with the report of Schneerson et al. (49). The above-described results suggested that the lipid A portion of the LOS may play a role as an adjuvant in stimulating antibody production.

A number of studies (35, 44, 47, 58, 59) have suggested that immunotypes L10 and L11 are prevalent in meningococcal serogroup A organisms, while in serogroup B and C organisms, L3 and L7 are the most prevalent, followed by L2, L1, and L8. Since L2, L3, and L7 LOSs often have an

LNnT structure, which is also present in human cell glycolipid (54), we selected strain A1 LOS (L8, 10, 11) as a vaccine candidate component because it does not have this structure (24) and because it shares epitopes with strains carrying other LOS immunotypes (Fig. 3A and B) (25); in doing so, we obtained good coverage of the above-listed immunotypes. In an analysis of the binding reactivities of rabbit antisera to LOS antigens (Fig. 3), A1 LOS antisera differed from A1 whole-cell antisera in reactivities with L2, L5, and L11 and showed higher levels of cross-reactivity with 12 prototype LOSs. This result was probably due to differences in the expression of the epitopes on purified LOS and on whole cells. Although OS-TT conjugate antisera showed some specificity, antisera induced by the conjugates plus Ribi-700 adjuvant bound most of the 12 prototype LOSs. The cross-reactivity of the antisera may have been due to the presence of some antibodies to certain common epitopes in the OS structures of the meningococcal LOSs (12, 28, 29, 40) and the presence of multiple LOS immunotypes on the same strain (21, 44, 55, 58, 59).

It is interesting that the OS-TT conjugates were more immunogenic in rabbits than in mice. In our experiments, the IgG antibody levels induced by the conjugates in rabbits showed about a 160-fold increase after two injections (Table 5), while the IgG antibody levels in mice only showed about a 20-fold increase after three injections (Table 3). However, many studies have shown that PS-protein conjugates are usually effective immunogens in mice and rabbits (2, 7, 48, 53) and in humans (9, 11, 46). These observations suggest that the OS-protein conjugates and PS-protein conjugates result in different responses in vivo. The correlation in immunogenicity between animal and human responses to OS-TT conjugates is not known. Gotschlich et al. (22a) suggested that the immune response of mice is about 10 times lower than the human response to pneumococcal PS.

For investigation of the protective capacity of the rabbit conjugate antisera in vitro, the bactericidal activities of the rabbit antisera were examined with homologous group A strain A1 and two heterologous group B strains, BB431 ($L8_a$) (25) and 44/76 (L3,8). The conjugate antisera not only killed the homologous strain but also killed the heterologous strains, although the levels of bactericidal activity of the antisera were low. The level of bactericidal activity needed for effective protection against meningococcal disease appears to be low, since it has been documented that protection can be achieved when human sera show bactericidal activity at a titer of 1:4 (21a, 21b). For evaluation of the LOS-derived OS-TT conjugate as a potential vaccine for human use, further studies are needed to investigate the conjugate antisera in terms of opsonization, endotoxin neutralization, and protection in animal models.

REFERENCES

1. **Achtman, M., B. Kusecek, G. Morelli, K. Elickmann, J. Wang, B. Crowe, R. A. Wall, M. Hassan-king, P. S. Moore, and W. Zollinger.** 1992. A comparison of the variable antigens expressed by clone IV-1 and subgroup III of *Neisseria meningitidis* serogroup A. J. Infect. Dis. **165:**53–68.
2. **Anderson, P.** 1983. Antibody responses to *Haemophilus influenzae* type b and diphtheria toxin induced by conjugates of oligosaccharides of the type b capsule with the nontoxic protein CRM_{197}. Infect. Immun. **39:**233–238.
3. **Beuvery, E. C., A. Kaaden, V. Kanhai, and A. B. Leussink.** 1983. Physicochemical and immunological characterization of meningococcal group A polysaccharide-tetanus conjugates prepared by two methods. Vaccine **1:**31–36.
4. **Beuvery, E. C., F. Miedema, R. Van Delft, and K. Haverkamp.** 1983. Preparation and immunochemical characterization of meningococcal group C polysaccharide-tetanus conjugates as a new generation of vaccines. Infect. Immun. **40:**39–45.
5. **Brandtzaeg, P., P. Kierulf, P. Gaustad, A. Skulberg, J. N. Bruun, S. Halvorsen, and E. Sorensen.** 1989. Plasma endotoxin as a predictor of multiple organ failure and death in systemic meningococcal disease. J. Infect. Dis. **159:**195–204.
6. **Chu, C., B. K. Liu, D. Watson, S. S. Szu, D. Bryla, J. Shiloach, R. Schneerson, and J. B. Robbins.** 1991. Preparation, characterization, and immunogenicity of conjugates composed of the O-specific polysaccharide of *Shigella dysenteriae* type 1 (Shiga's bacillus) bound to tetanus toxoid. Infect. Immun. **59:**4450–4458.
7. **Chu, C., R. Schneerson, J. B. Robbins, and S. C. Rastogi.** 1983. Further studies on the immunogenicity of *Haemophilus influenzae* type b and pneumococcal type 6A polysaccharide-protein conjugates. Infect. Immun. **40:**245–256.
8. **Cryz, S. J., A. S. Cross, J. C. Sadoff, and E. Furer.** 1990. Synthesis and characterization of *Escherichia coli* O18 O-polysaccharide conjugate vaccines. Infect. Immun. **58:**373–377.
9. **Cryz, S. J., E. Furer, A. S. Cross, A. Wegmann, R. Germanier, and J. C. Sadoff.** 1987. Safety and immunogenicity of a *Pseudomonas aeruginosa* O-polysaccharide toxin A conjugate vaccine in humans. J. Clin. Invest. **80:**51–56.
10. **Cryz, S. J., A. B. Lang, J. C. Sadoff, R. Germanier, and E. Furer.** 1987. Vaccine potential of *Pseudomonas aeruginosa* O-polysaccharide–toxin A conjugates. Infect. Immun. **55:**1547–1551.
11. **Decker, M. D., K. M. Edwards, R. Bradley, and B. Palmer.** 1990. Four conjugate Haemophilus b vaccines in infants: a comparative trial, abstr. 61. Program Abstr. 30th Intersci. Conf. Antimicrob. Agents Chemother.
12. **Difabio, J. L., F. Michon, J. R. Brisson, and H. J. Jennings.** 1990. Structure of the L1 and L6 core oligosaccharide of *Neisseria meningitidis*. Can. J. Chem. **86:**1029–1034.
13. **Dubois, M., H. Gillis, J. K. Hamilton, A. A. Rebers, and R. Smith.** 1956. Colorimetric method for the determination of sugars and related substances. Anal. Biochem. **28:**250–256.
14. **Fiine, J., M. Leinore, and P. H. Makela.** 1983. Antigenic similarities between brain components and bacteria causing meningitis. Lancet **ii:**355–357.
15. **Fox, J. D., and J. F. Robyt.** 1991. Miniaturization of three carbohydrate analyses using a microsample plate reader. Anal. Biochem. **195:**93–96.
16. **Frasch, C. E.** 1987. Prospects for prevention of meningococcal disease: special reference to group B. Vaccine **5:**3–4.
17. **Frasch, C. E.** 1989. Vaccines for the prevention of meningococcal disease. Clin. Microbiol. Rev. **2**(Suppl.)**:**134–138.
18. **Frasch, C. E.** 1990. Production and control of *Neisseria meningitidis* vaccines, p. 123–145. *In* A. Mizrahi (ed.), Bacterial vaccines. Wiley-Liss, New York.
19. **Frasch, C. E., L. F. Mocca, and A. B. Karpas.** 1988. Appearance of new strains associated with group B meningococcal disease and their use for rapid vaccine development, p. 97–104. *In* J. T. Poolman, C. Beuvery, and H. Zanen (ed.), Gonococci and meningococci. Martinus Nijhoff Publishers, Dordrecht, The Netherlands.
20. **Frasch, C. E., and J. D. Robbins.** 1978. Protection against group B meningococcal disease. J. Exp. Med. **147:**629–644.
21. **Frasch, C. E., W. D. Zollinger, and J. T. Poolman.** 1985. Serotyping of *Neisseria meningitidis* and a proposed scheme for designation of serotypes. Rev. Infect. Dis. **7:**504–510.

21a. **Goldschneider, I., E. C. Gotschlich, and M. S. Artenstein.** 1969. Human immunity to the meningococcus. I. The role of humoral immunity. J. Exp. Med. **129:**1307–1326.

21b. **Goldschneider, I., E. C. Gotschlich, and M. S. Artenstein.** 1969. Human immunity to the meningococcus. II. Development of natural immunity. J. Exp. Med. **129:**1327–1349.

22. **Gotschlich, E. C.** 1990. Neisseria, p. 551–560. *In* B. D. David et al. (ed.), Microbiology. J. B. Lippincott, Philadelphia.

22a. **Gotschlich, E. C., I. Goldschneider, and M. S. Artenstein.** 1969. Human immunity to the meningococcus. IV. Immunogenicity of group A and group C meningococcal polysaccharides in human

volunteers. J. Exp. Med. **129:**1367–1384.

23. **Gu, X. X., and C. M. Tsai.** 1991. Purification of rough-type lipopolysaccharides of *Neisseria meningitidis* from cells and outer membrane vesicles in spent media. Anal. Biochem. **196:** 311–318.
24. **Gu, X. X., and C. M. Tsai.** Unpublished data.
25. **Gu, X. X., C. M. Tsai, and A. B. Karpas.** 1992. Production and characterization of monoclonal antibodies to type 8 lipopolysaccharide of *Neisseria meningitidis*. J. Clin. Microbiol. **30:**2047–2053.
26. **Hardy, M. R., R. R. Townsend, and Y. C. Lee.** 1988. Monosaccharide analysis of glycoconjugates by anion exchange chromatography with pulsed amperometric detection. Anal. Biochem. **170:**54–62.
27. **Holten, E.** 1979. Serotypes of *Neisseria meningitidis* isolated from patients in Norway during the first six months of 1978. J. Clin. Microbiol. **9:**186–188.
28. **Jennings, H. J., M. Beurret, A. Gamian, and F. Michon.** 1987. Structure and immunochemistry of meningococcal lipopolysaccharides. Antonie van Leeuwenhoek **53:**519–522.
29. **Jennings, H. J., A. K. Bhattacharjee, L. Kenny, and G. Calver.** 1980. The R-type lipopolysaccharides of *Neisseria meningitidis*. Can. J. Chem. **58:**128–136.
30. **Jennings, H. J., A. Gamian, and F. E. Ashton.** 1987. N-propionylated group B meningococcal polysaccharide mimics a unique epitope on group B *Neisseria meningitidis*. J. Exp. Med. **165:**1207–1221.
31. **Jennings, H. J., and C. Lugowski.** 1981. Immunochemistry of group A, B, and C meningococcal polysaccharide-tetanus toxoid conjugates. J. Immunol. **127:**1011–1018.
32. **Jennings, H. J., C. Lugowski, and F. E. Ashton.** 1984. Conjugation of meningococcal lipopolysaccharide R-type oligosaccharides to tetanus toxoid as a route to a potential vaccine against group B *Neisseria meningitidis*. Infect. Immun. **43:**407–412.
33. **Jennings, H. J., R. Roy, and A. Gamian.** 1986. Induction of meningococcal group B polysaccharide-specific IgG antibodies in mice using an N-propionylated B polysaccharide-tetanus toxoid conjugate vaccine. J. Immunol. **137:**1708–1713.
34. **Kemp, A. H., and M. R. A. Morgan.** 1986. Studies on the detrimental effects of bivalent binding in a microtiter plate ELISA and possible remedies. J. Immunol. Methods **94:**65–72.
35. **Kim, J. J., R. E. Mandrell, Z. Hu, M. A. J. Westerink, J. T. Poolman, and J. M. Griffiss.** 1988. Electromorphic characterization and description of conserved epitopes of the lipooligosaccharides of group A *Neisseria meningitidis*. Infect. Immun. **56:**2631–2638.
36. **Lambden, P. R., and J. E. Heckels.** 1982. Synthesis of immunogenic oligosaccharide-protein conjugates from lipopolysaccharide of *Neisseria gonorrhoeae* P9. J. Immunol. Methods **48:**233–240.
37. **Lepow, M. L., J. Beeler, M. Randolph, J. S. Samuelson, and W. A. Hankins.** 1986. Reactogenicity and immunogenicity of a quadravalent combined meningococcal polysaccharide vaccine in children. J. Infect. Dis. **154:**1033–1036.
38. **Mandrell, R. E., J. M. Griffiss, and B. A. Macher.** 1988. Lipooligosaccharides (LOS) of *Neisseria gonorrhoeae* and *Neisseria meningitidis* have components that are immunochemically similar to precursors of human blood group antigens. J. Exp. Med. **168:**107–126.
39. **Mandrell, R. E., and W. D. Zollinger.** 1977. Lipopolysaccharide serotyping of *Neisseria meningitidis* by hemagglutination inhibition. Infect. Immun. **16:**471–475.
40. **Michon, F., M. Beurret, A. Gamian, J. R. Brisson, and H. J. Jennings.** 1990. Structure of the L5 lipopolysaccharide core oligosaccharide of *Neisseria meningitidis*. J. Biol. Chem. **256:** 7243–7247.
41. **Peltola, H.** 1983. Meningococcal disease: still with us. Rev. Infect. Dis. **5:**71–91.
42. **Peltola, H., A. Safary, H. Kayhty, and F. E. Andre.** 1985. Evaluation of 2 tetravalent (ACYW135) meningococcal vaccines in infants and small children—a clinical study comparing immunogenicity of O-acetyl-negative and O-acetyl-positive group C polysaccharides. Pediatrics **76:**91–96.
43. **Poolman, J. T.** 1990. Polysaccharides and membrane vaccines, p. 57–86. *In* A. Mizrahi (ed.), Bacterial vaccines. Wiley-Liss, New York.
44. **Poolman, J. T., C. T. P. Hopman, and H. C. Zanen.** 1982. Problems in the definition of meningococcal serotypes. FEMS Microbiol. Lett. **13:**339–348.
45. **Robbins, A., and P. Freeman.** 1988. Obstacles to developing vaccines for the Third World. Sci. Am. **259**(11)**:**90–95.
46. **Robbins, J. B., and R. Schneerson.** 1990. Polysaccharide-protein conjugates: a new generation of vaccines. J. Infect. Dis. **161:** 821–832.
47. **Salih, M. A. M., D. Danielsson, A. Backman, D. A. Caugant, M. Achtman, and P. Olcen.** 1990. Characterization of epidemic and nonepidemic *Neisseria meningitidis* serogroup A strains from Sudan and Sweden. J. Clin. Microbiol. **28:**1711–1719.
48. **Schneerson, R., O. Barrera, A. Sutton, and J. B. Robbins.** 1980. Preparation, characterization, and immunogenicity of *Haemophilus influenzae* type b polysaccharide-protein conjugates. J. Exp. Med. **152:**361–376.
49. **Schneerson, R., A. Fattom, S. C. Szu, D. Bryla, J. T. Ulrich, J. A. Rudbach, G. Schiffman, and J. B. Robbins.** 1991. Enhancement of the serum antibody response in mice to polysaccharide-protein conjugates by concurrent injection with MPL. J. Immunol. **147:**2136–2140.
50. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150:**76–85.
51. **Staros, J. V., R. W. Wright, and D. Swingle.** 1986. Enhancement by N-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions. Anal. Biochem. **156:**220–222.
52. **Svenson, S. B., and A. A. Lindberg.** 1979. Coupling of acid labile salmonella specific oligosaccharides to macromolecules. J. Immunol. Methods **25:**323–335.
53. **Svenson, S. B., and A. A. Lindberg.** 1981. Artificial *Salmonella* vaccines: *Salmonella typhimurium* O-antigen-specific oligosaccharide-protein conjugates elicit protective antibodies in rabbits and mice. Infect. Immun. **32:**490–496.
54. **Tsai, C. M., and C. V. Civin.** 1991. Eight lipooligosaccharides of *Neisseria meningitidis* react with a monoclonal antibody which binds lacto-*N*-neotetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc). Infect. Immun. **59:**3604–3609.
55. **Tsai, C. M., L. F. Mocca, and C. E. Frasch.** 1987. Immunotype epitopes of *Neisseria meningitidis* lipopolysaccharide types 1 through 8. Infect. Immun. **55:**1652–1656.
56. **Verheul, A. F. M., A. K. Braat, J. M. Leenhouts, P. Hoogerhout, J. T. Poolman, H. Snippe, and J. Verhoef.** 1991. Preparation, characterization, and immunogenicity of meningococcal immunotype L2 and L3,7,9 phosphoethanolamide group-containing oligosaccharide-protein conjugates. Infect. Immun. **59:**843–851.
57. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharide extraction with phenol:water and further application of the procedure. Methods Carbohydr. Chem. **5:**83–91.
58. **Zollinger, W. D., and R. E. Mandrell.** 1977. Outer membrane protein and lipopolysaccharide serotyping of *Neisseria meningitidis* by inhibition of a solid-phase radioimmunoassay. Infect. Immun. **18:**424–433.
59. **Zollinger, W. D., and R. E. Mandrell.** 1980. Type-specific antigens of group A *Neisseria meningitidis*: lipopolysaccharide and heat-modifiable outer membrane proteins. Infect. Immun. **28:**451–458.
60. **Zollinger, W. D., R. E. Mandrell, J. M. Griffiss, P. Altieri, and S. Berman.** 1979. Complex of meningococcal group B polysaccharide and type 2 outer membrane protein is immunogenic in man. J. Clin. Invest. **63:**836–846.