INFECTION AND IMMUNITY, Dec. 1991, p. 4450–4458
0019-9567/91/124450-09$02.00/0

Vol. 59, No. 12

Preparation, Characterization, and Immunogenicity of Conjugates Composed of the O-Specific Polysaccharide of *Shigella dysenteriae* Type 1 (Shiga's Bacillus) Bound to Tetanus Toxoid

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Received 4 June 1991/Accepted 5 September 1991

The background for developing conjugate vaccines for shigellosis composed of the O-specific polysaccharide (O-SP) bound to a protein is described elsewhere (C. Y. Chu, R. Schneerson, and J. B. Robbins, submitted for publication). Briefly, there is direct evidence for type (lipopolysaccharide [LPS])-specific protection after infection with the wild type or with attenuated strains of shigellae. Prospective studies of Israeli armed forces recruits show a correlation between preexisting serum immunoglobulin G (IgG) LPS antibodies and resistance to shigellosis (D. Cohen, M. S. Green, C. Block, R. Slephon, and I. Ofek, J. Clin. Microbiol. 29:386–389, 1991). In order to elicit IgG LPS-specific antibodies to *Shigella dysenteriae* type 1, the O-SP of this pathogen was purified and bound to tetanus toxoid (TT) by three schemes. The most immunogenic used a modification of a published method (C. Y. Chu, R. Schneerson, J. B. Robbins, and S. C. Rastogi, Infect. Immun. 40:245–256, 1983). The resultant O-SP–TT conjugates were stable and elicited high levels of IgG O-SP antibodies and booster responses in young mice when injected subcutaneously in saline at 1/10 the proposed human dose. Adsorption onto alum or concurrent administration with monophosphoryl lipid A enhanced both the IgG and IgM antibody responses to the O-SP of the conjugate; both the nonadsorbed and adsorbed conjugates elicited higher rises of IgG than of IgM antibodies. Clinical evaluations of *S. dysenteriae* type 1 O-SP–TT conjugates are planned.

Another article outlines the need and rationale for developing conjugate vaccines composed of the O-specific polysaccharides (O-SP) of nontyphoidal salmonellae and shigellae for the prevention of enteric diseases caused by these pathogens (8). In that article, we cite evidence, albeit indirect, that serum immunoglobulin G (IgG) lipopolysaccharide (LPS) antibodies (Ab) may confer protective immunity to shigellosis.

Shigella dysenteriae type 1 (Shiga's bacillus) was isolated in 1897 from patients with dysentery in Japan (48). Subsequently, bacilli with similar properties isolated from patients with dysentery were also referred to as shigellae (16, 19, 28, 49). Later, Kauffmann classified shigellae into groups A, B, C, and D on the basis of "distinctive" antigens and biochemical traits (28). Group A shigellae, which include *S. dysenteriae* type 1, do not produce acid from mannitol. The 10 LPS types of *S. dysenteriae* are structurally and serologically distinct, and only a few cause shigellosis (16, 20, 22, 28, 32, 38, 49, 55, 64).

S. dysenteriae type 1 is the most virulent of the shigellae, with a low infectious dose, high attack rate, high mortality, and extraintestinal manifestations such as bacteremia, hemolytic uremic syndrome, and postinfection arthritis (Reiter's syndrome) (19, 22, 32, 38–42, 49, 55, 61, 64). Infection with *S. dysenteriae* type 1 is rare in developed countries, but epidemic and endemic dysenteries caused by this pathogen are still present in developing nations (2, 3, 32, 38–43, 64). The most recent pandemic in the Western Hemisphere began in Guatemala in 1969 and spread to all of Central America over the next 4 years (22, 61). About 5×10^5 individuals contracted disease due to this pathogen, and ~20,000 (4%) died. Recently, a fivefold rise in the number of infections due to *S. dysenteriae* type 1 occurred in travelers returning to the United States from southern Mexico (39). Clinical data from 42 of 44 of these patients were available: 26 (64%) were hospitalized, 3 (7%) had bacteremia, and 3 (7%) had hemolytic uremic syndrome, but none died. The antibiotic resistance and plasmid profiles of strains isolated from these patients were similar to those from the pandemic of 1969 to 1973. These findings indicate that a reservoir of this pathogen has retained its virulence and remains in Central America.

The structure, synthesis, and insertion of the LPS in *S. dysenteriae* type 1 are encoded in the *his*$^+$ locus in the chromosome and on a 6-MDa plasmid (4, 20, 23, 51, 55, 59, 60). Variants which lack these DNA segments do not express the LPS antigen and are avirulent in in vitro models (4, 21, 23, 59, 60, 64). Dmitriev et al. reported the following structure for the repeat unit of the O-SP (14): →3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-α-D-Gal*p*-(1→3)-α-D-Glc*p*NAc-(1-. Sturm et al. proposed that the biosynthetic unit is as follows (51): →3)-α-D-Glc*p*NAc-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→2)-α-D-Gal*p*-(1-.

Here, we describe the synthesis, characterization, stability, and comparative immunogenicity of *S. dysenteriae* type 1 O-SP bound to tetanus toxoid (TT) by three different methods.

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MATERIALS AND METHODS

Reagents. Sterile pyrogen-free water and pyrogen-free saline (Travenol Laboratories, Deerfield, Ill.) were used in all experiments. G-25 Sephadex, CL-6B Sepharose, and dextrans for calibration of Superose 12 were from Pharmacia LKB Biotechnology Inc., Piscataway, N.J. P-10 BioGel was from Bio-Rad, Richmond, Calif. Imject Alum and *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) were from Pierce Chemical Co., Rockford, Ill. Dithiodipropionic acid was from Fluka, Buchs, Switzerland. DNase, RNase, protease, bovine serum albumin, ketodeoxyoctonate (KDO), sodium cyanoborohydride ($NaCNBH_3$), adipic acid dihydrazide (ADH), cyanogen bromide (CNBr), 1-ethyl-1-3-(3-dimethylaminopropyl)carbodiimide (EDAC), and dithiothreitol were from Sigma Chemical Co., St. Louis, Mo. Tryptic soy broth and MacConkey's medium were from Difco Laboratories, Detroit, Mich. Monophosphoryl lipid A (MPL) in oil-in-water emulsion (O/W) with squalene was from Ribi Immunochemical, Hamilton, Minn. (46). Affinity-purified, alkaline phosphatase-labeled goat antibodies to mouse Ig, IgG, IgA, and IgM were from Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.

Assays. Protein was measured with bovine serum albumin as a standard (65). Polysaccharide (PS) was measured with O-SP as a standard (47). Nucleic acids were measured by their A_{260} (65). Sulfhydryl groups were measured by using cysteamine as a standard (18). KDO was measured by the thiobarbituric acid reaction with KDO as a standard (58). The adipic hydrazide (AH) content of derivatized O-SP was assayed with ADH as a standard (9). LPS was assayed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (56) and by the rabbit thermal induction and the *Limulus* amebocyte gelation assays (24). ^{13}C and ^{1}H nuclear magnetic resonance spectra were obtained on a General Electric/Brucker hybrid spectrometer for which the nominal ^{1}H and ^{13}C resonance frequencies were 300 and 75 MHz, respectively. ^{1}H broad-band decoupling was used during the acquisition of the ^{13}C spectrum. ^{13}C chemical shifts are referenced to the *N*-acetyl methyl group, set to 22.9 ppm. The molecular weight of the O-SP was estimated by gel filtration through a Superose 12 HR 10/30 column in 0.1 M Na_2SO_4–0.02 M sodium phosphate (pH 6.98) at 1 ml/min. The effluent was monitored by a Pharmacia model UV-1 280-nm detector and a Waters R401 refractometer. The void volume was determined with T2000 dextran, and the total volume was determined with glycine. The K_ds of the dextrans, from 10 to 500 kDa, were fitted to a least-square line by plotting the $\log_{10}$ molecular weight versus the K_d with a correlation coefficient of 0.985. The conjugates were assayed for blood group A substance by using the standard of the Food and Drug Administration (36).

Bacterial strains. *S. dysenteriae* type 1 strain 1617, *Shigella flexneri* 2a strain 2457T0, and *Shigella sonnei* 53G WRAIR were kindly donated by Sam Formal, Walter Reed Army Institute of Research. *Salmonella paratyphi* A strain NDPG was obtained from Iswar Acharya, Kathmandu, Nepal. The serological and metabolic identifications of these strains were confirmed by Vee Gill and James MacLowry, Microbiology Service, Clinical Center, National Institutes of Health. These strains were maintained in the freeze-dried state at −20°C.

Purification of LPS and O-SP. The procedure followed that of Westphal and Jann with several modifications (62). *S. dysenteriae* type 1 was cultivated in a 100-liter fermentor with tryptic soy broth at 37°C with vigorous aeration and was harvested when the pH started to rise and the oxygen consumption ceased (the optical density at 550 nm was ~8.0). The identity and purity of the bacteria were confirmed by Gram stain and culture on MacConkey's medium. The bacteria were harvested by centrifugation, and the pellet was divided into ~200-g aliquots and stored at −20°C. For a typical purification, ~200 g was mixed with 2 liters of a 95% aqueous phenol solution, and H_2O was added to a final volume of 4 liters. The suspension was stirred at 68°C for 30 min, cooled in an ice bath, and centrifuged at 7,300 × *g* and 10°C for 1 h. The upper layer was withdrawn, and the bottom layer was brought to 4 liters with water. The suspension was stirred again at 68°C for 30 min and centrifuged as described above. The upper layer was withdrawn and combined with the upper layer from the first centrifugation.

The combined supernatants were brought to 10 mM sodium acetate–2 mM $CaCl_2$–25% ethanol, mixed, and stored overnight at 3 to 8°C. This suspension was centrifuged at 7,300 × *g* and 4°C for 1 h, and the supernatant was brought to 70% ethanol and stored overnight at 3 to 8°C. This suspension was centrifuged at 7,300 × *g* and 4°C for 1 h, and the pellet was suspended in 300 ml of 2 mM $MgSO_4$–50 mM Tris, pH 7.6. The material was added to a dialysis bag and equilibrated against this buffer at 37°C. DNase and RNase (50 mg each) were added, and 6 h later, 100 mg of protease was added for 24 h at 37°C (9). The contents were dialyzed against H_2O at 3 to 8°C for 2 days with four changes of the outer fluid and then centrifuged at 5,000 × *g* and 3 to 8°C for 30 min. The supernatant was ultracentrifuged at 64,000 × *g* and 10°C for 5 h, and the pellet was drained, suspended in 200 ml of water, and reextracted by hot phenol (62). The top phase was dialyzed against H_2O at 3 to 8°C for 2 days with two changes daily and then freeze-dried.

The LPS was dissolved at 10 mg/ml in 1% glacial acetic acid and placed in a boiling water bath for 1 h. After being cooled, the reaction mixture was ultracentrifuged at 64,000 × *g* for 5 h at 10°C, and the supernatant was passed through a 0.2-μm-pore-size filter, brought to pH 7.0 with 1.0 M NH_4OH, and freeze-dried. This product was dissolved in H_2O at 10 mg/ml and passed through a G-25 Sephadex column (5 by 30 cm) in H_2O. Fractions (10 ml each) were collected, and the major peak adjacent to the void volume was freeze-dried and stored at −20°C. This product is referred to as the O-SP.

Proteins. TT from clinical lot XI, kindly donated by Ivan Heron, Statens Seruminstitut, Copenhagen, Denmark, contained 1,732 Lf and >100 IU of nitrogen per mg of total protein. Cross-reacting mutant *Pseudomonas aeruginosa* exotoxin A (designated rexoprotein A, or rEPA) was prepared (49a).

Derivatization and conjugation of O-SP. Conjugates were prepared by three methods with ADH as a linker. The first two methods used ADH bound to the carbonyls of KDO at the reducing end of the core (26, 45), and the third method used ADH bound to CNBr-activated O-SP (9). The O-SP was derivatized for the first two methods as follows. To 100 mg of the O-SP in 5.0 ml of 0.1 M sodium borate (pH 8.5) were added 100 mg of ADH and then 100 mg of $NaCNBH_3$. The tube was flushed with N_2, sealed, and slowly tumbled at room temperature for 7 days. The reaction mixture was dialyzed extensively against H_2O at 3 to 8°C and passed through a G-25 Sephadex column (5 by 30 cm) in H_2O, and the void volume fractions were freeze-dried. This material, designated O-SP–AH_{RE}, contained 0.52% (wt/wt) AH or ~1 mol of AH per mol of O-SP.

(i) Method 1. O-SP–AH_{RE} and TT were dissolved in 0.2 M

NaCl to a final concentration of 20 mg/ml of each. The pH was brought to 5.6 with 0.1 M HCl at 4°C, and EDAC was added to 50 mM. The pH was maintained at 5.6 for 4 h, the reaction mixture was dialyzed against 0.2 M NaCl at 3 to 8°C for 2 days and passed through a CL-6B Sepharose column (2.5 by 90 cm) in 0.2 M NaCl, and the void volume fractions were pooled. Conjugates prepared by method 1 are denoted O-SP–$TT_{88\text{-}1}$ and O-SP–$TT_{88\text{-}3}$.

(ii) Method 2. O-SP–AH_{RE} (35 mg) was dissolved in 3.5 ml of H_2O. Dithiodipropionic acid (0.1 M) was adjusted to pH 6.0 with 0.1 M HCl, and 30 mg was added to the O-SP–AH_{RE}. Then, 70 mg of EDAC was added, and the pH was maintained between 5.75 and 6.0 in a pH stat (Radiometer, Copenhagen, Denmark). After 1 h, the reaction mixture was passed through a column of P10 (5 by 30 cm) in H_2O, and the void volume fractions were freeze-dried. TT was thiolated with SPDP (7, 54) and passed through a column of P-10 (5 by 30 cm) in phosphate-buffered saline. The dithiodipropionic acid-derivatized O-SP–AH_{RE} was reduced with 100 mM dithiothreitol at room temperature for 1 h, dialyzed against 1 mM EDTA in phosphate-buffered saline, and passed through a column of P-10 (5 by 30 cm) in 1 mM EDTA. The sulfhydryl contents of the void volume fractions were determined and then mixed with the SPDP-derivatized TT, flushed with N_2, sealed, and tumbled for 2 h at room temperature and overnight at 3 to 8°C. The reaction mixture was passed through a column of CL-4B Sepharose (2.5 by 90 cm) in saline, and the fractions were assayed for O-SP and protein (see above). The void volume contained ~18% of the conjugate (O-SP–$TT_{SPDP\text{-}1}$), and the remainder emerged as a broad peak with a K_d of 0.40 (O-SP–$TT_{SPDP\text{-}2}$).

(iii) Method 3. Multipoint attachment of the two reactants was achieved as described elsewhere for *Haemophilus influenzae* type b PS (9). *S. dysenteriae* type 1 O-SP (5.0 mg/ml of H_2O) was brought to pH 11.0 with 1 N NaOH in a pH stat, and an equal weight of CNBr (1 g/ml of acetonitrile) was added. The pH was maintained at 11.0 with 1.0 N NaOH for 6 min at 4°C. An equal volume of 0.5 M ADH in 0.5 M $NaHCO_3$ was added, and the pH was adjusted to 8.5 with 1.0 M HCl. The reaction mixture was tumbled overnight at 3 to 8°C and dialyzed against 0.2 M NaCl at 3 to 8°C. The outer fluid was changed to H_2O, and the dialysis was continued for 2 days with four changes of H_2O. The contents of the dialysis bag were freeze-dried, dissolved in H_2O (20 mg/ml), and passed through a G-25 Sephadex column (5 by 50 cm) in H_2O, and the void volume was freeze-dried. The AH content was 3.9% (wt/wt). This derivative was dissolved in H_2O to 20 mg/ml, added to an equal volume of TT–20-mg/ml saline, and placed in an ice bath, and the pH was adjusted to 5.6 with 0.1 M HCl. EDAC was added to 0.05 M, and the pH was maintained at 5.6 in a pH stat for 4 h. The reaction mixture was dialyzed against 0.2 M NaCl for 2 days at 3 to 8°C with two changes of the outer fluid daily. This material was centrifuged at 14,700 × *g* and 10°C for 1 h, and the supernatant was applied to a CL-6B Sepharose column, (5 by 95 cm) in 0.2 M NaCl–0.01% thimerosal. The void volume was stored at 3 to 8°C. Conjugates prepared by method 3 are designated O-SP–$TT_{88\text{-}4}$, O-SP–$TT_{89\text{-}1}$, and O-SP–TT_{49782}.

The preparation and characterization of *S. flexneri* type 2a and *S. sonnei* conjugates will be described elsewhere (7a).

Immunization. Hyperimmune sera were prepared by two methods. (i) Mice were injected intraperitoneally with heat-killed *S. dysenteriae* type 1 (1/10 of the dose recommended for rabbits [37]). (ii) *S. dysenteriae* type 1-TT conjugates emulsified in complete Freund's adjuvant (5.0 μg of O-SP per animal) were injected intraperitoneally. Four weeks later, the mice were reinjected with the same dose in incomplete Freund's adjuvant, and 2 weeks later, they were exsanguinated. Each immunogen was injected into 10 mice, and their postimmunization sera were pooled, sterile filtered, and stored at −20°C.

For evaluation of immunogenicity, 5-week-old general purpose mice from the National Institutes of Health were injected subcutaneously (s.c.) with 2.5 μg of O-SP alone or as a conjugate in saline three times 2 weeks apart. Ten mice were randomly chosen from each group and exsanguinated 2 weeks after the first injection, 10 mice were exsanguinated 10 days after the second injection, and 10 mice were exsanguinated 10 days after the third injection.

Serology. *S. dysenteriae* type 1 O-SP antibodies in hyperimmune sera were measured by precipitin analysis (27). Serum (0.2 ml) was added to 3.0-ml conical glass centrifuge tubes. Antigens or controls, in 0.1-ml saline aliquots, were added, and the solutions were gently mixed, capped, and incubated at 37°C for 1 h. The solutions were mixed again and incubated at 3 to 8°C for 1 week, with gentle shaking each day. The tubes were centrifuged at 5,000 × *g* and 4°C for 1 h, the supernatants were decanted, and the tubes were drained. The precipitates were suspended in 1.0 ml of cold saline and centrifuged three times at 5,000 × *g* for 1 h each time. The precipitates were dissolved in 1.0 ml of 1% SDS, and their A_{280}s were recorded. Murine IgG served as a reference standard, and the results were expressed as micrograms of Ab per milliliter of serum. Immunodiffusion was performed with 0.9% agarose in phosphate-buffered saline–0.01 M EDTA.

The enzyme-linked immunosorbent assay (ELISA) used biotinylated O-SP of *S. dysenteriae* type 1, *S. flexneri* type 2a, and *S. sonnei* as described elsewhere (10, 52). The Ab level was calculated by parallel-line analysis with hyerimmune mouse serum, assayed for its precipitin content, as a standard (see above). TT Abs were measured by ELISA and expressed in units per milliliter.

Statistical. Logarithms of the Ab concentrations were used for all calculations. Concentrations that were below the sensitivity of the ELISA were assigned values equal to one-half of the sensitivity cutoff point of the ELISA. Comparisons of geometric means (GM) were performed with the two-sided unpaired *t* test. Data analyses were performed by using the Statistical Analysis System.

RESULTS

Characterization of LPS and O-SP. The yield of LPS was ~100 mg/liter of culture. Multiple bands, mostly in the intermediate zone of the SDS-PAGE, were demonstrable in samples containing as little as 10 ng of LPS (Fig. 1, lane 2). Following acid hydrolysis of the LPS, the gel filtration pattern through G-25 Sephadex showed the major peak in the void volume with a shoulder. These two fractions precipitated with the *S. dysenteriae* type 1 hyperimmune antiserum. The remaining fractions showed no reactivity with this antiserum. The void volume fractions were freeze-dried and designated O-SP. In multiple purifications, the yield of O-SP was ~50% of the LPS. The O-SP (1.0 mg/ml) showed only trace absorption at 260 and 280 nm (65). There were no detectable bands in SDS-PAGE of samples of the O-SP containing 200 μg, indicating 2,000-fold reduction of lipid A achieved by the purification. The *Limulus* lysate assay showed 0.03 endotoxin unit (EU)/μg of O-SP and 3,000 EU/μg of LPS (24), indicating a ~10^5-fold reduction of

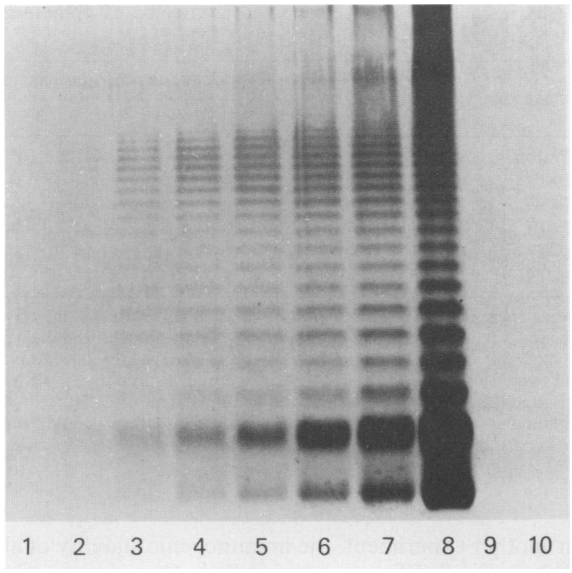

FIG. 1. Silver-stained SDS-PAGE 14% gel of *S. dysenteriae* type 1 LPS and O-SP. Lane 1, saline; lanes 2 through 8, 50 ng, 100 ng, 200 ng, 500 ng, 1 μg, 5 μg, and 10 μg, respectively, of LPS; lanes 9 and 10, 100 and 200 μg, respectively, of O-SP.

bioactive LPS that resulted in ~1.25 EU per proposed human dose of 25 μg of O-SP (9, 45).

The LPS and O-SP of *S. dysenteriae* yielded an identity reaction with the *S. dysenteriae* type 1 hyperimmune antiserum by double immunodiffusion (Fig. 2). Neither the LPS nor the O-SP of *S. flexneri* type 2a, *S. sonnei*, or *S. paratyphi* A reacted with this *S. dysenteriae* type 1 antiserum.

High-pressure liquid chromatography of the O-SP through Superose 12 HR showed a minor peak of ~65,200 Da and a major peak of ~18,700 Da. There was a small UV-absorbing peak with a molecular mass of 5,700 Da (not shown).

Nuclear magnetic resonance spectroscopy. Consistent with the proposed structure of the repeat unit, four sugar residues are indicated by the ^{13}C anomeric resonances at 94.4, 97.8, 101.5, and 102.2 ppm (these observed resonances are close to those predicted for this structure by the computer program CASPER) (Fig. 3) (14, 25). That one of the residues is a 2-acetamido-2-deoxy sugar is indicated by the presence of CH_3CO resonances at 22.9 (CH_3-) and 175.1 [C(O)] ppm and the C-2 resonance of the hexose at 53.7 ppm. The ^1H spectrum shows a characteristic doublet for the CH_3 groups of the two rhamnose residues at 1.31 ppm and a singlet for the *N*-acetyl group at 2.05 ppm (not shown).

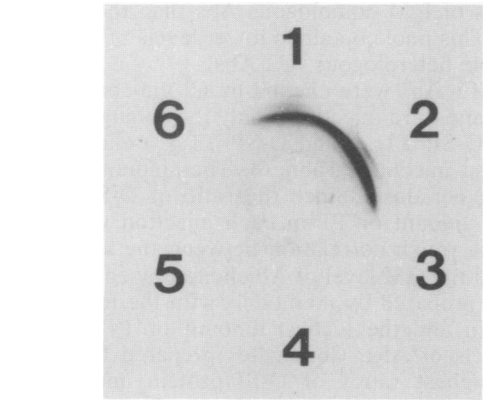

FIG. 2. Double immunodiffusion. Center well, *S. dysenteriae* type 1 hyperimmune serum; outer wells, antigen at 100 μg/ml. Well 1, *S. dysenteriae* type 1 LPS; well 2, *S. dysenteriae* type 1 O-SP; well 3, *S. flexneri* type 2a LPS; well 4, *S. flexneri* type 2a O-SP; well 6, *S. sonnei* LPS; well 6, *S. sonnei* O-SP.

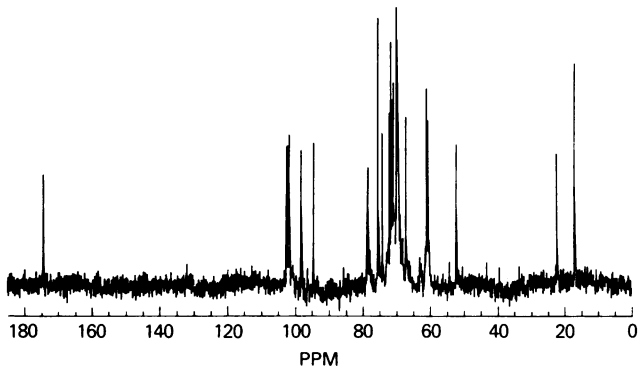

FIG. 3. ^{13}C nuclear magnetic resonance spectroscopy of *S. dysenteriae* type 1 O-SP.

Characterization of conjugates. The yields, calculated by recovery of the O-SP in the conjugate and the ratios of O-SP to protein in the conjugates, varied (Table 1). The first process bound ADH to the KDO of the O-SP by reductive amination; maximal binding was achieved at pH 8.5 in 0.1 M $NaCNBH_3$–0.1 M borate buffer held at room temperature for 3 days. About 5% of the O-SP–AH_{RE} bound to the protein with EDAC, resulting in PS/protein ratios of 0.065 for O-SP–$TT_{88\text{-}1}$ and 0.081 for O-SP–$TT_{88\text{-}3}$, or ~0.5 mol of O-SP per mol of TT. The second method bound dithiopropionic acid to the AH and then reduced the AH. The sulfhydryl derivative was then bound to the TT by SPDP (54). The ratios of O-SP to TT were 0.69 and 0.14, indicating 1.1 to 5.6 mol of O-SP per mol of TT for this type of conjugate.

The third method of attachment used CNBr to bind ADH to the O-SP. The AH derivative was then bound to TT by using EDAC to form dihydrazido bonds (8, 9). The average PS/protein ratio for these conjugates was 0.30, indicating that there were ~2.4 mol of O-SP per mol of TT.

TABLE 1. Composition of *S. dysenteriae* type 1 O-SP–TT conjugates

Synthetic scheme and conjugate	% Yield	O-SP (μg/ml)	Protein (μg/ml)	O-SP/protein ratio	
				wt/wt	mol/mol
Method 1					
O-SP–$TT_{88\text{-}1}$	13.2	66.1	1,020	0.06	0.49
O-SP–$TT_{88\text{-}3}$	11.3	112.9	1,380	0.08	0.65
Method 2					
O-SP–$TT_{SPDP\text{-}1}$	14.0	72.0	105.0	0.69	5.62
O-SP–$TT_{SPDP\text{-}2}$	41.9	93.6	670.0	0.14	1.14
Method 3					
O-SP–$TT_{88\text{-}4}$	23.6	235.9	868.8	0.27	2.20
O-SP–$TT_{89\text{-}1}$	31.0	310.5	1,088	0.29	2.37
O-SP–TT_{49782}[a]	32.1	422.9	1,270	0.33	2.69

[a] *S. dysenteriae* type 1-TT_{49782} was prepared for clinical investigation. Calculation of the molar ratios assumed molecular masses of 18,800 Da for the O-SP and 155,000 Da for the TT.

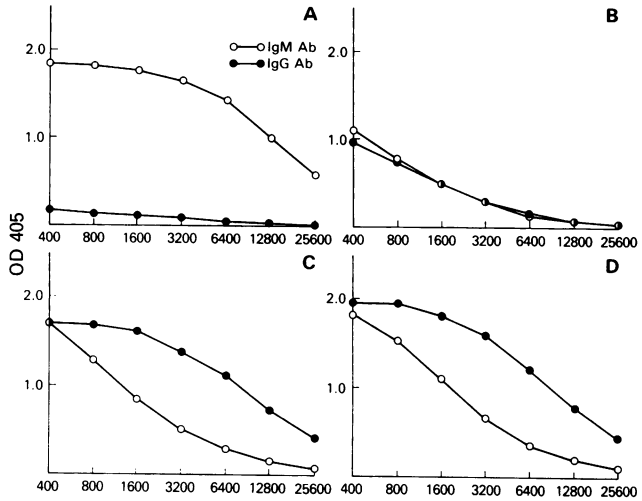

FIG. 4. IgG and IgM compositions of *S. dysenteriae* type 1 Abs in immunized mice as assayed by ELISA. The Ab levels are depicted as A_{405}. Pooled sera from mice were immunized with heat-killed *S. dysenteriae* type 1 cells (A) O-SP–$TT_{88\text{-}4}$ (B) O-SP–TT_{49782} (C), and O-SP–TT_{49782} adsorbed onto alum (D). OD 405, optical density at 405 nm.

The stability of conjugate O-SP–TT_{49782} in five dose vials prepared for clinical evaluation and stored at 3 to 8°C, room temperature, or 37°C was assayed by gel filtration through Superose 6 at 3 and 6 months. A single peak containing both protein and O-SP emerged in the void volume at all times and under all temperatures (data not shown).

Abs in hyperimmune sera. The highest content of precipitins (998 μg of Ab per ml) to the O-SP was elicited by multiple injections of heat-killed *S. dysenteriae* type 1; most were IgM Abs (Fig. 4). The next highest was elicited by O-SP–$TT_{88\text{-}4}$ in complete Freund adjuvant (747 μg of Ab per ml); ~70% was IgG, and the remainder was IgM. The shapes of the precipitin curves were similar for both serum pools (not shown). IgA Abs were not detected in these antisera.

Conjugate-induced Abs. The O-SP alone did not elicit detectable serum Abs; only O-SP–$TT_{SPDP\text{-}1}$ and O-SP–$TT_{88\text{-}4}$ elicited a statistically significant rise of the GM Ab level after each injection ($P < 0.05$ for each injection). O-SP–$TT_{88\text{-}4}$, prepared by method 3, elicited the highest GM Ab level after each of the three injections. Accordingly, more-detailed studies of the serum Ab response and interaction with adjuvants of this type of conjugate were made. Another lot of this conjugate was prepared by method 3 for clinical evaluation (O-SP–TT_{49782}) (Table 2). Table 3 shows that O-SP injected alone or adsorbed onto alum or in an O/W with MPL did not elicit serum IgG Abs. Only the O-SP with MPL elicited low but statistically significant levels of IgM Abs after the first injection, which persisted without change after subsequent injections. O-SP–TT_{49782}, injected as a saline solution, elicited a statistically significant rise in both IgM and IgG Abs after each injection. All the mice had both IgG and IgM Abs after the third injection. Adsorption of O-SP–TT_{49782} onto alum or incorporated with MPL in an O/W increased the serum IgM and IgG Ab levels over those elicited by the conjugate alone ($P < 0.01$ for each injection). The enhancement of O-SP Ab synthesis was higher with the alum-adsorbed conjugate than with the MPL O/W for IgG Abs after the second injection ($P = 0.04$). IgA antibodies were not detected in these sera.

TABLE 2. Total Ab levels elicited in mice by *S. dysenteriae* type 1 O-SP–TT[a]

Immunogen	GM μg of Ab/ml (25th–75th centiles after injection)		
	1	2	3
O-SP alone	<0.10	<0.10	<0.10[b]
O-SP–$TT_{88\text{-}1}$	0.52 (0.42–1.02)	0.88 (0.48–2.11)	0.91[c] (0.53–1.20)
O-SP–$TT_{88\text{-}3}$	0.44 (0.26–0.43)	0.53 (0.39–0.66)	0.70[c] (0.50–0.93)
O-SP–$TT_{SPDP\text{-}1}$	0.43 (0.32–0.56)	0.66 (0.54–0.81)	1.24[c] (0.88–1.49)
O-SP–$TT_{SPDP\text{-}2}$	0.26 (0.05–0.53)	0.73 (0.48–1.05)	1.18[c] (0.54–2.25)
O-SP–$TT_{88\text{-}4}$	0.98[d] (0.54–1.85)	5.87[e] (2.03–11.8)	92.2[f] (32.8–309)

[a] Mice were injected three times 2 weeks apart with 0.1 ml of saline containing 2.5 μg of PS alone or as a conjugate. Ten mice from each group were randomly chosen before injection 2 and at 10 days after injections 2 and 3 and were exsanguinated. Total Abs were measured by ELISA with hyperimmune serum that was calibrated for its content of O-SP Ab by using precipitin analysis as a standard (see Materials and Methods). For *f* versus *e* and *e* versus *d*, $P < 0.001$. For *c* versus *b* and *f* versus *c*, $P = 0.0001$.

In another experiment, the immunogenic stability of alum-adsorbed O-SP–TT_{49782} was studied. One group of mice received the conjugate adsorbed onto alum prior to each injection. Another group of mice received this conjugate adsorbed onto alum and then stored for 3 months at 3 to 8°C. Table 4 shows that there was no difference in the levels of serum Abs elicited by the alum-adsorbed conjugates injected immediately or stored for 3 months. In another experiment, O-SP–TT_{49782} stored for 6 months at 3 to 8, 22, or 37°C was injected s.c. three times into mice. All preparations elicited significant IgM and IgG Ab rises after each injection ($P < 0.01$). The only significant difference after each injection was a higher GM level of IgG elicited by the third injection of the vaccine stored at 3 to 8°C (2.97 μg of Ab per ml versus 0.83 μg/ml at 22°C and 0.81 μg/ml at 37°C; $P < 0.01$; data not shown).

Cross-reactions between *S. dysenteriae* and *S. flexneri*. Pooled sera taken after the third injection of either *S. dysenteriae* type 1-TT or *S. flexneri* type 2a-rEPA had both IgM and IgG Abs to the O-SP of the homologous immunogen (Table 5). Both pools of antiserum also had IgM Abs but no detectable IgG to the heterologous O-SP. The sera elicited by the alum-adsorbed *S. dysenteriae* type 1-TT had higher levels of IgM Abs and low but detectable levels of IgG Abs to *S. flexneri* type 2a. The alum-adsorbed *S. flexneri* type 2a-rEPA conjugate elicited higher levels of IgG Abs and lower levels of IgM homologous Abs than the unadsorbed conjugate. This pool contained lower levels of IgM Abs and no detectable heterologous IgG Abs.

TT Abs. TT Abs were elicited by all the conjugates after the second and third injections: only the conjugates made by method 1 (O-SP–$TT_{88\text{-}1}$ and O-SP–$TT_{88\text{-}3}$) elicited TT Abs after the first injection (Table 6). The amount of the O-SP was 2.5 μg per dose. Since the ratio of O-SP to protein varied, the amount of TT in each injection was different. There was a rough correlation between the amount of TT injected and the GM level of Ab elicited by each conjugate. Conjugates prepared by method 1, with the lowest ratio of O-SP/protein and the highest amount of TT, elicited the highest levels of Abs. Conjugates prepared by method 3, with the highest ratios of O-SP/protein and the lowest amount of TT, elicited the lowest levels of Abs. On the basis of our previous experience, the levels of Abs to TT elicited by our conjugates could be expected to be greater than the estimated protective level (0.01 IU/ml). As expected, the O-SP alone did not elicit TT Abs.

TABLE 3. Serum IgM and IgG O-SP Abs elicited in mice by conjugate O-SP–TT_{49782}[a]

Vaccine	GM μg of Ab/ml (25th–75th centiles)					
	IgG after injection:			IgM after injection:		
	1	2	3	1	2	3
O-SP	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
O-SP + alum	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
O-SP + MPL	<0.1	<0.1	<0.1	0.38 (0.18–1.00)	0.35 (0.05–0.31)	0.34 (0.16–0.62)
O-SP–TT	0.21 (0.05–0.54)	0.91 (0.45–2.62)	2.86 (1.05–4.80)	0.16 (0.05–0.54)	0.33 (0.19–0.67)	4.45 (0.61–48.9)
O-SP–TT + alum	0.42 (0.22–0.53)	3.44 (1.67–11.4)	67.3[b] (9.66–549)	0.64 (0.32–1.17)	1.73 (0.72–5.28)	8.62 (1.46–36.0)
O-SP–TT + MPL	0.51 (0.20–0.88)	4.25 (1.25–23.4)	8.63[c] (1.33–49.4)	0.98 (0.52–1.54)	3.75 (0.94–33.1)	9.61 (2.48–16.7)

[a] Mice were injected s.c. three times 2 weeks apart with 0.1 ml of 2.5 μg of O-PS alone, as a conjugate in saline, admixed with MPL in an O/W, or with alum (see Materials and Methods). Ten mice from each group were exsanguinated before injection 2 and 10 days after injections 2 and 3. For *b* versus *c*, $P = 0.04$

DISCUSSION

We assume, mostly from studies of *S. flexneri* type 2a, that either immunization with attenuated strains or natural infection induces type (LPS)-specific protection (19–21, 32, 35, 64). In one report from China, the etiology of shigellosis was recorded during two epidemics several years apart: each was caused by multiple groups (3). This study showed ~85% type protection against reinfection with *S. flexneri* and ~60% protection against *S. dysenteriae* type 1 and *S. sonnei*. This partial type-specific protection waned 3 years after the second outbreak. The nature of this type-specific protective immunity elicited by experimental challenge, immunization with attenuated strains, or convalescence from shigellosis is not known. Data from prospective surveys of armed forces recruits in Israel show that serum IgG LPS Abs are correlated with resistance to shigellosis (11). Using these data and an analogy with the pathogenesis of and protective immunity to salmonellosis, we have designed conjugate vaccines to elicit O-SP Abs in humans (8).

The immunogenicity of O-SP conjugates prepared by three synthetic schemes was compared in young outbred mice. The immunization protocol, which used ~1/10 the human dose injected in saline by the s.c. route, was predictive of the immunogenicity of *H. influenzae* type b, pneumococcus types 6B and 12F, and *Staphylococcus aureus* capsular PS conjugates in humans (44). The LPS content of the conjugates was low and could not account for the increased immunogenicity and acquisition of the T-cell-dependent properties of the O-SP. The superior immunogenicity of O-SP conjugates prepared by multipoint attachment with AH as a spacer is consistent with two clinical evaluations of *H. influenzae* type b conjugates: higher levels of capsular PS antibodies were elicited in infants by an *H. influenzae* type b-TT conjugate prepared by method 3 than by conjugates prepared by other methods (1, 9, 13). The mechanism(s) of this increased immunogenicity is not yet understood.

S. dysenteriae type 1 is an inhabitant of and a pathogen for humans only. In monkeys, doses of 10^{10} to 10^{11} organisms are required to induce dysentery (5, 21, 34). In contrast, dysentery is regularly induced in volunteers fed ~10^3 organisms of *S. dysenteriae* type 1 (15, 31). For this reason, we question the validity of using monkeys as a model for disease caused by this pathogen. One method of evaluating the secondary biological activities of LPS Abs, which may be correlated with protective immunity, is to measure the bactericidal activity of sera. IgG LPS Abs have been shown to induce complement-dependent bacteriolysis in a variety of gram-negative pathogens. But shigellae, including *S. dysenteriae* type 1, are susceptible to the bactericidal action of serum alone (43). Clinical trials will be required to demonstrate whether our O-SP conjugate vaccines will confer protective immunity. Because there are no in vivo or in vitro correlates of protection, we plan to study the region of the O-SP that determines type (LPS)-specificity in human sera by inhibition reactions with oligosaccharides corresponding to the repeat unit (14, 51). In this way, we hope to measure the amount and fine specificity of Abs elicited by our O-SP conjugate vaccines.

As described for hyperimmune antiserum prepared by immunization of rabbits with whole inactivated bacteria (16),

TABLE 4. Stability of alum-adsorbed *S. dysenteriae* type 1 O-SP–TT_{49872}[a]

Vaccine	GM μg of Ab/ml of serum (25th–75th centiles)					
	IgG after injection:			IgM after injection:		
	1	2	3	1	2	3
Saline	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
O-SP–TT_A	0.18 (0.10–0.24)	1.88 (0.64–4.66)	25.3 (6.22–171)	0.28 (0.09–0.23)	1.67 (0.40–4.66)	10.4 (3.45–19.3)
O-SP–TT_{A3}	0.13 (0.10–0.19)	10.6 (3.54–40.3)	18.1 (7.98–39.6)	0.57 (0.04–1.20)	6.04 (1.84–32.6)	2.06 (0.50–3.76)

[a] *S. dysenteriae* type 1 O-SP–TT_{49782} was injected s.c. alone or adsorbed to alum at a dose of 2.5 μg of O-SP three times 2 weeks apart. Representative mice [$n = 10$] were exsanguinated from each group 14 days after injection 1 and 10 days after injections 2 and 3. The conjugate was either adsorbed prior to each injection (O-SP–TT_A) or adsorbed and stored for 3 months at 3 to 8°C before injection (O-SP–TT_{A3}).

TABLE 5. Cross-reactions between Abs (μg/ml) induced by O-SP of *S. dysenteriae* type 1-TT_{49782} and *S. flexneri* type 2a-*P. aeruginosa* rEPA[a]

Immunogen[b]	Antigen used for ELISA			
	S. dysenteriae		*S. flexneri*	
	IgG	IgM	IgG	IgM
S. dysenteriae type 1-TT	2.64	1.50	<0.1	0.99
S. dysenteriae type 1-TT (ads)	5.32	1.19	0.22	1.85
S. flexneri type 2a-rEPA	<0.1	0.74	1.63	6.23
S. flexneri type 2a-rEPA (ads)	<0.1	0.1	2.65	2.32

[a] Groups of 10 mice were injected three times 2 weeks apart, and their pooled sera were assayed for O-SP antibodies 7 days after the last injection.
[b] The conjugates were injected in saline or adsorbed to alum (ads) as described in Materials and Methods.

a cross-reaction with the O-SP of *S. flexneri* type 2a was observed in sera taken after the third injection of *S. dysenteriae* type 1-TT. A similar cross-reaction was noted in sera elicited by the *S. flexneri* type 2a-rEPA. These cross-reacting Abs were mostly IgM. These cross-reactions are probably due to the -α-L-Rha*p*-(1→3)-α-L-Rha*p*- moiety in the repeat units of *S. dysenteriae* type 1 and *S. flexneri* type 2a and will have to be accounted for during clinical evaluation of these conjugates (6, 29, 33).

Conjugates of O-SP from other gram-negative pathogens have been synthesized. *Salmonella typhimurium* conjugates emulsified in complete Freund's adjuvant conferred on mice passive protection against lethal challenge (53). Conjugates have been prepared with lipooligosaccharides from neisseriae (26, 30, 57). The reducing ends of these saccharides were bound to the protein by single point attachments. The O-SP of *P. aeruginosa* and the O18 LPS of *Escherichia coli* have been conjugated with ADH as a spacer (12, 13). In all, the conjugated O-SP was more immunogenic than the saccharide alone.

Epidemics of *S. dysenteriae* type 1 are likely to occur in young children where there are less-than-optimal nutrition, exposure to multiple infections, and other conditions which depress Ab responsiveness to vaccines (8, 19, 22, 38–42, 49, 50, 55, 63, 64). For this reason, we evaluated the adjuvancy of alum and MPL in an O/W on our *S. dysenteriae* type 1 conjugates. Reports of the effect of alum on Ab synthesis elicited by conjugates are confusing. Adsorption of *H. influenzae* type b-TT increased the immunogenicity of the PS component in rhesus monkeys; adsorption of a similar conjugate onto alum and injection several months later of 18-month-old children resulted in depressed PS and TT Ab responses (10). Einhorn et al. showed PS Ab responses in infants elicited by one injection of *H. influenzae* type b capsular PS bound to an outer membrane preparation of group B *Neisseria meningitidis* adsorbed onto alum and injected on the same day (17). Evaluation of the adjuvancy of the alum was not possible, because this conjugate had high levels of LPS and was not injected alone into infants. Presumably, the LPS toxicity of this conjugate was rendered nonreactive by adsorption onto alum. O-SP–TT_{49782} showed enhanced immunogenicity in mice whether injected the same day or 3 months after adsorption onto alum. Because of the stability of its adjuvant effect in mice and its widespread use in human infants, we plan to evaluate alum-adsorbed *S. dysenteriae* type 1 O-SP–TT in volunteers.

TABLE 6. TT Abs elicited in mice by *S. dysenteriae* type 1 O-SP–TT

Immunogen	μg of TT/dose	GM (range) (U/ml) after injection:		
		1	2	3
O-SP	None	<0.10	<0.10	<0.10[a]
O-SP–$TT_{88\text{-}1}$	40.9	0.43 (0.1–1.3)	7.28 (5.4–10)	20.9[b] (12.5–34)
O-SP–$TT_{88\text{-}3}$	30.6	0.16 (0.1–0.3)	0.94 (0.1–5.4)	8.49[c] (4.5–22)
O-SP–$TT_{SPDP\text{-}1}$	3.60	<0.10	0.51 (0.1–1.4)	2.53[d] (1.2–4.9)
O-SP–$TT_{SPDP\text{-}2}$	17.9	<0.10	2.66 (1.7–5.4)	8.30[c] (4.9–21)
O-SP–$TT_{88\text{-}4}$	9.20	<0.10	0.43 (0.1–1.4)	0.83[d] (0.4–2.3)
O-SP–TT_{49872}	7.50	<0.10	0.36 (0.3–0.6)	2.47[d] (1.6–4.1)

[a,b,c,d] For *d*, *c*, and *b* versus *a*, $P < 0.001$. For *b* versus *c*, $P < 0.02$. For *c* versus *d*, $P < 0.03$.

Most adults in both developing and industrialized nations have serum Abs to the O-SP of *S. dysenteriae* type 1 (8, 33, 49). It is likely that the stimuli for these Abs in developed countries are nonpathogenic cross-reacting bacteria of the respiratory and intestinal tracts (8, 44). The immunoglobulin compositions of serum LPS Abs were assayed in patients during an outbreak of dysentery in Vietnam caused by *S. dysenteriae* type 1 (33). Controls were otherwise healthy adults in Vietnam and Sweden; all had low levels of IgM, IgA, and IgG Abs. The patients had significantly elevated titers of both IgA and IgG Abs. The IgG Abs in the patients remained elevated longer than either the IgM or IgA titers. Absorption experiments indicated that Abs to both the core and O-SP regions of the LPS were present in almost all the sera from patients as well as controls. O-SP–TT conjugates, especially those synthesized by method 3, elicited high levels of IgG Abs in mice, thus mimicking the serum Ab response to infection with *S. dysenteriae* type 1.

In summary, conjugates of O-SP of *S. dysenteriae* type 1 bound to TT, which were prepared by three synthetic schemes, were evaluated in mice. Injected s.c. in saline solutions, all elicited both IgM and IgG Abs. Conjugates prepared with multipoint attachments with ADH as a spacer were the most immunogenic. Both alum and MPL in an O/W enhanced the serum Ab responses to the O-SP of the conjugate. The adjuvant effect of alum was stable for at least 3 months. Clinical evaluation of this *S. dysenteriae* type 1 conjugate is planned.

ACKNOWLEDGMENTS

We are grateful to William Egan, Larry Philips, and Willie Vann, OBER, FDA, and Ivan Heron, Statens Seruminstitut, Copenhagen, Denmark, for their assistance and cooperation in this research.

REFERENCES

1. **Anderson, P. W., M. E. Pichichero, E. C. Stein, S. Porcalli, R. F. Betts, D. M. Connuck, D. Lorones, R. A. Insel, J. M. Zahradnik, and R. Eby.** 1989. Effect of oligosaccharide chain length, exposed terminal group, and hapten loading on the antibody response of human adults and infants to vaccines consisting of *Haemophilus influenzae* type b capsular antigen unitermally coupled to the diphtheria protein CRM_{197}. J. Immunol. **142:** 2464–2468.
2. **Bhattacharya, S. K., D. Sen, G. B. Nair, M. K. Bhattacharya, P. Darta, and D. Darta.** 1986. Multiply drug-resistant *Shigella dysenteriae* type 1 and travelers' diarrhea. J. Infect. Dis. **154:**729–730.
3. **Bing-nan, F., G. R. Zhang, H. C. Yang, D. B. Zhao, and M. X. Liu.** Epidemiological analysis of the duration of intestinal immunity after natural infection with Shigella. Chin. Med. J., in press.
4. **Binns, M. W., M. S. Vaughan, and K. N. Timmis.** 1985. *O*

antigens are essential virulence factors of *S. sonnei* and *S. dysenteriae* type 1. Zentrabl. Bakteriol. Hyg. Abt. I Orig. Reihe B **181**:197–205.

5. **Branham, S. E., K. Habel, and R. D. Lillie.** 1949. Studies with Shigella dysenteriae (SHIGA). III. Infection and intoxication in *Maccacus mulatta* monkeys. J. Infect. Dis. **85**:295–303.
6. **Carlin, N. I. A., and A. A. Lindberg.** 1987. Monoclonal antibodies specific for *Shigella flexneri* lipolysaccharides: clones binding to type IV, V, and VI antigens, group 3,4 antigen, and an epitope common to all *Shigella flexneri* and *Shigella dysenteriae* type 1 strains. Infect. Immun. **55**:1412–1420.
7. **Carlsson, J., H. Drevin, and R. Axen.** 1978. Protein thiolation and reversible protein-protein conjugation. N-succinimidyl 3-(2-pyridyldithio)propionate, a new heterofunctional reagent. Biochem. J. **173**:723–737.

7a. **Chu, C. Y., B. Liu, D. Watson, S. Szu, R. Schneerson, and J. B. Robbins.** Personal communication.

8. **Chu, C. Y., R. Schneerson, and J. B. Robbins.** Submitted for publication.
9. **Chu, C. Y., 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.
10. **Claesson, B. A., B. Trollfors, T. Lagergard, J. Taranger, D. Bryla, G. Otterman, T. Cramton, Y. Yang, C. R. Reimer, J. B. Robbins, and R. Schneerson.** 1988. Clinical and immunological responses to the capsular polysaccharide of *Haemophilus influenzae* type b alone or conjugated to tetanus toxoid in 18- to 23-month-old children. J. Pediatr. **112**:695–702.
11. **Cohen, D., M. S. Green, C. Block, R. Slephon, and I. Ofek.** 1991. Prospective study of the association between serum antibodies to lipopolysaccharide O antigen and the attack rate of shigellosis. J. Clin. Microbiol. **29**:386–389.
12. **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.
13. **Decker, M. D., K. M. Edwards, R. Bradley, and B. Palmer.** 1990. Four conjugate Haemophilus b vaccines in infants: a comparative trial, abstr. 61, 30th Intersci. Program Abstr. Conf. Antimicrob. Agents Chemother. 1990.
14. **Dmitriev, B. A., N. Knirel, N. K. Kochetkov, and I. L. Hofman.** 1976. Somatic antigens of *Shigella*. Structural investigations on the O-specific polysaccharide chain of *Shigella dysenterae* type 1 lipolysaccharide. Eur. J. Biochem. **66**:559–566.
15. **Dupont, H. L., M. M. Levine, R. B. Hornick, and S. B. Formal.** 1989. Inoculum size in shigellosis and implications for expected mode of transmission. J. Infect. Dis. **159**:1126–1128.
16. **Edwards, P. R., and W. H. Ewing.** 1972. Identification of Enterobacteriaceae, 3rd ed., p. 121. Burgess Publishing Co., Minneapolis.
17. **Einhorn, M. S., G. A. Weinberg, E. L. Anderson, P. D. Granoff, and D. M. Granoff.** 1986. Immunogenicity in infants of *Haemophilus influenzae* type B polysaccharide in a conjugate vaccine with *Neisseria meningitidis* outer-membrane protein. Lancet **ii**:299–302.
18. **Ellman, G. L.** 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. **82**:70–77.
19. **Felsen, J.** 1945. Bacillary dysentery. Colitis and enteritis. The W. B. Saunders Co., Philadelphia.
20. **Formal, S. B., T. L. Hale, and C. Kapfer.** 1989. Shigella vaccines. Rev. Infect. Dis. **11**(Suppl.):S547–S551.
21. **Formal, S. B., T. H. Kent, H. C. May, A. Palmer, S. Falkow, and E. H. LaBrec.** 1966. Protection of monkeys against experimental shigellosis with a living oral polyvalent dysentery vaccine. J. Bacteriol. **92**:17–22.
22. **Gordon, J. E., W. Ascoli, V. Pierce, M. A. Guzman, and L. J. Mata.** 1965. Studies of diarrheal diseases in Central America. VI. An epidemic of diarrhea in a Guatemalan highland village with a component due to *Shigella dysenteriae* type 1. Am. J. Trop. Med. Hyg. **14**:404–411.
23. **Hales, T. L., P. Guerry, R. C. Seid, C. Kapfer, M. E. Wingfield, C. B. Reaves, L. S. Baron, and S. M. Formal.** 1984. Expression of lipopolysaccharide O antigen in *Escherichia coli* K-12 hybrids containing plasmid and chromosomal genes from *Shigella dysenteriae* 1. Infect. Immun. **46**:470–475.
24. **Hochstein, H. D., D. F. Mills, A. S. Outschoorn, and S. C. Rastogi.** 1983. The processing and collaborative assay of a reference endotoxin. J. Biol. Stand. **11**:251–260.
25. **Jansson, P.-E., L. Kenne, and G. Widmalm.** 1987. CASPER—a computerised approach to structure determination of polysaccharides using information from N.M.R. spectroscopy and simple chemical analyses. Carbohydr. Res. **168**:67–77.
26. **Jennings, H. J., C. Lugowski, and F. E. Ashton.** 1984. Conjugation of meningococcal lipopolysaccharide R-type oligosaccharides to tetanus toxoid as route to a potential vaccine against group B *Neisseria meningitidis*. Infect. Immun. **43**:407–412.
27. **Kabat, E. A., and M. M. Mayer.** 1964. Experimental immunochemistry, p. 22. Charles C Thomas, Publisher, Springfield, Ill.
28. **Kauffmann, F.** 1954. Enterobacteriaceae. Munksgaard, Copenhagen.
29. **Kenne, L., B. Lindberg, K. Petersson, E. Katzenellenbogen, and E. Romanowska.** 1978. Structural studies of *Shigella flexneri* O-antigens. Eur. J. Biochem. **91**:279–284.
30. **Lambden, P. R., and J. E. Heckels.** 1982. Synthesis of immunogenic oligosaccharide-protein conjugates from the lipopolysaccharide of *Neisseria gonorrhoeae* P9. J. Immunol. Methods **48**:233–240.
31. **Levine, M. M., H. L. Dupont, S. B. Formal, R. B. Hornick, A. Takeuchi, M. J. Snyder, and J. P. Libonati.** 1973. Pathogenesis of *Shigella dysenteriae* type 1 (Shiga) dysentery. J. Infect. Dis. **127**:261–270.
32. **Levine, M. M., J. B. Kaper, R. E. Black, and M. L. Clements.** 1983. New knowledge on pathogenesis of bacterial enteric infections as applied to bacterial vaccine development. Microbiol. Rev. **47**:510–550.
33. **Lindberg, A. A., S. Haeggman, K. Karlsson, P. DacCam, and D. DucTrach.** 1984. The humoral antibody response to *Shigella dysenteriae* type 1 as determined by ELISA. Bull. W.H.O. **62**:597–606.
34. **McIver, J., G. F. Grady, and S. B. Formal.** 1977. Immunization with *Shigella dysenteriae* type 1: evaluation of antitoxic immunity in prevention of experimental diseases in Rhesus monkeys (*Macaca mulatta*). J. Infect. Dis. **136**:416–421.
35. **Mel, D. M., A. L. Terzin, and I. Vuksic.** 1965. Studies on vaccination against bacillary dysentery. 3. Effective oral immunization against *Shigella flexneri* 2a in a field trial. Bull. W.H.O. **32**:647–655.
36. **Oravec, L. S., C. J. Lee, A. Hoppe, and C. Santos.** 1984. Detection of blood group substances in vaccines by countercurrent immunoelectrophoresis. J. Biol. Stand. **12**:159–166.
37. **Orskov, F., and I. Orskov.** 1978. Serotyping of Enterobacteriacae, with special emphasis on K antigen determination, p. 1–77. *In* T. Bergman and J. R. Norris (ed.), Methods in microbiology, vol. 11. Academic Press Ltd., London.
38. **Pal, S. C.** 1984. Epidemic bacillary dysentery in West Bengal, India. Lancet **i**:1462.
39. **Parsonnet, J., K. D. Greene, A. Russell Gerber, R. V. Tauxe, O. J. Vallejo Aguilar, and P. A. Blake.** 1989. *Shigella dysenteriae* type 1 infections in US travellers to Mexico. Lancet **ii**:543–545.
40. **Raghupathy, P., A. Date, J. C. M. Shastry, A. Sudarsanam, and M. Jadhav.** 1978. Haemolytic-uraemic syndrome complicating Shigella dysentery in south Indian children. Br. Med. J. **1**:1518–1521.
41. **Rahaman, M. M., K. Haider, H. Baru, and N. Rahman.** 1985. Re-emergence of epidemic due to resistant Shiga bacillus (*S. dysenteriae* type 1) and *S. flexneri* in Bangladesh. J. Infect. Dis. **152**:1114–1120.
42. **Rahaman, M. M., M. M. Khan, K. M. S. Aziz, N. S. Islam, and A. K. M. G. Kibriya.** 1973. An outbreak of dysentery caused by *Shigella dysenteriae* type 1 on a coral island in the Bay of Bengal. J. Infect. Dis. **132**:15–19.
43. **Reed, W. P., and E. L. Albright.** 1974. Serum factors responsible for killing of Shigella. Immunology **26**:205–215.
44. **Robbins, J. B., and R. Schneerson.** 1990. Polysaccharide-protein

conjugates: a new generation of vaccines. J. Infect. Dis. **161:** 821–832.

45. **Roy, R., E. Katzenellenbogen, and H. J. Jennings.** 1984. Improved procedures for the conjugation of oligosaccharides to protein by reductive amination. Can. J. Biochem. Cell Biol. **62:**270–280.
46. **Schneerson, R., A. Fattom, S. C. Szu, D. Bryla, J. T. Ulrich, J. A. Rudbach, G. Schiffman, and J. B. Robbins.** Enhancement of the serum antibody response to the polysaccharide component of conjugates in mice by concurrent injection with monophosphoryl lipid A and squalene. J. Immunol., in press.
47. **Scott, T. A., and E. H. Melvin.** 1953. Determination of dextran with anthrone. Anal. Chem. **25:**1656–1661.
48. **Shiga, K.** 1898. Ueber den Dysenteriebacillus (*Bacillus dysenteriae*). Zentralbl. Bakteriol. **24:**817–828.
49. **Shiga, K.** 1936. The trend of prevention, therapy, and epidemiology of dysentery since the discovery of its causative organism. N. Engl. J. Med. **215:**1205–1211.

49a. **Shiloach, J., A. Fattom, I. Pastan, and D. Fitzgerald.** Personal communication.

50. **Siber, G. R., M. Santosham, G. R. Reid, C. Thompson, J. Almeido-Hill, A. Morell, G. deLange, J. K. Ketcham, and E. H. Callahan.** 1990. Impaired antibody response to *Haemophilus influenzae* type b polysaccharide and low IgG2 and IgG4 concentrations in Apache children. N. Engl. J. Med. **323:**1387–1392.
51. **Sturm, S., B. Jann, K. Jann, P. Fortnagel, and K. N. Timmis.** 1986. Genetic and biochemical analysis of *Shigella dysenteriae* 1 *O* antigen polysaccharide biosynthesis in *Escherichia coli* K-12: structure and functions of the rfb gene cluster. Microb. Pathog. **1:**307–324.
52. **Sutton, A., W. F. Vann, A. Karpas, K. E. Stein, and R. Schneerson.** 1985. An avidin-biotin-based ELISA for quantitation of antibody to bacterial polysaccharides. J. Immunol. Methods **82:**215–224.
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. **Szu, S. C., A. L. Stone, J. D. Robbins, R. Schneerson, and J. B. Robbins.** 1987. Vi capsular polysaccharide-protein conjugates for prevention of typhoid fever. J. Exp. Med. **166:**1510–1524.
55. **Taylor, D. V., P. Echeverria, O. Sethabutr, C. Pitarangsi, U. Leksomboon, N. R. Blacklow, B. Rowe, R. Gross, and J. Cross.** 1988. Clinical and microbiologic features of *Shigella* and enteroinvasive *Escherichia coli* infections detected by DNA hybridization. J. Clin. Microbiol. **26:**1362–1366.
56. **Tsai, C.-M.** 1986. The analysis of lipopolysaccharide (endotoxin) in meningococcal polysaccharide vaccines by silver staining following SDS-polyacrylamide gel electrophoresis. J. Biol. Stand. **17:**249–258.
57. **Verheul, A. F. M., A. K. Braat, L. 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 phosphoethanolamine group-containing oligosaccharide-protein conjugates. Infect. Immun. **59:** 843–851.
58. **Warren, L.** 1959. The thiobarbituric acid reaction for the measurement of sialic acid. J. Biol. Chem. **234:**1971–1975.
59. **Watanabe, H., A. Nakamura, and K. N. Timmis.** 1984. Small virulence plasmid of *Shigella dysenteriae* 1 strain W30864 encodes a 41,000-dalton protein involved in formation of specific lipopolysaccharide side chains of serotype 1 isolates. Infect. Immun. **46:**55–63.
60. **Watanabe, H., and K. N. Timmis.** 1984. A small plasmid in *Shigella dysenteriae* 1 specifies one or more functions essential for O antigen production and bacterial virulence. Infect. Immun. **43:**391–396.
61. **Weissman, J. B., K. I. Marton, J. N. Lewis, and C. T. H. Friedman.** 1974. Impact in the United States of the Shiga dysentery pandemic of Central American and Mexico: a review of the surveillance data through 1972. J. Infect. Dis. **129:**218–223.
62. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. Methods Carbohydr. Chem. **5:**83–91.
63. **Williamson, W. A., and B. M. Greenwood.** 1978. Impairment of the immune response to vaccination after acute malaria. Lancet **i:**1328–1329.
64. **World Health Organization.** 1988. Guidelines for control of epidemics due to *Shigella dysenteriae* type 1. WHO/CDD/SER/88.12. World Health Organization, Geneva.
65. **World Health Organization Expert Committee on Biological Standardization.** 1977. Technical report series no. 610. World Health Organization, Geneva.