INFECTION AND IMMUNITY, Mar. 2001, p. 1351–1357 Vol. 69, No. 3
0019-9567/01/$04.00+0 DOI: 10.1128/IAI.69.3.1351–1357.2001

Safety and Immunogenicity of Improved *Shigella* O-Specific Polysaccharide-Protein Conjugate Vaccines in Adults in Israel

JUSTEN H. PASSWELL,[1]* EFRAT HARLEV,[1] SHAI ASHKENAZI,[2] CHIAYUNG CHU,[3] DAN MIRON,[1] REUT RAMON,[1] NAHEED FARZAN,[1] JOSEPH SHILOACH,[4] DOLORES A. BRYLA,[3] FATHY MAJADLY,[3] ROBIN ROBERSON,[3] JOHN B. ROBBINS,[3] AND RACHEL SCHNEERSON[3]

Samuel Jared Pediatric Immunology Laboratory, Sheba Medical Center,[1] and Schneider's Children's Hospital,[2] Tel-Aviv, Israel, and National Institute of Child Health and Human Development[3] and National Institute of Diabetes and Digestive and Kidney Diseases,[4] National Institutes of Health, Bethesda, Maryland 20892

Received 11 August 2000/Returned for modification 12 October 2000/Accepted 28 November 2000

Data suggest that the O-specific polysaccharide (O-SP) domain of the lipopolysaccharide (LPS) of *Shigella* species is both an essential virulence factor and a protective antigen and that a critical level of serum immunoglobulin G (IgG) to this antigen will confer immunity to shigellosis. Because covalent attachment of polysaccharides to proteins increases their immunogenicity, especially in infants and in young children, the O-SP of *Shigella* species were bound to medically useful proteins, and the safety and immunogenicity of the resultant conjugates were confirmed in adults and 4- to 7-year-old children. Succinylation of the carrier protein improved the immunogenicity of *Shigella* conjugates in mice and increased their yield. Based on these results, a clinical trial of O-SP conjugates of *Shigella sonnei* and *Shigella flexneri* 2a bound to succinylated mutant *Pseudomonas aeruginosa* exotoxin A (*r*EPA$_{succ}$) or native or succinylated *Corynebacterium diphtheriae* toxin mutant (CRM9 or CRM9$_{succ}$) was conducted in healthy adults. The conjugates were safe and immunogenic. *S. sonnei*-CRM9, *S. sonnei*-CRM9$_{succ}$, and *S. sonnei*-*r*EPA$_{succ}$ elicited significant rises of geometric mean (GM) IgG anti-LPS within 1 week of injection ($P < 0.001$). At 26 weeks, the GM anti-LPS levels elicited by these three conjugates were similar and higher than their prevaccination levels ($P < 0.0001$). GM IgG anti-LPS levels elicited by *S. flexneri* 2a-*r*EPA$_{succ}$ were significantly higher than those elicited by *S. flexneri* 2a-rCRM9$_{succ}$ at all intervals after injection. At 26 weeks, the levels of IgG anti-LPS in vaccinees were higher than their prevaccination levels ($P < 0.0001$). The serum antibody responses were specific, as there was no significant rise of anti-LPS to the heterologous O-SP in any vaccinee. Both conjugates elicited statistically significant rises of serum antibodies to the injected carrier protein. At 6 months, these five *Shigella* conjugates elicited higher fold rises than similar conjugates (D. N. Taylor et al., Infect. Immun. 61:3678–3687, 1993). Based on these data, we chose *S. sonnei*-CRM9 and *S. flexneri* 2a-*r*EPA$_{succ}$ for evaluation in children.

Shigellosis remains a serious and common disease (16, 19, 23, 26, 28, 38, 46, 55, 56). In addition to causing watery diarrhea, shigellae are a major cause of dysentery (fever, cramps, and blood and/or mucus in the stool) (19, 46, 49, 55, 56). Not commonly appreciated is that dysentery, not watery diarrhea, retards growth in children (7, 9, 19, 30, 38, 46, 49, 55, 56).

Although *Shigella dysenteriae* type 1 was discovered as the cause of epidemic dysentery in Japan in 1898 (53), there is neither a licensed vaccine for it nor a consensus as to the mechanism(s) of host immunity to *Shigella* (11, 18, 31, 38, 46, 47). Vaccine development has been hampered by three factors: (i) the ineffectiveness of parenterally injected inactivated whole-cell vaccines which led to the belief that serum antibodies do not confer immunity (25, 32); (ii) the lack of a suitable animal model (46); and (iii) only indirect evidence of immune mechanism(s) in humans (11, 14, 38, 46–48).

The O-specific polysaccharide (O-SP) domain of lipopolysaccharide (LPS) is both an essential virulence factor and a protective antigen of *Shigella* (46). Convalescence from shigellosis confers LPS-specific immunity, although incomplete and of limited duration (6, 18, 31, 38, 46). The following data indicate serum immunoglobulin G (IgG) anti-O-SP confers immunity to shigellosis. (i) Correlation was found between the level of IgG LPS antibodies and resistance to shigellosis among Israeli solders (11, 14). (ii) There is an inverse relationship between the age incidence of shigellosis and the presence of IgG antibodies to the LPS of *Shigella* (41, 46). The peak incidence of shigellosis is in children and young adults; the disease is rare in infants and in older adults (14, 15, 20, 21, 23, 26, 28, 38, 46). Most newborns and adults have serum LPS antibodies that may be stimulated by cross-reacting bacteria (46–48). (iii) In a double-blind, vaccine-controlled randomized trial of our *S. sonnei*-*r*EPA (*Pseudomonas aeruginosa* recombinant mutant exoprotein A) conjugate (15), 1,447 Israel Defense Force (IDF) recruits from seven companies at separate field sites were vaccinated with *S. sonnei*-*r*EPA or one of two control vaccines. Shigellosis occurred in three units 2 to 3 months after vaccination; *S. sonnei*-*r*EPA induced an overall efficacy of 74% ($P = 0.001$). In one company, infection with *S. sonnei* occurred within 1 to 17 days of injection. Nevertheless, *S. sonnei*-*r*EPA conferred 43% ($P = 0.04$) protection, suggesting that our conjugates may be of value when administered during epidemics. A correlation was demonstrated only between the level of IgG anti-LPS and protection (15). Since serum antibodies are the main, if not the only, host mechanism induced by polysaccha-

* Corresponding author. Mailing address: Lea and Arie Pickel Chair in Pediatric Research, The Chaim Sheba Medical Center, Tel-Hashomer 5261, Israel. Phone: 972 3530 2439. Fax: 972 3530 2562. E-mail: jpasswel@post.tau.ac.il.

TABLE 1. Compositions of *Shigella* conjugate vaccines

Conjugate	Protein (μg/ml)	Carbohydrate (μg/ml)	Protein/ saccharide	LAL (EU/ml)
S. sonnei-CRM9, lot 68210	80.7	~40	1.35	3.38
S. sonnei-$CRM9_{succ}$, lot 68211	108	52.9	2.06	0.86
S. sonnei-*r*EPA_{succ}, lot 68212	93.4	46.4	2.02	0.20
S. flexneri 2a-*r*EPA_{succ}, lot 68208	115	45.3	2.58	0.01
S. flexneri 2a-$CRM9_{succ}$, lot 68209	73.2	44.2	1.66	0.09

ride-protein conjugates, these data provide evidence that a critical level of IgG anti-LPS confers immunity to shigellosis (11, 46–48).

The immunogenicity of polysaccharide-based vaccines, including conjugates, is age dependent (46–48). We improved the immunogenicity of *Shigella* conjugates as assayed in mice by introduction of another carrier protein, *Corynebacterium diphtheriae* CRM9 (a genetically derived nontoxic mutant of *C. diphtheriae*) (34) and by succinylation of the proteins prior to binding to the polysaccharide (27, 42, 43). Succinylation of a mutant *Clostridium difficile* toxin increased its solubility and its effectiveness for conjugates (43). Succinylation has been proposed for inactivating diphtheria and tetanus toxins and stabilizing the resultant toxoids (51).

We evaluated the safety and immunogenicity of *S. sonnei* and *S. flexneri* type 2a conjugates in adults prepared with these two carrier proteins, native or treated with succinic anhydride. These agents were approved for investigation by the National Institutes of Health (OH98-CH-N009), Food and Drug Administration (BB IND 7443), Office for Protection against Research Risks (SPA SF-5900-09), and Ministry of Health, Israel.

MATERIALS AND METHODS

Clinical protocol. Healthy 18- to 40-year olds comprising workers in the clinics of the participating institutions, medical students, and some outsiders were recruited. Individuals were questioned about their health and whether they had been hospitalized or were receiving medication. Their vaccination histories were reviewed, and informed consent was obtained before admission to the study. Volunteers were excluded if they were or planned to be pregnant within 6 months of injection of the investigational vaccines, hospitalized for a chronic disease, had infection with human immunodeficiency virus type 1 (AIDS) or hepatitis, were taking medication on a continual basis, had multiple allergies, or had a febrile disease at the time of immunization or another infection that required medication. The oral temperature of each volunteer and a blood sample were taken before vaccination. Female volunteers underwent a urine pregnancy test.

Volunteers were randomized to receive one of five experimental vaccines. Each received a 0.5-ml injection, containing 25 μg of saccharide, into the left deltoid. Blood samples were also taken on day 3 and at 1, 4, and 26 weeks following vaccination. Liver function tests were assayed on days 0, 3, and 7. Serum antibodies to the O-SPs and carrier proteins were assayed at all intervals. The volunteers examined their injection sites in two dimensions and took their temperatures at 6, 24, and 48 h after the injection. They entered these data on a form for each of these intervals. Volunteers were asked to return to the clinic if they had a temperature higher than 38°C or induration (swelling) greater than 3.0 cm in diameter at the injection site. Each volunteer was called by a nurse or a physician at about 6 h and the next two mornings after the injection and questioned about their demeanor, temperature, and local reactions.

Analyses. *S. flexneri* type 2a O-SP was assayed by the anthrone method (52). *S. sonnei* O-SP was assayed by the 2-phenylphenol assay for uronic acids (8). Protein was assayed by the Lowry method with bovine serum albumin as a standard (10), and nucleic acids were measured by A_{260}. Derivatization with adipic acid dihydrazide was estimated with the trinitrobenzene sulfonic acid assay (10). ADP ribosyltransferase activity, with purified diphtheria and *Shigella* toxin as standards, was assayed as described elsewhere (40) by Allison O'Brien and Stephen Darnell, Uniformed Services University of the Health Sciences, Bethesda, Md., with Vero cells as the substrate. CRM9 had about 10^4, and *S. sonnei*-*r*EPA had 1.6×10^5, lower toxicity compared to diphtheria toxin.

Bacterial antigens. O-SPs from *Plesiomonas shigelloides* O7 (possessing a structure identical to that of the O-SP of *S. sonnei*) and *S. flexneri* type 2a were purified as described elsewhere (57). Both lots, containing less than 1% protein and nucleic acids, were passed through a 2.5- by 100- cm column in 0.2 M NaCl. The void-volume fractions were dialyzed extensively against pyrogen-free water and freeze-dried.

For production of CRM9, strain C7 $(\beta)^{(tox\text{-}201,\ tox\text{-}9)}$ was obtained from Randall Holmes, Uniform Services University of the Health Sciences, and David M. Neville, Jr., National Institute of Mental Health, National Institutes of Health. C7 $(\beta)^{(tox\text{-}201,\ tox\text{-}9)}$ was cultivated in modified CY medium without deferration and purified as described with the additional step of gel filtration through Superdex 200 (42, 54). Fractions containing CRM9, located by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were pooled, sterile filtered, concentrated to ~30 mg/ml, and stored at −70°C (54). *r*EPA was prepared as described elsewhere (22, 57).

Succinylation of CRM9 and *r*EPA. Succinic anhydride was added to the protein at ratios of 1:10 for CRM9 and 1:5 for *r*EPA (42). Residual lysine was used as an indirect measure of succinylation; CRM9 had 15.65 lysine units versus 2.65 for its succinylated derivative ($CRM9_{succ}$) and *r*EPA had 25.4 lysine units versus 17.3 for its succinylated derivative (*r*EPA_{succ}) (24). Double immunodiffusion against the corresponding antisera showed a line of identity between the succinylated and native proteins (not shown). SDS-PAGE showed a slight decrease in migration of both succinylated proteins compared to the native proteins (not shown). By Limulus amoebocyte lysate (LAL) assay, both proteins contained <0.04 endotoxin units of (EU) protein μg.

Derivatization of *S. sonnei* O-SP with adipic acid hydrazide. *S. sonnei* O-SP was prepared as described elsewhere (57). The final product (*S. sonnei*-AH) contained 5.6% adipic acid hydrazide, and LAL assay showed 0.125 EU of polysaccharide μg. Double diffusion in agar showed an identity reaction with the O-SP (not shown).

Derivatization of *S. flexneri* type 2a O-SP with adipic acid dihydrazide. *S. flexneri* 2a O-SP was prepared as described elsewhere (57). The final product had 4.4% adipic acid hydrazide, and LAL assay showed 0.006 EU per μg of polysaccharide. Double diffusion in agar showed an identity reaction with the O-SP (not shown).

Conjugation of *S. sonnei* O-SP. *S. sonnei*-AH was added to either CRM9, $CRM9_{succ}$, or *r*EPA_{succ}, and the mixture was stirred at room temperature in a pH stat. EDC was added to 0.1 M, and the pH was maintained at 5.8 (33). The reaction mixture was dialyzed against 0.2 M NaCl–0.25 mM sodium phosphate–0.01% thimerosal (pH 7.0) at 4°C for 3 days and passed through Sepharose CL-4B; the void-volume peak was diluted with pyrogen-free saline to about 40 μg of saccharide/ml.

Conjugation of *S. flexneri* 2a–AH–O-SP. Conjugation was performed as described previously (42). The conjugate was diluted with pyrogen-free saline to about 40 μg of saccharide/ml/.

The five conjugates passed the Food and Drug Administration requirements for sterility, general safety, and pyrogenicity (58) (Table 1).

Serology. Sera from the volunteers were separated immediately and stored at −70°C until assayed. Serum IgG, IgA, and IgM to the LPS of *S. sonnei* and of *S. flexneri* 2a were measured by enzyme-linked immunosorbent assay (ELISA) (57). Monoclonal antibodies HP6043 (anti-human IgG), HP6084 (anti-human IgM), and HP6107 (anti-human IgA), from George Carlone, Centers for Disease Control and Prevention, were used to assay both LPS and carrier protein antibodies. Antibody levels were calculated by parallel line comparison to a standard serum (included on each plate) assigned a value of 100 U (44). Serum IgG to *P. aeruginosa* exotoxin A and diphtheria toxin were also measured by ELISA (4,

TABLE 2. Serum LPS antibodies in adults injected with *S. sonnei* O-SP–protein conjugates containing CRM9 or *r*EPA

Conjugate (*n*)	GM ELISA titer (25th–75th centiles)					Fold rise at 26 wks over prevaccination level
	Prevaccination	Postvaccination				
		3 days	1 wk	4 wks	26 wks	
IgG						
S. sonnei-CRM9 (22)	1.13 (0.3–3.5)	1.19 (0.3–3.4)	42.5 (19–172)	76.1 (28–178)	27.1 (17–64)	24
S. sonnei-CRM9$_{succ}$ (30)	1.84 (0.6–3.7)	1.94 (0.6–4.2)	36.0 (15–115)	68.5 (61–97)	31.1 (22–48)	17
*S. sonnei-r*EPA$_{succ}$ (30)	1.34 (0.5–3.0)	1.47 (0.5–4.4)	15.4 (4.2–56)	65.3 (25–140)	35.7 (24–58)	27
IgM						
S. sonnei-CRM9	109 (61–218)	196 (92–403)	2115 (704–7,010)	1044 (519–2,020)	139 (76–216)	1.3
S. sonnei-CRM9$_{succ}$	82.4 (43–153)	101 (49–258)	1260 (653–2,906)	620 (342–1,176)	97 (62–158)	1.2
*S. sonnei-r*EPA$_{succ}$	100 (57–160)	129 (82–183)	739 (274–1,640)	700 (371–1,249)	110 (80–163)	1.1
IgA						
S. sonnei-CRM9	0.33 (0.2–0.7)	0.54 (0.2–1.4)	56.7 (51–164)	19.4 (14–42)	5.80 (2.2–19)	18
S. sonnei-CRM9$_{succ}$	0.26 (0.1–0.7)	0.35 (0.1–1.0)	22.3 (7.3–124)	22.8 (14–50)	5.86 (5.1–16)	23
*S. sonnei-r*EPA$_{succ}$	0.26 (0.1–0.6)	0.40 (0.2–0.7)	16.7 (6–44)	36.4 (20–60)	10.5 (7.1–17)	40

57). A positive antibody response was defined as a fourfold or greater rise above the prevaccination level.

Immunogenicity in mice. Serum antibodies elicited in mice by the five conjugates have been reported elsewhere (42). Briefly, saline solutions containing 2.5 of μg O-SP/100 μl were injected subcutaneously into 5-week-old outbred female mice; IgG antibodies were detected in all mice after the third injection. CRM9 served as a more immunogenic carrier for serum anti-LPS than *r*EPA. Conjugates composed of succinylated proteins elicited higher levels of IgG anti-LPS than did those prepared from native components. Conjugates prepared with native proteins elicited higher levels of IgG protein antibodies than did conjugates composed of succinylated proteins.

Statistical analysis. Antibody levels, expressed as geometric means (GM), were calculated by using log transformation data and compared by paired and unpaired *t* test or Wilcoxon rank sum test; levels of less than the sensitivity of the ELISA were assigned one-half of that level. We used the chi-square test for contingency tables (e.g., proportion of adverse effects) and the Wilcoxon rank sum test for continuous variables. *P* values of <0.05 were considered statistically significant.

RESULTS

Clinical reactions. None of the 152 volunteers had serious local reactions or a temperature above 37.6°C up to 48 h after injection. Four volunteers, each injected with a different conjugate, had mild and uncomplicated diarrhea starting 1 to 2 days after injection, likely due to an intercurrent infection.

One volunteer, a medical student who received *S. sonnei*-CRM9, had acute onset of urticaria and shortness of breath 2 days after injection. These symptoms abated shortly after corticosteroid treatment, and she has remained well to date. She had no history of allergies, and no cause for this episode was established.

One week after injection, three volunteers had palpable lymph nodes and five had localized redness and swelling of 0.1 to 1.9 cm. A recipient of *S. flexneri*-CRM9$_{succ}$ had local swelling of 5 cm.

A pediatrician who received *S. sonnei-r*EPA$_{succ}$ had normal creatine and liver enzymes prior to vaccination. Three days after vaccination, his alkaline phosphatase, serum glutamic oxaloacetic transaminase, and serum glutamic pyruvic transaminase levels were 87, 112, and 240 U/liter; respectively. At 1 week, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, and lactate dehydrogenase levels were 174, 339, and 494 U/liter, respectively. The vaccinee was asymptomatic, tested negative for hepatitis A, B, and C and Epstein-Barr virus but tested positive for cytomegalovirus (CMV) both IgG and IgM anti-CMV titers were elevated: prevaccination, 1.21 (IgG) and 3.54 (IgM); 3 days postvaccination, 2.11 and 4.71; 1 month postvaccination, 3.16 and 1.86. He remained asymptomatic, and his enzyme levels returned to normal within 2 weeks. All other volunteers had normal levels of liver enzymes.

Composition of conjugates (Table 1). The ratios of protein to polysaccharide of the five conjugates were similar, ranging from 1.35 for *S. sonnei*-CRM9 to 2.58 for *S. flexneri* 2a-*r*EPA$_{succ}$. The pyrogen contents of all five conjugates were low: all passed the rabbit thermal induction assay undiluted (58).

Serum LPS antibodies (homologous response; Tables 2 and 3). All volunteers had preexisting anti-LPS of the three Ig classes to both *Shigella* O-SPs, and there were no significant differences in these levels between the conjugate groups. None of the conjugates elicited a significant GM, antibody rise at 3 days following injection.

***Shigella sonnei* (Table 2).** All three conjugates elicited a significant rise of anti-LPS of the three Ig classes at 1 and 4 weeks after injection ($P < 0.0001$).

(i) IgG. All three conjugates elicited a statistically significant rise of the GM anti-LPS 1 week after immunization ($P = 0.0001$): 66 of 81 volunteers (82%) had a ≥4-fold rise over their prevaccination levels. The GM levels at 4 weeks were higher than those at 1 week ($P = 0.01$). The peak level of IgG anti-LPS was at the 4-week interval for all three conjugates: 81 of 82 volunteers (99%) had a ≥4-fold rise over the preimmunization levels. At 26 weeks, these levels declined about twofold and were not significantly different from each other: 70 of 77 volunteers (91%) had anti-LPS levels ≥4-fold higher than before vaccination ($P = 0.0001$).

Except for the IgG levels at 1 week (*S. sonnei*-CRM9, 42.5 EU > *S. sonnei-r*EPA$_{succ}$, 15.4; $P = 0.03$), the GM IgG levels at each interval were not statistically different.

(ii) IgM and IgA. The peak GM levels of IgM and IgA occurred at the first week with the exception of IgA anti-LPS elicited by *S. sonnei-r*EPA$_{succ}$, which was at 4 weeks. The level elicited by *S. sonnei*-CRM9 was higher than those elicited by the other two conjugates (56.7 versus 16.7, $P = 0.02$). At the 26-week interval, IgM levels declined to the prevaccination

TABLE 3. Serum LPS antibodies in adults injected with *S. flexneri* type 2a O-SP–-protein conjugates containing CRM9 or *r*EPA

Conjugate (*n*)	GM ELISA level (25th–75th centiles)					Fold rise at 26 wk over prevaccination level
	Prevaccination	Postvaccination				
		3 days	1 wk	4 wk	26 wk	
IgG						
S. flexneri 2a-*r*EPA$_{succ}$ (31)	14.0 (9.2–26)	15.1 (8.6–35)	77.5 (35–175)	420 (322–959)	179 (89–365)	13
S. flexneri 2a-CRM9$_{succ}$ (33)	10.6 (6.5–16)	11.0 (7.1–16)	50.3 (26–88)	221 (139–370)	94.6 (53–150)	9
IgM						
S. flexneri 2a-*r*EPA$_{succ}$	10.6 (6.4–17)	11.0 (6.3–17)	35.6 (22–51)	56.3 (29–103)	25.9 (17–37)	2.4
S. flexneri 2a-CRM9$_{succ}$	10.6 (7.3–15)	11.1 (7.4–17)	36.6 (18–72)	50.0 (25–86)	28.8 (18–39)	2.7
IgA						
S. flexneri 2a-*r*EPA$_{succ}$	0.76 (0.5–1.3)	0.96 (0.5–1.6)	16.4 (5.7–33)	31.9 (15–79)	12.1 (5.1–30)	16
S. flexneri 2a-CRM9$_{succ}$	0.80 (0.4–1.9)	0.80 (0.3–1.9)	9.39 (6.0–34)	9.09 (3.7–21)	3.96 (1.1–9.5)	5

level. IgA anti-LPS at 26 weeks declined at least twofold from their peak levels but remained higher than the preinjection levels ($P = 0.0001$).

***S. flexneri* type 2a (Table 3).** Both conjugates elicited a rise of anti-LPS of the three Ig isotypes 1 week following injection ($P = 0.0001$).

(i) IgG. *S. flexneri* 2a-*r*EPA$_{succ}$ elicited higher GM levels than *S. flexneri* type 2a-CRM9$_{succ}$ at all intervals. Both conjugates elicited a statistically significant rise of the GM anti-LPS 1 week after immunization. For *S. flexneri* 2a-*r*EPA$_{succ}$, the GM was 77.5 versus 14.0 ($P = 0.0001$), and 14 of 29 volunteers (48%) had a ≥4-fold rise above the prevaccination level. For *S. flexneri* 2a-CRM9$_{succ}$, the GM was 50.3 versus 10.6 ($P = 0.0001$), and 14 of 30 volunteers (47%) had a ≥4-fold rise over their prevaccination levels.

A statistically significant rise over the level at the 1-week interval was elicited by both conjugates at 4 weeks. For *S. flexneri* 2a-*r*EPA$_{succ}$, the GM was 420 versus 77.5 ($P = 0.0001$), with 28 of 30 volunteers (93%) having ≥4-fold rise above their preimmunization levels. For *S. flexneri* 2a-CRM9$_{succ}$, 1 the GM was 221 versus 50.3 ($P = 0.0001$), and 28 of 32 volunteers (88%) had ≥4-fold or higher levels compared to those before immunization.

At the 26-week interval, the GM levels in both groups had declined about 60% from their maximal levels: the GM of the *S. flexneri* 2a-*r*EPA$_{succ}$ group was higher than that of the *S. flexneri* 2a-CRM9$_{succ}$ group (179 versus 94.6, $P = 0.045$). For *S. flexneri* 2a-*r*EPA$_{succ}$, 22 of 28 volunteers (75%) had a ≥4-fold rise over their preimmunization levels; 20 of 29 volunteers (69%) had a ≥4-fold rise for *S. flexneri* 2a-CRM9$_{succ}$. The GM levels of the two conjugate vaccine groups were higher than those prior to injection ($P = 0.0001$).

(ii) IgM. Both conjugates elicited comparatively low but statistically significant rises of anti-LPS at weeks 1 and 4 after immunization ($P = 0.0001$). The peak levels of IgM anti-LPS were at week 4; these levels declined to about 2.5 times those prevaccination but at 26 weeks were significantly higher than those prior to injection ($P = 0.0001$).

(iii) IgA. Both conjugates elicited a statistically significant rise of IgA anti-LPS 1 week after vaccination. Only *S. flexneri* 2a-*r*EPA$_{succ}$ elicited an additional rise from week 1 to week 4 (31.9 versus 16.4, $P = 0.02$). At the 26-week interval, the GM levels declined about 60% for both groups: the GM elicited by *S. flexneri* 2a-*r*EPA$_{succ}$ was higher than that of elicited by *S. flexneri* 2a-CRM9 (12.1 versus 3.96, $P = 0.005$).

Serum heterologous LPS antibodies. None of the conjugates elicited statistically significant GM rises of any isotype at any interval to the heterologous O-SP (not shown).

Comparative immunogenicity of "improved" *Shigella* conjugates with previous conjugates (Table 4). The GM serum levels and increases of IgG anti-LPS after 26 weeks following immunization of volunteers in this study were compared to those elicited by similar conjugates in IDF recruits in a previous study (57). All three *S. sonnei* conjugates in this study

TABLE 4. GM IgG LPS antibodies and fold differences at 26 wk elicited by *Shigella* conjugates in this study and a 1992 study of IDF recruits (57)

Conjugate	*n*	GM ELISA titer			Fold rise at 26 wk over prevaccination level
		Prevaccination	Postvaccination		
			6 wk	26 wk	
IDF: *S. sonnei*-*r*EPA	49	3.76	66.5	34.7	9.2
S. sonnei-CRM9, lot 68210	22	1.19	76.1	27.1	23.4
S. sonnei-CRM9$_{succ}$, lot 68211	30	1.84	68.5	31.1	16.9
S. sonnei-*r*EPA$_{succ}$, lot 68212	30	1.34	65.3	35.7	26.6
IDF: *S. flexneri* 2a-*r*EPA	47	25.0	126	93.7	3.7
S. flexneri 2a-*r*EPA$_{succ}$, lot 68208	30	14.0	420	179	12.8
S. flexneri 2a-CRM9$_{succ}$, lot 68209	32	10.6	221	94.6	8.9

TABLE 5. IgG antibodies to proteins elicited by conjugates of *S. sonnei* and *S. flexneri* 2a O-SPs bound to CRM9 or *r*EPA

Protein (lot, *n*)	Gm ELISA titer (25th–75th centiles)					Fold rise at 26 wk over prevaccination level
	Prevaccination	Postvaccination				
		3 days	1 wk	4 wk	26 wk	
CRM9						
S. sonnei-CRM9 (68210, 22)	1.10 (0.5–1.8)	1.28 (0.7–2.6)	6.37 (2.4–16)	8.89 (3.0–22)	2.34 (1.1–5.4)	2.1
S. sonnei-CRM9$_{succ}$ (68211, 30)	1.05 (0.5–2.6)	1.23 (0.5–2.7)	5.03 (2.9–11)	6.08 (2.8–11)	2.03 (1.3–4.4)	1.9
S. flexneri-CRM9$_{succ}$ (68208, 33)	0.90 (0.4–1.4)	0.94 (0.5–1.3)	2.54 (1.1–7.5)	4.28 (2.3–12)	1.83 (0.7–4.1)	2.0
*r*EPA						
S. sonnei-*r*EPA$_{succ}$ (68212, 30)	0.44 (0.2–0.9)	0.42 (0.2–0.9)	0.87 (0.3–2.4)	1.65 (0.5–4.9)	0.92 (0.5–2.0)	2.1
S. flexneri-*r*EPA$_{succ}$ (68209, 31)	0.43 (0.3–0.9)	0.54 (0.3–1.1)	1.30 (0.5–4.8)	1.80 (0.6–6.5)	1.05 (0.5–2.3)	2.4

elicited similar levels of anti-LPS at 6 and 26 weeks after immunization as *S. sonnei*-*r*EPA (IDF). The three conjugates in this study also elicited similar fold rises (23.4, 16.9, and 26.6) at 26 weeks, all of which were higher than values for the *S. sonnei*-*r*EPA used in the previous study (9.2 EU) (57).

In this study, *S. flexneri* 2a-*r*EPA$_{succ}$ was more immunogenic than *S. flexneri* 2a-CRM9$_{succ}$ (Table 3) and *S. flexneri*-*r*EPA (IDF), as shown by the higher anti-LPS levels at both 6 (420 EU versus 126) and 26 (179 EU versus 93.7) weeks and by the fold rise at 26 weeks (12.8 versus 3.7).

Carrier protein antibodies (Table 5). All vaccinees had preexisting levels of antibodies to the two toxins, and none of the conjugates elicited a significant antibody response to the homologous carrier protein at the 3-day interval following injection.

Diphtheria toxin. All volunteers had >0.01 U of antitoxin prior to injection, and there was no statistically significant difference between the three groups. All three conjugates containing CRM9 elicited a significant rise of diphtheria toxin antibodies 1 week after injection, with peak levels at 4 weeks. *S. sonnei*-CRM9, the conjugate with the native protein carrier, elicited the highest level (8.89 EU), but this was not significantly different from the response elicited by conjugates with the succinylated protein (6.08 for *S. sonnei*-CRM9$_{succ}$ and 4.28 for *S. flexneri*-CRM9$_{succ}$). Both *S. sonnei* conjugates with CRM9 as the carrier elicited higher levels than *S. flexneri*-CRM9$_{succ}$ at 1 week (6.37 and 5.03 versus 2.54, $P = <0.05$). At 26 weeks, although the level was highest in the recipients of *S. sonnei*-CRM9 (2.34), there was no significant difference between the GM elicited by the CRM9-containing conjugates, and all had about twice the preinjection levels.

***P. aeruginosa* exotoxin A (ETA).** Both the *S. sonnei* and *S. flexneri* type 2a conjugates contained *r*EPA$_{succ}$ as the carrier, and both elicited a lesser response (about 3.7-fold rise at the 4-week interval) to the ETA compared to the fold rise to the carrier. The GM level of anti-*r*EPA declined at 26 weeks to about twice the preinjection level; there was no difference in the fold rises between the two conjugates.

None of the conjugates elicited a significant GM rise in antibodies to the heterologous carrier protein (not shown).

DISCUSSION

None of the vaccinees developed fever within 48 h of injection, and local reactions were minor and infrequent; no volunteer missed a day of work or of school because of the vaccination.

We cannot explain the episode of shortness of breath and urticaria in one vaccinee 2 days after injection of *S. sonnei*-CRM9; she responded quickly to an injection of corticosteroid. The vaccinee had not experienced such a reaction following other vaccinations and has remained well since that episode. Her pre and postimmunization antibody levels were not unique. A serologically documented asymptomatic CMV infection provides an explanation for the transient rise in liver enzymes in a recipient of *S. sonnei*-*r*EPA$_{succ}$.

The safety and immunogenicity of our *Shigella* conjugates in U.S. Army and IDF recruits and in 4- to 7-year-old Israelis have been reported elsewhere (4, 12, 15, 57). The safety of our O-SP conjugates for *Salmonella paratyphi* A, *Vibrio cholerae* O1, and *Escherichia coli* O157 has been documented elsewhere (29, 38, 39).

Reinjection of *S. flexneri* 2a O-SP conjugates induced a booster response in adults and in 4- to 7-year-old children not observed with other polysaccharide conjugates (4, 48, 57). Although it is not possible to compare the levels of the IgG anti-LPS in mass units, the differences in fold rises suggest that the O-SP of *S. sonnei* is more immunogenic than that of *S. flexneri* 2a. Another difference is that the IgG responses elicited by our O-SP conjugates persisted for at least 2 years, considerably longer than after disease (4, 12, 13, 20, 21, 37).

The immunogenicity of polysaccharide conjugates in mice or in human adults may not always predict their activity in infants or young children (1, 2). Our choice for the five conjugates was based on their immunogenicity in young outbred mice (41). The three *S. sonnei* O-SP conjugates, *S. sonnei*-CRM9, *S. sonnei*-CRM9$_{succ}$, and *S. sonnei*-*r*EPA$_{succ}$, induced similar IgG anti-LPS responses in adults. We chose *S. sonnei*-CRM9 to evaluate in young children because it is the simplest of the three conjugates to synthesize. *S. flexneri* 2a containing *r*EPA$_{succ}$ was the more immunogenic of the two conjugates and was chosen for study in young children.

Shigellosis induced a rise of anti-LPS of all three Ig isotypes; the increase in IgG anti-LPS is the highest and has the longest duration (11, 13, 20, 21, 37, 57). The magnitude of the anti-LPS response is related to the severity of symptoms, and anti-LPS levels of the three isotypes decline to those in acute-phase sera in less than 1 year (13, 39). This comparatively rapid decline of serum anti-LPS provides an explanation for the limited duration of disease-induced immunity (6, 18). The higher and long-

er-lasting levels of IgG anti-LPS suggest that our conjugates will induce a more complete and long-lasting immunity than shigellosis.

Finally, we reported preparation of highly immunogenic conjugates with synthetic saccharides corresponding to the O-SP of *S. dysenteriae* type 1 (45). Both the length and the density of the saccharide were related to the immunogenicity of the conjugate in mice (1, 2). The use of synthetic saccharides may permit the development of more immunogenic polysaccharide conjugates (48).

ACKNOWLEDGMENT

We gratefully acknowledge Sylvia Koo of the Immunology Service, Department of Laboratory Medicine, National Institutes of Health, for assay of CMV antibodies.

REFERENCES

1. **Anderson, P., M. E. Pichichero, and R. A. Insel.** 1985. Immunogens consisting of oligosaccharides from the capsule of *Haemophilus influenzae* type b coupled to diphtheria toxoid or the toxin protein CRM197. J. Clin. Investig. **76:**52–59.
2. **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 unterminally coupled to the diphtheria protein CRM197. J. Immunol. **142:**2464–2468.
3. **Ashkenazi, S., G. Dinari, A. Zevulunov, and M. Nitzan.** 1987. Convulsions in childhood shigellosis. Clinical and laboratory features in 153 children. Am. J. Dis. Child. **141:**208–210.
4. **Ashkenazi, S., J. H. Passwell, E. Harlev, D. Miron, R. Dagan, N. Farzan, R. Ramon, F. Majadly, D. A. Bryla, A. B. Karpas, J. B. Robbins, R. Schneerson, and the Israel Pediatric Shigella Study Group.** 1999. Safety and immunogenicity of *Shigella sonnei* and *Shigella flexneri* 2a O-specific polysaccharide conjugates in children. J. Infect. Dis. **179:**1565–1568.
5. **Baumgartner, J. D., D. Heumann, T. Calandra, and M. P. Glauser.** 1991. Antibodies to lipopolysaccharide after immunization of humans with rough mutant *Escherichia coli*. J. Infect. Dis. **163:**769–772.
6. **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.
7. **Black, R. E., K. H. Brown, and S. Becker.** 1984. Effects of diarrhea associated with specific enteropathogens on the growth of children in rural Bangladesh. Pediatrics **73:**799–805.
8. **Blumenkrantz, R., and G. Asboe-Hansen.** 1973. New method for quantitative determination of uronic acids. Anal. Biochem. **54:**484–489.
9. **Briend, A., H. K. Hasan, K. M. Aziz, and B. A. Hoque.** 1989. Are diarrhoea programmes likely to reduce childhood malnutrition? Observations from Bangladesh. Lancet **ii:**319–322.
10. **Chu, C.-Y., R. Schneerson, J. B. Robbins, and S. C. Rastogi.** 1983. Further studies on immunogenicity of *Haemophilus influenzae* type b and pneumococcal 6A polysaccharide-protein conjugates. Infect. Immun. **40:**245–256.
11. **Cohen, D., M. S. Green, C. Block, T. Rouach, and T. Ofek.** 1988. Serum antibodies to lipopolysaccharide and natural immunity to Shigellosis in an Israeli military population. J. Infect. Dis. **157:**1068–1071.
12. **Cohen, D., S. Ashkenazi, M. Green, Y. Lerman, R. Slepon, G. Robin, N. Orr, D. N. Taylor, J. C. Sadoff, C. Chu, J. Shiloach, R. Schneerson, and J. B. Robbins.** 1996. Safety and immunogenicity of investigational *Shigella* conjugate vaccines in Israeli volunteers. Infect. Immun. **64:**4074–4077.
13. **Cohen, D., C. Block, M. S. Green, G. Lowell, and I. Ofek.** 1989. Immunoglobulin M, A, and G antibody response to lipopolysaccharide O antigen in symptomatic and asymptomatic *Shigella* infections. J. Clin. Microbiol. **27:** 162–167.
14. **Cohen, D., M. S. Green, C. Block, R. Slepon, and I. Ofek.** 1991. A prospective study of the association between serum antibodies to lipopolysaccharide O antigen and the attack rate of shigellosis. J. Clin. Microbiol. **29:**386–389.
15. **Cohen, D., S. Ashkenazi, M. S. Green, M. Gdalevich, G. Robin, R. Slepon, M. Yavzori, N. Orr, C. Block, I. Ashkenazi, J. Shemer, D. N. Taylor, T. L. Hale, J. C. Sadoff, D. Pavliakova, R. Schneerson, and J. B. Robbins.** 1997. Double-blind vaccine-controlled randomized efficacy trial of an investigational *Shigella sonnei* conjugate vaccine in young adults. Lancet **349:**155–159.
16. **Dan, M., D. Michaeli, and J. Treistman.** 1988. The epidemiology of shigellosis in Israel. Ann. Trop. Med. Parasitol. **82:**159–162.
17. **DeMaria, A., M. A. Johns, H. Berbeerich, and W. R. McCabe.** 1988. Immunization with rough mutants of *Salmonella minnesota*: initial studies in human subjects. J. Infect. Dis. **158:**301–311.
18. **DuPont, H. L., R. B. Hornick, M. J. Synder, J. P. Libonati, S. B. Formal, and E. J. Gangarosa.** 1972. Immunity in shigellosis. II. Protection induced by oral live vaccine or primary infection. J. Infect. Dis. **125:**12–16.
19. **Echeverria, P., O. Sethabutr, O. Serichantalergs, U. Lexomboon, and K. Tamura.** 1992. *Shigella* and enteroinvasive *Escherichia coli* infections in households of children with dysentery in Bangkok. J. Infect. Dis. **165:**144–147.
20. **Ekwall, E., P. D. Cam, N. Chan, L. K. Phu, D. D. Trach, and A. A. Lindberg.** 1988. *Shigella flexneri* O-antigen specific enzyme immunoassay: a prospective study of class-specific antibody titres against lipopolysaccharide antigens in Vietnamese children and adults with serotype 1b or 2a dysentery. Serodiagn. Immunother. Infect. Dis. **2:**171–182.
21. **Ekwall, E., P. D. Cam, D. D. Trach, A. Taube, and A. A. Lindberg.** 1988. *Shigella flexneri* O-antigen-specific immunoassay: class-specific antibody titres against lipopolysaccharide antigens in healthy Vietnamese and Swedish populations. Serodiagn. Immunother. Infect. Dis. **2:**47–61.
22. **Fattom, A., R. Schneerson, S. C. Szu, W. F. Vann, J. Shiloach, W. W. Karakawa, and J. B. Robbins.** 1990. Synthesis and immunologic properties in mice of vaccines composed of *Staphylococcus aureus* type 5 and type 8 capsular polysaccharides conjugated to *Pseudomonas aeruginosa* recombinant exoprotein A. Infect. Immun. **58:**2367–2374.
23. **Ferreccio, C., V. Prado, A. Ojeda, M. Cayyazo, P. Abrego, L. Guers, and M. M. Levine.** 1991. Epidemiologic patterns of acute diarrhea and endemic *Shigella* infections in children in a poor periurban setting in Santiago, Chile. Am. J. Epidemiol. **134:**614–627.
24. **Fields, R.** 1971. The measurment of amino groups in proteins and peptides. Biochem. J. **124:**581–590.
25. **Formal, S. B., R. M. Maenza, S. Austin, and E. H. LaBrec.** 1967. Failure of parenteral vaccines to protect monkeys against experimental shigellosis. Proc. Soc. Exp. Biol. Med. **125:**347–349.
26. **Ghosh, A. R., and S. C. Sehgal.** 1996. Existing status of shigellois in Andaman & Nicobar Islands. Indian J. Med. Res. **103:**134–137.
27. **Gounaris, A. D., and G. E. Perlmann.** 1967. Succinylation of pepsinogen. J. Biol. Chem. **242:**2739–2745.
28. **Green, M. S., C. Block, D. Cohen, and P. Slater.** 1991. Four decades of shigellosis in Israel—the epidemiology of a growing public health problem. Rev. Infect. Dis. **13:**248–253.
29. **Gupta, R. K., D. N. Taylor, D. A. Bryla, J. B. Robbins, and S. C. Szu.** 1998. Phase 1 evaluation of *Vibrio cholerae* O1, serotype Inaba, polysaccharide-cholera toxin conjugates in adult volunteers. Infect. Immun. **66:**3095–3099.
30. **Henry, F. J., N. Alam, K. M. S. Aziz, and M. M. Rahaman.** 1987. Dysentery, not watery diarrhoea, is associated with stunting in Bangladeshi children. Human Nutr. Clin. Nutr. **41C:**243–249.
31. **Herrington, D. A., L. van de Verg, S. B. Formal, T. L. Hale, B. D. Tall, S. J. Cryz, E. C. Tramont, and M. M. Levine.** 1990. Studies in volunteers to evaluate candidate Shigella vaccines: further experience with a bivalent *Salmonella typhi-Shigella sonnei* vaccine and protection conferred by previous *Shigella sonnei* disease. Vaccine **8:**353–357.
32. **Higgins, A. R., T. M. Floyd, and M. A. Kader.** 1955. Studies in shigellosis. III. A controlled evaluation of a monovalent Shigella vaccine in a highly endemic environment. Am. J. Trop. Med. **4:**281–288.
33. **Hoare, D. G., D. E. Koshland, Jr.** 1967. A method for the quantitative modification and estimation of carboxylic acid groups in proteins. J. Biol. Chem. **22:**2417–2453.
34. **Hu, V. W., and R. K. Holmes.** 1987. Single mutation in the A domain of diphtheria toxin results in a protein with altered membrane insertion behavior. Biochim. Biophys. Acta **902:**24–30.
35. **Islam, D., B. Wretlind, M. Ryd, A. A. Lindberg, and B. Christensson.** 1995. Immunoglobulin subclass distribution and dynamics of *Shigella*-specific antibody responses in serum and stool samples in shigellosis. Infect. Immun. **63:**2045–2061.
36. **Konadu, E., J. Shiloach, D. A. Bryla, J. B. Robbins, and S. C. Szu.** 1996. Synthesis, characterization and immunological properties in mice of conjugates composed of detoxified lipopolysaccharide of *Salmonella paratyphi* A bound to tetanus toxoid, with emphasis on the role of *O*-acetyls. Infect. Immun. **64:**2709–2715.
37. **Konadu, E. Y., J. C. Parke, Jr., H. T. Tran, D. A. Bryla, J. B. Robbins, and S. C. Szu.** 1998. Investigational vaccine for *Escherichia coli* O157: phase 1 study of O157 O-specific polysaccharide-*Pseudomonas aeruginosa* recombinant exoprotein A (*r*EPA) conjugates in adults. J. Infect. Dis. **177:**383–387.
38. **Kotloff, K. L., J. P. Winickoff, B. Ivanoff, J. D. Clemens, D. L. Swerdlow, P. J. Sansonetti, G. K. Adak, and M. M. Levine.** 1999. Global burden of *Shigella* infections: implications for vaccine development and implementation of control strategies. Bull. W. H. O. **77:**651–666.
39. **Mackowski, P. A., S. S. Wasserman, and M. M. Levine.** 1993. Relationship between humoral immune responses and oral temperature during human shigellosis. J. Infect. Dis. **167:**1449–1452.
40. **Oku, T., T. Tutsudo, A. Hirayama, A. D. O'Brien, and T. Takeda.** 1989. Purification and some properties of a verotoxin from a human strain of *Escherichia coli* that is immunologically related to Shiga-like toxin II (VT2). Microb. Pathog. **6:**113–122.
41. **Passwell, J. H., S. Freier, R. Shor, N. Farzam, C. Block, M. Lison, E. Shiff,**

and S. Ashkenazi. 1995. *Shigella* lipopolysacchride antibodies in pediatric populations. Pediatr. Infect. Dis. J. **14:**859–865.
42. **Pavliakova, D., C.-Y. Chu, S. Bystricky, N. Tolson, F. Majadly, J. Shiloach, J. B. Kauffman, D. A. Bryla, J. B. Robbins, and R. Schneerson.** 1999. Treatment with succinic anhydride improves the immunogenicity of *Shigella flexneri* type 2a O-specific polysaccharide-protein conjugates in mice. Infect. Immun. **67:**5526–5529.
43. **Pavliakova, D., J. S. Moncrief, D. M. Lyerly, G. Schiffman, D. A. Bryla, J. B. Robbins, and R. Schneerson.** 2000. *Clostridium difficile* recombinant toxin A repeating units as a carrier protein for conjugate vaccines: studies of pneumococcal type 14, *Escherichia coli* K1, and *Shigella flexneri* type 2a polysaccharides in mice. Infect. Immun. **68:**2161–2166.
44. **Plikaytis, B. D., P. F. Holder, and G. Carlone.** 1996. Program ELISA for *Windows*. User's Manual 12, version 1.00. Centers for Disease Control and Prevention, Atlanta, Ga.
45. **Pozsgay, V., C.-Y. Chu, L. Pannell, J. Wolfe, J. B. Robbins, and R. Schneerson.** 1999. Protein conjugates of synthetic saccharides elicit higher levels of serum IgG lipopolysaccharide antibodies in mice than do those of the O-specific polysaccharide from *Shigella dysenteriae* type .1. Proc. Natl. Acad. Sci. USA **96:**5194–5197.
46. **Robbins, J. B., C.-Y. Chu, and R. Schneerson.** 1992. Hypothesis for vaccine development: protective immunity to enteric diseases caused by nontyphoidal salmonellae and shigellae may be conferred by serum IgG antibodies to the O-specific polysaccharide of their lipopolysaccharides. Clin. Infect. Dis. **15:**346–361.
47. **Robbins, J. B., R. Schneerson, and S. C. Szu.** 1995. Perspective: hypothesis: serum IgG antibody is sufficient to confer protection against infectious disease by inactivating the inoculum. J. Infect. Dis. **171:**1378–1398.
48. **Robbins, J. B., R. Schneerson, P. W. Anderson, and D. H. Smith.** 1996. Prevention of systemic infections, especially meningitis, caused by *Haemophilus influenzae* type b: impact on public health and implications for other polysaccharide-based vaccines. J AMA **276:**1181–1185.
49. **Ronsman, C., M. L. Bennish, and T. Wierzba.** 1988. Diagnosis and management of dysentery by community health workers. Lancet **ii:**552–555.
50. **Schneerson, R., O. Barrera, A. Sutton, J. B. Robbins.** 1980. Preparation, characterization and immunogenicity of *Haemophilus influenzae* type b polysaccharide-protein conjugates. J. Exp. Med. **152:**361–376.
51. **Schwendeman, S. P., H. R. Costantino, R. K. Gupta, G. R. Siber, A. M. Klibanov, and R. Langer.** 1995. Stabilization of tetanus and diphtheria toxoids against moisture-induced aggregation. Proc. Natl. Acad. Sci. USA **92:** 11234–11238.
52. **Shields, R., and W. Burnett.** 1960. Determination of protein-bound carbohydrate by an anthrone method. Anal. Chem. **32:**885.
53. **Shiga, K.** 1898. Ueber den Dysenteriebacillus (*Bacillus dysenteriae*). Zentbl. Bakteriol. **24:**817–828.
54. **Shiloach, J., and J. B. Kaufman.** 1999. The combined use of expanded bed volume adsorption and gradient elution for capture and partial purification of mutant diphtheria toxin (CRM*9*) from *Corynebacterium diphtheriae*. Sep. Sci. Technol. **34:**29–40.
55. **Taylor, D., P. Echeverria, T. Pal, O. Sethabutr, S. Saiborisuth, S. Sricharmorn, B. Rowe, and J. Cross.** 1986. The role of *Shigella* spp., enteroinvasive *Escherichia coli*, and other enteropathogens as causes of childhood dysentery in Thailand. J. Infect. Dis. **153:**1132–1138.
56. **Taylor, D. V., P. Echeverria, O. Sethabutr, C. Pitarangsi, U. Leksomboon, N. R. Blacklow, B. Rowe, R. Gross, and J. Cross.** 1988. Clinical and microbiological features of *Shigella* and enteroinvasive *Escherichia coli* infections detected by DNA hybridization. J. Clin. Microbiol. **26:**1362–1366.
57. **Taylor, D. N., A. C. Trofa, J. Sadoff, C. Chu, D. Bryla, J. Shiloach, D. Cohen, S. Ashkenazi, Y. Lerman, W. Egan, R. Schneerson, and J. B. Robbins.** 1993. Synthesis, characterization and clinical evaluation of conjugate vaccines composed of the O-specific polysaccharides of *Shigella dysenteriae* type 1, *Shigella flexneri* type 2a, and *Shigella sonnei* (*Plesiomonas shigelloides*) bound to bacterial toxoids. Infect. Immun. **61:**3678–3687.
58. **U.S. Department of Health and Human Services.** 2000. Code of Federal Regulations, title 21, vol. 7, parts 600 to 799, revised as of April 1. U.S. Government Printing Office, Washington, D.C.

Editor: A. D. O'Brien