INFECTION AND IMMUNITY, Oct. 1996, p. 4074–4077 Vol. 64, No. 10
0019-9567/96/$04.00+0

Safety and Immunogenicity of Investigational *Shigella* Conjugate Vaccines in Israeli Volunteers

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Received 1 April 1996/Returned for modification 17 May 1996/Accepted 2 July 1996

The safety and immunogenicity of investigational conjugates, composed of the O-specific polysaccharides of *Shigella sonnei* and *Shigella flexneri* type 2a covalently bound to *Pseudomonas aeruginosa* recombinant exoprotein A (*r*EPA), were evaluated in 192 Israeli soldiers. None had significant local reactions or fever. Fourteen days after injection, 90% of *S. sonnei*-*r*EPA recipients and 73 to 77% of *S. flexneri*-*r*EPA recipients had a fourfold or greater increase in serum immunoglobulin G (IgG) and IgA anti-lipopolysaccharide (anti-LPS) levels; at 2 years, these remained higher than at prevaccination ($P < 0.01$). There was a fourfold or greater increase in IgM anti-LPS in 20% of vaccinees at 2 weeks, but levels returned to prevaccination values at 6 to 12 months. IgG was the highest and most sustained class of LPS antibodies. Reinjection at day 42 did not boost antibody levels. Eighteen of 23 (78%) who received *S. sonnei*-*r*EPA and 13 of 19 (68%) who received *S. flexneri*-*r*EPA had significant IgA-secreting cell responses. Significant IgG antibody-secreting cell responses were detected in 19 of 23 (83%) and 11 of 19 (58%) volunteers following vaccination with *S. sonnei*-*r*EPA and *S. flexneri* 2a-*r*EPA, respectively. On the basis of these data, further evaluation of the *Shigella* conjugates for protective efficacy in field trials in Israel was started.

Shigellosis is a common and serious disease throughout the world (16). Shigellae are the most important cause of dysentery (blood and mucus in stool, cramps, fever), which is associated with arrested growth and malnutrition (14, 16, 24). Infection can be transmitted by a low dose (10^2 to 10^3 organisms) (9), explaining the difficulty of routine sanitary and hygienic measures to prevent shigellosis (4, 13, 16). Shigellosis is endemic in Israel, with high rates among children aged 1 to 4 years and soldiers serving under field conditions (12, 13). Treatment of the disease is difficult and expensive because of increasing antibiotic resistance of *Shigella* spp. (1, 23). An effective vaccine is needed to prevent shigellosis, especially in high-risk groups.

The nature of protective immunity to shigellosis is unclear. Convalescence confers type (lipopolysaccharide [LPS])-specific immunity of limited duration (8, 15) and is accompanied by significant rises in serum antibodies to the homologous LPS (3, 10). We have reported a significant correlation between serum immunoglobulin G (IgG) anti-LPS and resistance to shigellosis in Israeli military recruits (5, 6). On the basis of these two observations, we proposed that serum IgG anti-LPS may confer protective immunity to shigellosis (20). Conjugates of *Shigella* O-specific polysaccharides were developed since they have the potential to elicit high levels of serum LPS antibodies, have an excellent record of safety, and their potency can be reliably predicted (2, 20). A phase 1 study of U.S. Army soldiers indicated that these conjugates were safe and immunogenic; the geometric mean levels of conjugate-induced anti-LPS were equal to those following convalescence from infection with *Shigella sonnei* or *Shigella flexneri* type 2a (25). We have now extended these studies to Israeli Defence Force soldiers, a population of young adults in an area of endemicity, and recorded in a phase 2 study the safety, immunogenicity, and duration of conjugate-induced LPS antibodies compared with natural infection.

MATERIALS AND METHODS

Conjugates. Conjugates of the O-specific polysaccharide of *S. sonnei* and of *S. flexneri* 2a bound to exoprotein A of *Pseudomonas aeruginosa* (*r*EPA) were prepared as described previously (2, 25). Each 0.5-ml dose contained 25 μg of polysaccharide and 75 μg of protein in saline–0.01% thimerosal.

Clinical protocol. Informed consent was obtained from male Israeli soldiers, 18 to 22 years old. One hundred ninety-two volunteers were randomly assigned to receive *S. flexneri* 2a-*r*EPA ($n = 64$), *S. sonnei*-*r*EPA ($n = 66$), or Engerix B hepatitis B vaccine (licensed in Israel and used as a control) ($n = 62$). The last 33 (16 in the *S. flexneri* 2a and 17 in the *S. sonnei* groups) received a second dose of the same conjugate 6 weeks later. Subjects vaccinated with the hepatitis B vaccine received a full course of three injections.

The volunteers were examined by a physician at 2 to 6, 24, and 48 h after each injection. Their temperatures were taken, and if present, erythema and induration at the injection site were measured. Blood was tested for urea, creatinine, serum glutamic oxalacetic transaminase, serum glutamic pyruvic transaminase, and alkaline phosphatase before and after each injection. Sera were obtained before and at 2 and 6 weeks and 6, 12, and 24 months after vaccination and were stored at −30°C.

The study was approved by the Committee for Research on Human Subjects of the Israel Defence Force Medical Corps, the Surgeon General's Human Subjects Research Review Board of the Department of the U.S. Army, the U.S. National Institutes of Health, and the Food and Drug Administration. Informed consent was obtained from all participants.

Serum LPS antibodies. An enzyme-linked immunosorbent assay (ELISA) was performed as described previously (3). Goat anti-human IgG, IgA, or IgM, conjugated to alkaline phosphatase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) were employed as second antibodies. Control sera were included in each microtiter plate. Adjusted optical densities, derived from a linear regression analysis of eight doubling dilutions, were expressed as end point (adjusted optical density, 0.3) titers, and geometric mean titers (GMT) were

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TABLE 1. Serum antibody response to *S. sonnei* LPS among recipients of *S. sonnei* conjugate

Time postvaccination (wk)	No. of subjects	IgG		IgA		IgM	
		GMT[a]	% Recipients with ≥4-fold rise	GMT	% Recipients with ≥4-fold rise	GMT	% Recipients with ≥4-fold rise
0	49	263		30		497	
2	42	12,741	90	5,164	90	1,058	17
6	41	9,558	85	1,692	88	783	5
26	40	5,438	78	775	80	580	0
52	16	3,831	75	735	81	540	0
104	29	2,436	66	500	72	ND[b]	ND

[a] Antibody levels are expressed as geometric mean adjusted titer for an optical density of 0.3 calculated from a slope based on eight double dilutions.
[b] ND, not determined.

calculated. An antibody response was defined as a fourfold or greater rise in titer, compared with that at day 0.

Peripheral blood ASCs to LPS. Solid-phase enzyme-linked immunospot (ELISpot) assays were carried out as described previously (19) on blood samples obtained 7 days after the first dose of the investigational and control vaccines. An antibody-secreting cell (ASC) response was defined as ≥14 (IgG) or ≥18 (IgA) ASCs per million cells on the basis of the mean ± 2 standard deviations of unimmunized volunteers.

Statistics. Statistical analysis was performed with SAS software for PC version 6.04. A two-tailed exact test was employed to evaluate differences in side effects between the treatment groups. Duncan's multiple-range test was used for multiple comparisons of GMTs among volunteer groups, where P values of <0.05 and <0.01 were considered to be statistically significant.

RESULTS

Safety. The first injections of the conjugates and the hepatitis B vaccine elicited similar rates of minor local reactions. Tenderness was detected in 9.7% (6 of 62), 8.1% (5 of 62), and 5.0% (3 of 60) of the volunteers, and redness was observed in 1.6% (1 of 62), 3.2% (2 of 62), and 3.3% (2 of 60) of the volunteers, following vaccination with the *S. sonnei*, *S. flexneri* 2a, and hepatitis B vaccines, respectively. The second injection of *S. sonnei-r*EPA induced pain at the injection site in 10 of 14 volunteers compared with 2 of 11 who received hepatitis B vaccine ($P = 0.02$). These symptoms were considered mild and did not interfere with the activities of the volunteers. Otherwise, there were no significant differences between the signs and symptoms after the first or second dose in the conjugate recipients and controls.

Two of the 130 volunteers who received a conjugate (one receiving *S. sonnei* and one receiving *S. flexneri*) had a minimal increase in temperature (37.6 and 37.7°C, respectively) within 24 h after vaccination, while one recipient of the hepatitis B vaccine had a temperature of 38.5°C 2 h after the first dose.

One volunteer developed mild self-limiting herpes zoster 48 h following the first injection with *S. flexneri* 2a-*r*EPA. Two volunteers who received *S. flexneri* 2a-*r*EPA had slight increases in serum glutamic oxalacetic transaminase (51 and 85 IU) and serum glutamic pyruvic transaminase (119 and 35 IU) 14 days after injection which returned to normal (5 to 35 IU) within a few days. One of these began an intensive physical training program on the day following vaccination; his creatinine phosphokinase level on day 14 was 621 IU. One recipient of *S. sonnei-r*EPA had slightly increased serum glutamic oxalacetic transaminase and serum glutamic pyruvic transaminase values (69 and 109 IU, respectively) on day 42 which were normal 6 months after vaccination. All other volunteers had normal hepatic and renal function tests.

Serum LPS antibodies. The serum antibody responses to a single injection of *S. sonnei-r*EPA or *S. flexneri* 2a are given in Tables 1 and 2. The *S. sonnei-r*EPA elicited significant rises of IgG and IgA anti-LPS in 90% of recipients 2 weeks after immunization. The GMT of IgG anti-LPS rose from 263 to 12,741 at 2 weeks ($P < 0.01$); this level decreased gradually after 26 and 52 weeks but was still 9 times higher than the prevaccination level ($P < 0.01$ compared with prevaccination and 2-week levels) 2 years after vaccination. The rise in GMT of IgA anti-LPS at 2 weeks from 30 to 5,164 was also highly significant ($P < 0.01$); the IgA level decreased to 500 after 2 years, which was still 16 times higher than the prevaccination level ($P < 0.01$ compared with prevaccination and 2-week levels). At 2 weeks, only 17% of the volunteers showed a fourfold or greater rise in IgM anti-LPS. The GMT rose from 497 on day 0 to 1,058 at 2 weeks and returned to 540 at 1 year, similar to the prevaccination level.

S. flexneri 2a-*r*EPA elicited lesser serum antibody responses than *S. sonnei-r*EPA. Two weeks after immunization, 73% showed a fourfold or greater rise of IgG anti-LPS and 77% had a fourfold or greater rise of IgA anti-LPS. The preimmunization GMT of IgG anti-LPS rose from 117 to 1,314 at 2 weeks ($P < 0.01$) and was 748 and 485 at 6 months and 2 years after vaccination ($P < 0.01$ compared with prevaccination level).

TABLE 2. Serum antibody response to *S. flexneri* 2a LPS among recipients of *S. flexneri* conjugate

Time postvaccination (wk)	No. of subjects	IgG		IgA		IgM	
		GMT[a]	% Recipients with ≥4-fold rise	GMT	% Recipients with ≥4-fold rise	GMT	% Recipients with ≥4-fold rise
0	48	117		32		410	
2	44	1,314	73	674	77	925	21
6	44	961	75	241	59	883	11
26	33	748	61	171	55	690	9
52	14	518	29	103	38	504	0
104	25	485	28	98	40	ND[b]	ND

[a] Antibody levels are expressed as geometric mean adjusted titer for an optical density of 0.3 calculated from a slope based on eight double dilutions.
[b] ND, not determined.

TABLE 3. IgG and IgA anti-LPS ASC response elicited by *S. sonnei* of *S. flexneri* conjugates or hepatitis B vaccine placebo

Isotype	Conjugate or vaccine	ASC response[a] [no of recipients/total (%)]		GMT of positive responses	
		S. sonnei LPS	*S. flexneri* LPS	*S. sonnei* LPS	*S. flexneri* LPS
IgG	*S. sonnei*	19/23 (83)	0/8 (0)	659.2	
	S. flexneri	0/6 (0)	11/19 (58)		121.0
	Hepatitis B	1/10 (10)	0/11 (0)		
IgA	*S. sonnei*	18/23 (78)	0/8 (0)	1,159.8	
	S. flexneri	0/6 (0)	13/19 (68)		242.3
	Hepatitis B	2/10 (20)	0/11 (0)		

[a] An ASC response was defined as ≥14 spots per 10^6 cells on the basis of the mean (2.86) + 2 standard deviations (2 × 5.36) found in nonvaccinees for IgG. An ASC response was defined as ≥18 spots as ≥18 spots per 10^6 cells on the basis of the mean (3.33) + standard deviations (2 × 6.97) found in nonvaccinees for IgA.

Two years after vaccination, 28% of the volunteers still had anti-*S. flexneri* 2a LPS at levels fourfold or higher than on day 0. The preimmunization GMT of IgA anti-LPS rose from 33 to 674 at 2 weeks ($P < 0.01$) and fell to 98 at 2 years postvaccination ($P < 0.01$ compared with prevaccination and 2-week levels). The IgM response elicited by *S. flexneri* type 2a-*r*EPA was lower than those of IgG and IgA. Only 21% of the volunteers showed a fourfold or greater rise in IgM antibodies at 2 weeks; the GMT rose from 410 to 925 ($P < 0.01$). Six months later, only 9% of the volunteers had fourfold or higher levels of IgM anti-LPS and the GMT was 690 ($P < 0.01$ compared with prevaccination level).

A second dose, 6 weeks later, of either *S. sonnei-r*EPA or *S. flexneri* 2a-*r*EPA neither elicited a GM booster response nor increased the number of responders. No differences in antibody levels were found between recipients of one and two doses during the 2-year follow-up. IgG was the highest and most sustained immunoglobulin level elicited by both conjugates. Of 13 volunteers who had received two doses of *S. flexneri-r*EPA and had serum samples taken after each dose, one did not respond in either IgA or IgG titer after the first dose; that volunteer did not have a response after the second dose either. Of nine volunteers who had received two doses of *S. sonnei-r*EPA and had serum samples taken after each dose, two did not respond in either IgA or IgG titer after the first dose; these two volunteers did not have a response after the second dose either.

Only 2% (3 of 158) of the volunteers produced a fourfold or greater antibody rise to the heterologous LPS. None of the recipients of the hepatitis B vaccine developed a significant antibody response to either LPS.

ASC responses. *S. sonnei-r*EPA elicited significant IgA and IgG ASC responses in 78 and 83%, respectively, of 23 recipients after the first dose (Table 3). *S. flexneri* 2a-*r*EPA induced significant IgA and IgG ASC rises in 68 and 58%, respectively, of 19 volunteers. The GM numbers of ASCs elicited by *S. sonnei-r*EPA were 1,160/10^6 cells for IgA and 659/10^6 cells for IgG, while *S. flexneri* type 2a-*r*EPA elicited 242/10^6 cells for IgA and 121/10^6 cells for IgG. None of the recipients had a significant ASC response to the heterologous LPS.

In both conjugate groups, there was a strong association between a fourfold or greater IgA or IgG antibody rise on day 14 and a positive IgA or IgG ASC response on day 7. In all but one case (of *S. flexneri* IgA), blood samples positive for ASC response were found to be positive by ELISA. However, the opposite was not true, with about 50% of those negative by ASC response being positive by ELISA (this was true for all groups, namely, *S. sonnei* IgA, *S. sonnei* IgG, *S. flexneri* 2a IgA, and *S. flexneri* 2a IgG).

DISCUSSION

The *S. sonnei* and *S. flexneri* type 2a conjugates were well tolerated: local reactions were mild, and none of the volunteers had systemic reactions or fever. Similar findings were observed in U.S. Army recruits (25) and confirm the excellent safety record of conjugates in general (18, 21). We feel that the case of herpes zoster that occurred in one recipient of *S. flexneri* type 2a-*r*EPA was coincidental.

The levels of serum IgG and IgA anti-LPS elicited by *S. flexneri* type 2a-*r*EPA and *S. sonnei-r*EPA were similar to or higher than those of Israeli soldiers following infection with those pathogens (3, 25). The levels of the serum IgG and IgA LPS antibodies remained significantly elevated 2 years after vaccination. The decrease in the titer of IgA anti-LPS between 2 weeks and 2 years after vaccination was more rapid than that of IgG. The IgM serum anti-LPS responses induced by the conjugates were lower than the IgG and IgA responses. The persistence of elevated IgG and IgA anti-*Shigella* LPS induced by the conjugates appears to be of longer duration than that following shigellosis (3).

Similar to that observed in the U.S. recruits, 6 and 11% of recipients of the *S. flexneri* and *S. sonnei* conjugates, respectively, failed to respond with a rise in serum anti-LPS IgA or IgG (25). A second injection of either conjugate did not elicit a booster response; this absence of a booster response by conjugates in adults, in contrast to that observed in infants, remains unexplained (7, 22, 25). Three of the volunteers who had no significant rise in serum antibodies to homologous LPS after the first injection of the conjugates remained nonresponders after the second dose too, suggesting a possible host-related failure to elicit antibodies against the polysaccharide antigens of the *Shigella* conjugates. It has been shown in humans that the immune response to *Haemophilus influenzae* type b polysaccharide vaccine may be associated with genetically determined factors such as immunoglobulin allotypes (11).

The *Shigella* conjugates induced increases in IgA anti-LPS ASCs similar to those observed after infection (19). In one study, immunization of adults with pneumococcus type 12F-diphtheria toxoid conjugates elicited serum antibody responses similar to those of the *Shigella* conjugates, a good ASC response 7 days after vaccination, but little or no secretory antibodies in the saliva (17). Further investigation of this effect of the conjugates could provide insight into the immune moieties elicited by the *Shigella* conjugates.

This study provided data that the *Shigella* conjugates are safe and induce long-lived IgG and IgA anti-LPS and served to stimulate further evaluation of the *Shigella* conjugates for protective efficacy in field trials in Israel. These immune responses will be correlated with the effect upon shigellosis exerted by our *Shigella* conjugate vaccines.

ACKNOWLEDGMENTS

This study was supported by grant DAMD17-93-V-3001 from the U.S. Army Medical Research and Materiel Command, Fort Detrick, Frederick, Md.

We thank Rachel Heller-Mendelziss, Ruhama Ambar, and Ita Marcus for very helpful technical assistance.

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Editor: S. H. E. Kaufmann