Age-related efficacy of *Shigella* O-specific-polysaccharide conjugates in 1 to 4 year-old Israeli children

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Abstract

Background—Despite its high worldwide morbidity and mortality, there is yet no licensed vaccine for shigellosis. We reported the safety and immunogenicity of Shigella O-specific polysaccharide-protein conjugates in adults and young children and efficacy of *Shigella sonnei* conjugate in young adults.

Methods—A double-blinded, randomized and vaccine-controlled Phase 3 evaluation of *S. sonnei* and *S. flexneri* 2a O-SP–rEPA conjugates, 25 μg, injected IM twice, 6 weeks apart, into healthy 1 to 4 year-olds, is reported. The children were followed for 2 years by telephone every other week and stool cultures were obtained for each episode of acute diarrhea (3 loose stools/day or a

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bloody/mucous stool). Sera were taken randomly from 10% of the participants for IgG anti-LPS and anti-carrier levels.

Results—Of the 2799 enrollees, 1433 received *S. sonnei* and 1366 *S. flexneri* 2a conjugates; 2699 (96.4%) completed the two-year follow up. Local reactions occurred in ~5% and ~4% had temperatures 38.0°C lasting 1-2 days. There were no serious adverse events attributable to the vaccines. Of the 3,295 stool cultures obtained, 125 yielded *S. sonnei* and 21 *S. flexneri* 2a. Immunogenicity and efficacy were age-related. The overall efficacy of the *S. sonnei* conjugate was 27.5%; 71.1% (*P*=0.043) in the 3-4 year-olds. The numbers for *S. flexneri* 2a were too few for meaningful analysis. Cross protection by *S. flexneri* 2a for non-vaccine *S. flexneri* types was found, but the numbers were too few for statistical significance. There was an age-related rise of vaccine-specific IgG anti-LPS in both groups, peaking at about 10 weeks and declining thereafter, but remaining 4 fold higher than in the controls 2 years after the second dose.

Conclusions—Shigella conjugates are safe and immunogenic in 1 to 4 year-olds. The *S. sonnei* conjugate elicited 71.1% efficacy in the 3 to 4 year-olds and can be predicted to be efficacious in individuals older than 3 years of age. These results urge studies with our improved conjugates.

INTRODUCTION

Shigellosis continues to be an important cause of dysentery and diarrhea worldwide. In the United States, about 18,000 cases/year are reported to the CDC, and it is estimated that about 180 million cases with 660,000 deaths occur annually in developing countries [1, 2]. It is unlikely that improvement in drinking water and sanitary conditions will occur in the foreseeable future in most developing areas of the world. Further, resistance to the most commonly used, cheap antibiotics has made treatment unavailable to many afflicted communities.

Despite its discovery over a century ago and the efforts of many laboratories, there is yet no vaccine for *Shigella* [3]. We proposed that a critical level of serum IgG, specific for the O-SP domain of the LPS of this pathogen, would confer immunity to shigellosis by inducing complement-mediated lysis of the inoculum on the epithelial surface of the small intestine [4-6]. Because the O-SP is not immunogenic, probably due to its comparatively low molecular weight, methods were developed to bind it covalently to carrier proteins [7, 8]. These conjugates were safe and immunogenic in adults and in young children [7-11]. Further, our *S. sonnei* conjugate conferred immunity to Israeli soldiers at high risk for shigellosis during their training [11]. Because the highest incidence, morbidity and mortality caused by *Shigella* occur in young children, we conducted a Phase 3 trial (safety, immunogenicity, and efficacy) in 1 to 4 year-olds of our *S. sonnei* and *S. flexneri* 2a conjugates at 15 sites in Israel.

METHODS AND MATERIALS

Study Protocol

The study was approved by the Institutional Review Board of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (OH-CH-N003), the US FDA (BB IND 7443), the Ethics Committee of the Sheba Medical Center (2633) and by the

National Ethics Committee of the Israeli Ministry of Health, and assigned a Single Project Assurance Number by the Office of Human Research Protection of the US Department Health and Human Services.

Participants were healthy 1 to 4 year-olds recruited from 15 clinics throughout Israel. The parents/guardians of the participants were given the information sheet, discussed the proposed study with the clinic directors and signed the consent form. Excluded were children with a chronic disease receiving medication, those who received systemic steroids during the month preceding vaccination, those who had severe side effects following vaccinations and those not available for follow-up. Vaccination was delayed for those who had a respiratory or enteric infection the previous week, those who were vaccinated the preceding month or who had planned to have a vaccination during the month following the administration of the investigational vaccine, or if the child had a temperature ($>38.0°C$) at the time of vaccination.

Randomization to vaccine "A" or "B" was done using the last digit of the National Identification Card number (given to all Israeli children at birth). Five of the numbers from 0-9 were randomized to vaccine A and the other 5 to vaccine B. This vaccine assignment was recorded both on the Physician's Examination and Vaccination form and on the volunteer's chart. Both vaccines were clear aqueous solutions in the same type of vial and label. The randomization scheme was kept by the Pharmacy Development Service, NIH, and given only to the members of the Data and Safety Monitoring Board. Vials that were opened were discarded at the end of the week without exception. The enrollment period was May 1, 2003 to January 31, 2006.

The vaccines were administered IM in 0.5 mL at each community clinic by the research nurse. Local and systemic reactions were sought at 30 minutes, 6, 24 and 48 hours after vaccination by a structured questionaire. Adverse reactions that occurred were sought for 48 hours after they were no longer detectable. Serious adverse events (SAEs) were recorded throughout the study period. Adverse reactions were recorded at each site by the research staff and transferred to the study headquarters.

Vaccines

Two lots of conjugates prepared by PDMI, similar to those used in the Phase 2 studies of 4-7 year-olds and in 1-4 year-olds [9, 12] were used. The investigational vaccines were composed of the O-SP of *S. sonnei* or of *S. flexneri* 2a covalently bound to recombinant exoprotein A of *Pseudomonas aeruginosa* (rEPA). To increase binding to the *S. flexneri* 2a O-SP, the rEPA was succinylated prior to conjugation [12-14]. Both conjugates were dissolved in saline to a final concentration of 50 μg/mL, 0.01% thimerosal added, dispensed in 5 dose vials and stored at 4-7°C. *S. sonnei* and *S. flexneri* 2a were chosen to serve as controls for each other because: 1. their O-SPs are structurally and antigenicity unrelated [15-17]; 2. infection with one does not confer immunity to the other [18].

Surveillance

Parents were contacted by telephone every other week. In addition, they were asked to report each episode of diarrhea to the clinic. Stool specimens were obtained for each episode of

diarrhea (3 loose stools/day or a single bloody/mucous stool), cultured for bacterial pathogens (*Shigella* Spp., *Salmonella* Spp. and *Campylobacter* Spp) and examined for viral pathogens (rotavirus and adenovirus) by antigen-detection assays at the Maccabi Healthcare Services Laboratories. All *Shigella* isolates were sent for confirmation and typing to the Department of Clinical Microbiology of the Sheba Medical Center and to the Reference Center for Shigella of the Israeli Ministry of Health. Blood cultures were obtained from children with acute diarrhea and fever of 38.5°C. The per-protocol follow-up period was 2 years; however, all vaccinees were followed until the last one completed the 2-year follow-up (January 31, 2008).

Antibody assay

Serum IgG anti O-SP of *S. sonnei* and of *S. flexneri* 2a and IgG anti *P. aeruginosa* exotoxin A (ETA) were measured by ELISA using standard reference sera. Levels less than the sensitivity of the ELISA were assigned one half of that value [7, 8].

Statistics

Statistical analysis was performed using SAS software version 9.1 (SAS institute Inc., Cary, NC USA). IgG anti-LPS concentrations were expressed as the geometric means (G.M.) and compared by the Wilcoxon rank sum test. $P<0.05$ was considered statistically significant. For multiple comparisons, $P<0.01$ was considered statistically significant.

Efficacy was calculated by the formula:

$$\frac{\text{Disease rate of controls} - \text{Disease rate of vaccinees}}{\text{Disease rate of controls}} \times 100$$

Comparison of rates between the study groups used χ^2 and Fisher's Exact tests with $P<0.05$ considered statistically significant. All tests were 2 tailed.

RESULTS

A total of 2,799 children, including Jews, Arabs, Druze, and Bedouins, were enrolled at 15 sites throughout Israel (Table 1). There were 1,455 males (52%) and 1,344 females (48%), 1,029 (36.8 %) were >1 to 2 years-old, 1,013 (36. 2 %) were >2 to 3 years-old, and 757 (27.0 %) were >3 years old. All enrolled children received the first dose of an investigational vaccine, 2,748 (98.2%) received 2 doses. Ten were excluded because of protocol violation (4 due to age >4 years, 3 due to more than 2 immunizations, 3 due to different vaccines in the same recruit), and 39 additional participants dropped out due to loss of contact, relocation or death; 2,699 (96.4%) completed the 2-year follow-up.

Safety

The acute adverse events to the investigational vaccines by vaccine type and dose are shown in Table 2. Local pain was noted in approximately 5% of the vaccinees; other reactions were less common. Fever was noted after each injection in approximately 4% of the vaccines (Table 2). None of the 309 SAEs, including 4 deaths, reported during the study, was

considered related to the vaccines. Causes of death were drowning, electrical injury, murder and thrombocytopenia with brain hemorrhage.

IgG LPS antibodies (Tables 3 & 4)

As observed for surface bacterial polysaccharides, including those of *Shigella*, there was a "natural" age-related development of IgG LPS antibodies in the control groups (about a 2.5-fold increase between 1-2 and 3-4 year olds) for both LPSs, likely independent of interaction with the homologous bacteria [19-21]. Overall both vaccines induced similar antibody levels to those of the Phase 2 study [9, 12]; an age related increase in vaccine-induced antibody levels was found when vaccinees' ages were stratified by year (Table 3.) Among the *S. sonnei* vaccinees, there was a 4.5-fold difference in the level of IgG *S. sonnei* antibodies between the ages of 1-2 and 3-4 (6.38 vs 1.40, *P*=0.002), and a 2.7 fold difference for the *S. flexneri* 2a antibodies in *S. flexneri* 2a vaccines (9.51 vs 3.43, p=0.0002). Significant age related rises to both O-SPs were also found in the controls (development of "natural immunity", reviewed in ref 5); *S.sonnei* antibodies in *S.flexneri* 2a recipients, *p* for trend=0.002 and *S. flexneri* 2a antibodies in *S.sonnei* recipients, *P* for trend=0.001, but the levels in the vaccinees were 9-fold (*S.sonnei*) and 4.5-fold (*S.flexneri* 2a) higher. Compared to the controls, both vaccines elicited statistically significant responses (each *P*<0.001).

Both vaccine-induced antibodies were short lived, peaking at around 10 weeks after the second injection and declining thereafter (Table 4).

IgG anti-ETA—A recombinant non toxic variant of *P. aeruginosa* exotoxin A was the carrier, but the antibodies were measured against the exotoxin A. This carrier has also been used successfully in the efficacy study of Vi-rEPA in 2-5 year olds and in infants [22, 23]. A rise in IgG anti-ETA was detected in almost all vaccinees, similar to that observed in the Phase 2 study [9, 12].

Stool cultures

The overall rate of diarrhea in the study population was 0.6 episodes/child/year, similarly distributed between the *S. sonnei* and *S. flexneri* 2a conjugate groups (0.61 and 0.59 respectively). Rates of diarrhea were significantly affected by age: 0.79, 0.56 and 0.35/child/year in the 1-2, 2-3 and 3-4 year olds, respectively (p<0.001).

Of 3295 stool cultures obtained, 716 were positive for pathogens. *Shigella* was the most common bacterial isolate, 202, followed by *Campylobacter* Spp, 140. Of the *Shigella*, there were 125 *S. sonnei* and 65 *S. flexneri*, of which 29 isolates were of type 6, 21 type 2a, 5 type 1b, 3 type 1a, 1 each of types 2b and 3a and 5 not identified.

There were 8 *S. boydii*, 3 *S. dysenteriae* and 1 *Shigella* Spp (not identified). There were no significant differences in isolation rates of the other pathogens between the vaccine groups.

Efficacy (Tables 5a & b)

The per-protocol efficacy analysis was based on 2699 children who received 2 doses of one of the investigational vaccines and were followed for 2 years.. The overall attack rate for *S.*

sonnei was 1.34%/yr and for *S. flexneri* type 2a 0.83%/yr. Most *S. sonnei* cases occurred in the 1-2 year-olds, declining in the 2-3 year-olds and further in the 3-4 year-olds. Two clusters of *S. sonnei* shigellosis occurred in 2 communities in June-July 2006; 8 cases occurred within 3 weeks, 7 of which in *S. flexnei* 2a vaccine receipients and 17 cases occurred within 5 weeks, 7 of which in *S. flexnei* 2a receipients, 22/25 were >3years old. Other than one positive culture for *Salmonella enterica*, there were no positive blood cultures among vaccinees that had fever in addition to diarrhea.
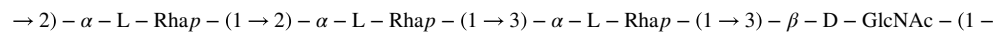
There was an age-related efficacy for recipients of the *S. sonnei* conjugate: 3.8% for the 1-2 year-olds, 35.5% for the 2–3 year-olds and 71.1% (*P*=0.043) for the 3-4-years old. Because of the small number of isolates during the first 10 weeks after the second injection when antibody levels were at their highest, no efficacy could be assessed for that time.

There were too few cases of the *S. flexneri* 2a infection for statistical significance. A reason for the small number of *shigella* isolates is that the study started at the descending part of the bi-annual incidence curve of shigellosis in Israel. As observed with other communicable diseases transmitted by human contact, there is a cyclic pattern to shigellosis in Israel with peaks every 2-3 years. A new epidemic occurs when the time limited herd immunity provided by recovery from disease wanes and a new, naïve cohort of infants and children develops[24]. Protection from non-vaccine types of *S. flexneri*, in *S. flexneri* 2a conjugate recipients, was noticed. These types included type 6, the most common *S. flexneri* isolate during the study. The overall efficacy of *S. flexneri* 2a vaccine against all *S. flexneri* non-type 2a was 44.9%, and against type 6 alone - 51.7%; both these values were not statistically significant. Intent to treat analysis of all enrolled children yielded almost identical results (not shown).
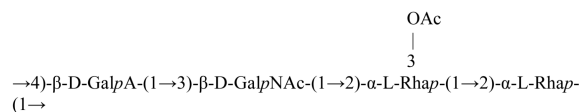
DISCUSSION

Protection was conferred by the *S. sonnei* O-SP-rEPA conjugate in 3-4 year-olds, 71.1% efficacy (*P*=0.04), 35.5% in the 2-3 year-olds (*P* not significant) but there was no efficacy in the 1-2 years-old group. Efficacy paralleled the age-related immunogenicity of the *S. sonnei* conjugate, demonstrated during the 2 year follow up, at the time when the peak antibody levels have declined (still significantly higher than in the controls, *P*<0.01). Homologous efficacy was not significant in recipients of the *S. flexneri* 2a conjugate likely due to the small number of cases. No serious adverse reactions related to the immunization were observed. These results extend our efficacy data in adults and provide a vaccine for *S. sonnei* shigellosis in individuals older than 3 years of age. Moreover, the data confirm our proposal that a critical (protective) level of serum IgG anti-O-SP antibodies confers immunity to shigellosis [5, 6]. Importantly, this information will allow a more precise prediction of the efficacy of our improved O-SP conjugates, including in those less than 3 years of age [25].

In developing countries, *S. flexneri* is the major cause of shigellosis. The structure and antigenicity of Group B Shigellae O-SPs are related [17]. All, except type 6, are composed of the tetrasaccharide repeat unit:

$\rightarrow 2) - \alpha - L - Rhap - (1 \rightarrow 2) - \alpha - L - Rhap - (1 \rightarrow 3) - \alpha - L - Rhap - (1 \rightarrow 3) - \beta - D - GlcNAc - (1 -$

Addition of glucose and OAc moieties to this tetrasaccharide backbone, under the control of phage infections, confers the fine antigenic specificities of the Group B Shigella O-SPs [15, 17].

The repeat unit of *S. flexneri* type 6 O-SP is:

$$
\begin{array}{c}
\text{OAc} \\
| \\
3 \\
\rightarrow 4)\text{-}\beta\text{-D-Gal}p\text{A-}(1\rightarrow 3)\text{-}\beta\text{-D-Gal}p\text{NAc-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-} \\
(1\rightarrow
\end{array}
$$

[26].

It is likely that the disaccharide Rha*p*-(1→2)-Rha*p* (O-acetylated in the same position in the type 2a O-SP) accounts for the cross-reactivity of the types 2a and the other *S. flexneri* types, particularly type 6. The data, showing efficacy of the *S. flexneri* 2a conjugate against the cross-reactive types of Group B shigellae, although not statistically significant, is consistent with previous studies in animals [27, 28].

This is the first Shigella vaccine candidate to demonstrate efficacy in young children older than 3 years of age. A vaccine of improved immunogenicity is considered for evaluation in infants. Orally-administered streptomycin-dependent strains of *S. flexneri* types 1, 2a, and *S. sonnei* induced type-specific immunity in 80% of 2 to 7 year-olds in hyperendemic regions of Yugoslavia [29]. Four doses of about 10^{10} viable organisms were administered at 3-day intervals. There were no reported serologic assays performed on the participants. The virulence of these strains was not attenuated and they were not used because of the high rate of severe adverse reactions they elicited.

Bacteriologists have concluded that *Shigella* and *Escherichia coli* should be considered as one Genus [30-32]. As an example, the virulent *E. coli* O157 and *S. dysenteriae* type 1 excrete the same exotoxin denoted as shigella toxin and cause similar diseases, including the hemolytic-uremic syndrome. Immunity to infection with both of these pathogens has also been proposed to be related to LPS-specific serum IgG [33]. On the basis of published data and the results of our clinical trials, we predict that a critical (protective) level of serum IgG anti O157-O-SP will confer immunity to this pathogen.

Acknowledgement

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Table 1

Enrollment by gender, age and vaccine type

Vaccine	Total	Male	Female	1-2 yr	>2-3 yr	>3-4 yr	>4 yr
S. sonnei							
N=	1433	732	701	533	514	385	1
%	51.2	51.1	48.9	37.2	35.9	26.8	0.1
S. flexneri 2a							
N=	1366	723	643	496	499	368	3
%	48.8	52.9	47.1	36.3	36.5	26.9	0.2
Total							
N=	2799	1455	1344	1029	1013	753	4
%	100	52.0	48.0	36.8	36.2	26.9	0.1

Table 2

Adverse events per vaccine type and dose

Adverse event	S. sonnei				S. flexneri 2a			
	Dose 1		Dose 2		Dose 1		Dose 2	
	N=1433		N=1405		N=1366		N=1343	
	N	%	N	%	N	%	N	%
Local pain	82	5.72	79	5.62	61	4.47	64	4.77
Swelling	11	0.77	19	1.35	11	0.81	9	0.67
Redness	7	0.49	15	1.07	6	0.44	6	0.45
Fever	56	3.91	36	2.56	72	5.27	51	3.80
Nausea	22	1.53	9	0.64	10	0.73	11	0.82
Vomiting	28	1.95	8	0.57	14	1.03	13	0.97

Table 3

Age-related IgG anti-LPS levels of sera drawn randomly >2 weeks after the second vaccine dose

Vaccine	Age (yr)	N=	S. sonnei	S. flexneri 2a
		G.M. IgG anti-LPS (EU)[*]		
S. sonnei	1-2	38	1.40	3.43
	>2-3	44	3.71	7.53
	>3-4	29	6.38	9.51
S. flexneri 2a	1-2	43	0.25	18.98
	>2-3	53	0.42	26.96
	>3-4	30	0.76	43.86

0.25 vs. 0.42 P=0.12; 0.42 vs. 0.76 P=0.05; 0.25 vs. 0.76 P=0.001; P for trend =0.002

3.43 vs. 7.53 P=0.02; 7.53 vs. 9.51 P=0.28; 3.43 vs. 9.51 P=0.0002; P for trend =0.001

18.98 vs 26.96 P=0.13; 26.96 vs, 43.86 P=0.09; 18.98 vs. 41.68 P=0.007; P for trend =0.005

6.38 vs. 0.76 P=0.0001

3.71 vs. 0.42 P<0.0001

1.40 vs. 0.25 P<0.0001

[*] 1.40 vs. 3.71 P=0.01; 3.71 vs. 6.38 P=0.25; 1.40 vs. 6.38 P=0.002; P for trend =0.001

Table 4

IgG anti-LPS of sera drawn randomly by time from the second vaccine dose

| Vaccine | Week | N= | G.M. IgG anti-LPS (EU)[*] | |
			S. sonnei	*S. flexneri* 2a
S. sonnei	2-10	24	12.93	6.67
	>10-30	29	3.98	5.52
	>30	58	1.48	6.20
S. flexneri 2a	2-10	34	0.74	52.89
	>10-30	40	0.25	28.62
	>30	52	0.38	16.42

[*] 12.93 vs. 3.98 P=0.02; 3.98 vs. 1.48 P=0.007; 12.93 vs. 1.48 P<0.0001; P for trend =0.0007 52.89 vs. 28.62 P=0.02; 28.62 vs. 16.42 P=0.02; 52.89 vs. 16.42 P<0.0001; P for trend =0.005

Table 5

Efficacy of 2 doses of *Shigella* conjugate vaccines by age, per-protocol

a. *Shigella sonnei*

	Vaccine administered						
	S. sonnei		*S. flexneri 2a*				
Age	N=	Cases	N=	Cases	Efficacy	(95% CI)	*P*
1–2 yr	516	18	476	16	3.8%	(101.1, 46.5)	0.91
>2–3 yr	497	8	481	12	35.5%	(−56.4, 73.4)	0.33
>3–4 yr	371	3	358	10	71.1%	(−4.43, 92.0)	0.04
All ages	1384	29	1315	38	27.5%	(−16.9, 54.0)	0.18

b. *Shigella flexneri 2a*

	Vaccine administered						
	S. sonnei		*S. flexneri 2a*				
Age	N=	Cases	N=	Cases	Efficacy	(95% CI)	*P*
1–2 yr	516	3	476	3	−8.4%	(−434.5, 78.0)	0.99
>2–3 yr	497	4	481	3	22.5%	(−244.4, 82.6)	0.99
>3–4 yr	371	1	358	1	−3.6%	(−1550, 93.5)	0.99
All ages	1384	8	1315	7	7.9%	(−153.2, 66.5)	0.87