CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, July 1996, p. 451–455 Vol. 3, No. 4
1071-412X/96/$04.00+0

Detection of Antibodies to *Shigella* Lipopolysaccharide in Urine after Natural *Shigella* Infection or Vaccination

DANI COHEN,[1,2]* NADAY ORR,[1] GUY ROBIN,[1] RAPHAEL SLEPON,[1] SHAI ASHKENAZI,[1,2] ISAAC ASHKENAZI,[1] AND JOSHUA SHEMER[1,2]

Medical Corps, Israel Defence Force, Military Post 02149,[1] and Sackler School of Medicine, Tel Aviv University, Ramat Aviv 69978, Tel Aviv,[2] Israel

Received 27 July 1995/Returned for modification 1 November 1995/Accepted 18 March 1996

The purpose of the present study was to explore the possibility of detecting antibodies to *Shigella sonnei* lipopolysaccharide (LPS) in urine after infection or vaccination. Urinary immunoglobulin A (IgA) and IgG antibodies and specific IgA secretory protein against *S. sonnei* LPS were measured by enzyme-linked immunosorbent assay (ELISA), after adjustment for urine concentration. A significant antibody level was defined as one above a cutoff value calculated from the geometric mean + 2 standard deviations of urinary anti-*S. sonnei* LPS levels in 43 healthy hepatitis B vaccinees (controls). Of 11 culture-proven cases of *S. sonnei* shigellosis, at convalescence 9 (82%) had significantly elevated levels of urinary antibodies to the homologous LPS. The *S. sonnei* conjugate vaccine, composed of *S. sonnei* O-specific polysaccharide covalently bound to recombinant exoprotein A of *Pseudomonas aeruginosa*, elicited a significant urine IgA or IgG anti-LPS response in 60% (6 of 10), 56% (9 of 16), 43% (16 of 37), and 14% (3 of 21) of the volunteers at 2 weeks, 6 weeks, 6 months, and 12 months after vaccination, respectively. The specificity of the urine antibody response to *S. sonnei* LPS was documented by the total lack of response in subjects who received parenteral *Shigella flexneri* 2a-recombinant exoprotein A conjugate (69 urine samples) or meningococcal tetravalent control vaccines (4 urine samples). All the volunteers who lacked a significant response to *S. sonnei* LPS in serum also lacked such response in urine samples. Seventy-four percent of the volunteers with a significant IgA or IgG anti-LPS response in serum at convalescence or 14 days after vaccination showed a similar response in urine. The ratio of the titer of secretory protein bound to IgA anti-*S. sonnei* LPS in urine to that in serum was 303 times higher than the ratio of anti-*S. sonnei* LPS total IgA titer in urine to that in serum, indicating that the urine IgA is of secretory origin. These findings suggest the possible use of urinary *Shigella* LPS antibodies as markers of systemic and secretory immune responses after natural infection or vaccination. At this stage, because of its limited sensitivity, the detection by ELISA of *Shigella* LPS antibodies in urine cannot replace the same assay in serum as a definitive test in an individual with a negative result.

Natural *Shigella* infection induces a mucosal and systemic immune response to *Shigella* antigens. Passive hemagglutination and enzyme-linked immunosorbent assay (ELISA) have been employed for detection of serum antibodies of various immunoglobulin classes developed against *Shigella* serogroup-specific lipopolysaccharide (LPS) (5, 7). It has been reported that the rise in serum immunoglobulin A (IgA) to *Shigella* LPS indicates recent infection with the homologous organism while IgG-specific antibodies are markers of more distant exposure to *Shigella* strains (5). A strong correlation between preexisting IgG anti-LPS serum antibodies and acquired natural immunity against shigellosis was demonstrated previously (6, 7). In immunogenicity studies of candidate *Shigella* vaccines, a significant rise in serum antibodies to homologous LPS was used as evidence for stimulation of immunocompetent cells (4, 13, 21). A few studies have shown that antibodies to various bacterial antigens can be detected in urine after natural infection of the urinary tract or other mucosal sites, or after vaccination (1, 8, 11, 14, 18, 20), and that at least part of these antibodies are of mucosal origin (11, 18). There is no report on any attempt to measure urinary antibodies to *Shigella* antigens after natural infection or vaccination. We assume that, if anti-*Shigella* antibodies can be detected in urine at high sensitivity and specificity, they may have the same applications as the serum anti-*Shigella* antibodies. Urine samples may replace blood samples among young children for whom vein punctures are problematic, and in addition, the urinary antibodies may be used as reliable and direct markers of mucosal stimulation. In the present study, we examined the possibility of detecting anti-*Shigella* LPS antibodies in urine by a simple ELISA system after *Shigella* natural infection or parenteral vaccination with the *Shigella sonnei*-recombinant exoprotein A (rEPA) conjugate vaccine. We also studied the nature of the urinary antibodies and the correlation between the levels of these antibodies and those of serum antibodies against the same LPS antigens.

MATERIALS AND METHODS

Study subjects. Israeli volunteers who received one injection of *S. sonnei*-rEPA conjugate investigational vaccine ($n = 50$), one injection of *Shigella flexneri* 2a-rEPA conjugate investigational vaccine ($n = 41$), a partial or full regimen of hepatitis B vaccine ($n = 43$), or one injection of meningococcal tetravalent vaccine ($n = 4$) or suffered from culture-proven *S. sonnei* shigellosis ($n = 11$) were included in the study. Parallel blood and urine specimens were obtained from the study subjects at different times after vaccination or onset of disease. No specific time during the day was designated for collection of urine samples. Measurement of creatinine was used to control for intersample variations in the urine concentration.

Vaccines. O-specific polysaccharide of *S. sonnei* or *S. flexneri* 2a covalently bound to exoprotein A of *Pseudomonas aeruginosa* (rEPA) to form conjugates was prepared at the National Institutes of Health (3, 21). Each 0.5-ml dose contained 25 μg of conjugate dissolved in saline with 0.01% thimerosal. One dose of these vaccines was injected into the deltoid muscle of the volunteers. Three doses of the licensed hepatitis B vaccine (Engerix; SmithKline Beecham)

* Corresponding author. Mailing address: Medical Corps, Israel Defence Force, Military Post 02149, Israel. Phone: 972-3-5307259. Fax: 972-3-9221962 or 9220333.

TABLE 1. Urine IgA and IgG anti-*S. sonnei* LPS antibodies (rate of response and GMT) among patients with culture-proven *S. sonnei* shigellosis and volunteers vaccinated with *S. sonnei* conjugate vaccine

Time after vaccination or diagnosis	Value for *S. sonnei* conjugate vaccinees					Value for *S. sonnei* natural infection							
	n	*n* positive[a] (%)		GMT		*n*	*n* positive[a] (%)		*n* with fourfold antibody rise (%)			GMT	
		IgA	IgG	IgA	IgG		IgA	IgG	IgA	IgG	IgA or IgG	IgA	IgG
0						11	1 (9)	1 (9)				1.5	1.8
2 wk	10	6 (60)	5 (50)	6.0	4.2	11	8 (73)	6 (55)	8 (73)	4 (36)	9 (82)	12.8	5.6
6 wk	16	8 (50)	9 (56)	5.4	7.3								
6 mo	37	11 (30)	16 (43)	3.6	7.0								
12 mo	21	2 (10)	3 (14)	2.7	3.1								

[a] Based on a cutoff of 4.1 calculated from the geometric mean + 2 standard deviations in hepatitis B vaccinees.

were injected intramuscularly into volunteers at the usual schedule (0, 1, and 6 months). One dose of the licensed meningococcal tetravalent vaccine was used (Mencevax; SmithKline Beecham).

Urinary and serum antibodies to *S. sonnei* LPS. Urinary and serum IgA and IgG antibodies to homologous and heterologous *Shigella* LPS were measured by means of ELISA in double dilutions starting with the 1:2 dilution for urine and 1:50 dilution for serum. The assays were performed in microtitration plates (Costar, Cambridge, Mass.) as previously described for serum antibody detection (4, 5). LPSs extracted by the phenol-water method (22) from clinical isolates of *S. sonnei* (form 1) and *S. flexneri* 2a were used as antigens. Goat anti-human IgG or anti-IgA, conjugated to alkaline phosphatase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.), was employed as second antibody. Optical density was read at 405 nm with an automatic ELISA reader (EL-340; Bio-Tek, Winooski, Vt.). The adjusted optical densities derived from a linear regression analysis of eight doubling dilutions were expressed as endpoint titers. The endpoint titers were adjusted for urine concentration by division by the concentration of urine creatinine in the urine sample, and these values were then used for all the calculations. Geometric mean titers (GMT) were calculated. Control serum or urine samples were included in every microtitration plate in each of the assays. A significant antibody level was defined as one above a cutoff value calculated from geometric mean + 2 standard deviations in hepatitis B vaccinees.

Secretory protein bound to IgA anti-*S. sonnei* LPS. The above-mentioned ELISA protocol was modified to measure the level of the secretory protein bound to IgA developed against *S. sonnei* LPS. Briefly, serum and urine samples in double dilutions starting with a 1:25 dilution for serum and a 1:2 dilution for urine were incubated in *Shigella* LPS-coated microtiter plates. A monoclonal antibody to human IgA secretory protein (Bio-Makor, Rehovot, Israel) was added to the plates and was followed by an alkaline phosphatase goat anti-mouse IgG conjugate (Sigma Chemical Co., St. Louis, Mo.) and substrate.

Statistical analysis. Statistical analysis was carried out on the SAS version 6.04 software for the PC, with correlation analysis for comparing urine and serum titers and paired *t* test for the assessment of the ratio of secretory protein to total anti-LPS IgA in serum and urine.

RESULTS

Urinary and serum immune responses to LPS after natural infection. Paired urine specimens were available from persons with culture-proven *S. sonnei* shigellosis at the acute and convalescent stages of the disease. Of 11 patients at convalescence (10 to 19 days after onset of disease), 8 (73%) and 6 (55%) showed a significant response of IgA and IgG, respectively, to *S. sonnei* LPS in urine. Nine of these patients (82%) had significantly elevated levels of urine antibodies, either IgA or IgG, to the homologous LPS. Only one had a similar titer of urinary anti-LPS antibodies at the acute stage of the disease (Table 1). The GMT of IgA and IgG at convalescence were 8.5 and 3.1 times, respectively, higher than the corresponding GMT at the acute stage of the disease. All 10 patients from whom blood samples were obtained at convalescence had a significant response (≥4-fold rise) to *S. sonnei* LPS in serum.

Urinary and serum immune responses to LPS after vaccination. *S. sonnei*-rEPA elicited a significant urine IgA anti-LPS response in 60% (6 of 10), 50% (8 of 16), 30% (11 of 37), and 10% (2 of 21) of the volunteers 2 weeks, 6 weeks, 6 months, and 12 months after vaccination, respectively (Table 1). The rates of significant urine IgG response at the same time intervals after vaccination were 50% (5 of 10), 56% (9 of 16), 43% (16 of 37), and 14% (3 of 21) (Table 1). The rates of significant response of either IgA or IgG were not higher than the separate rates of IgA and IgG. The highest GMT was reached 2 weeks after vaccination for IgA and 6 weeks after vaccination for IgG. The rates of serum antibody response of either IgG or IgA were 90% (9 of 10), 88% (14 of 16), and 83% (30 of 36) at 2 weeks, 6 weeks, and 6 months after vaccination, respectively. All the subjects lacking a significant serum antibody rise also lacked a urinary significant anti-LPS response. It follows, for example, that 14 days after vaccination the sensitivity of the IgA urinary antibody response compared with the IgA serum antibody response is 67% (six of nine).

The specificity of the urine antibody response to *S. sonnei* LPS was documented by the total lack of such response in subjects who received the parenteral *S. flexneri* 2a conjugate and meningococcal tetravalent vaccines. In none of 73 urine samples obtained 14 days to 12 months after vaccination were significant levels of urinary anti-*S. sonnei* LPS detected.

Correlation between urinary and serum anti-LPS responses. Overall, in 62% (39 of 63) of the volunteers with a fourfold or higher rise in serum anti-LPS, a significant level of urinary anti-LPS could be detected. At convalescence or 14 days after vaccination, the sensitivity of the urinary ELISA was higher. Seventy-four percent of the volunteers (14 of 19) with a significant IgA or IgG anti-LPS response in serum showed a similar response in urine. All the volunteers who lacked a significant response to *S. sonnei* LPS in serum also lacked such response in the parallel urine samples (100% specificity). The scatter diagrams (Fig. 1 and 2) show a high correlation between the urine and serum IgA ($r = 0.68$, $P < 0.001$) and IgG ($r = 0.59$, $P < 0.001$) anti-*S. sonnei* LPS titers after vaccination or natural infection. The scatter diagrams also show that the threshold concentration of antibodies to *S. sonnei* LPS detected by the ELISA used in this study was about 10- to 100-fold higher in urine than in serum.

Secretory protein bound to IgA anti-*S. sonnei* LPS. The titers of the secretory protein bound to IgA anti-*S. sonnei* LPS and anti-*S. sonnei* LPS total IgA were measured in 27 parallel urine and serum samples at different time intervals after vaccination. The geometric mean of the ratio of anti-*S. sonnei* LPS IgA secretory protein titer in urine to that in serum was 1.057, while the geometric mean of the ratio of anti-*S. sonnei* LPS total IgA titer in urine to that in serum was only 0.003. The geometric mean of the ratio of these two ratios, which under the null hypothesis is assumed to be 1, was thus 302.7 (95% confidence intervals = 208.5 to 441.4; $P < 0.001$), pointing to a much

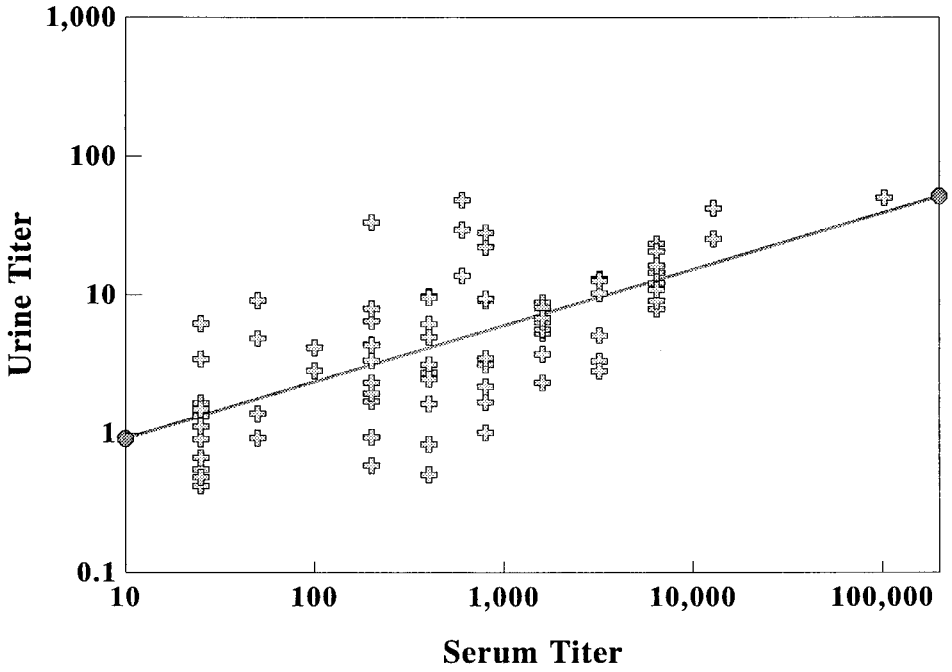

FIG. 1. Correlation between urinary and serum IgA anti-*S. sonnei* LPS titers. $r = 0.68$ ($P < 0.001$).

higher proportion of secretory protein to total IgA in urine than in serum.

DISCUSSION

In the present study, we have shown that natural, culture-proven *Shigella* infection induced a significant anti-LPS response in urine in more than 80% of the volunteers while parenteral vaccination with the *S. sonnei*-rEPA conjugate vaccine induced a significant urinary IgA or IgG response in 60% of the volunteers. The specificity of detection of homologous anti-*S. sonnei* LPS antibodies in urine was documented by the complete lack of detectable antibodies to *S. sonnei* LPS in volunteers who were vaccinated with *S. flexneri* 2a or meningococcal vaccines. Compared with serum antibody response, the sensitivity of the urinary antibody response was 62% over-

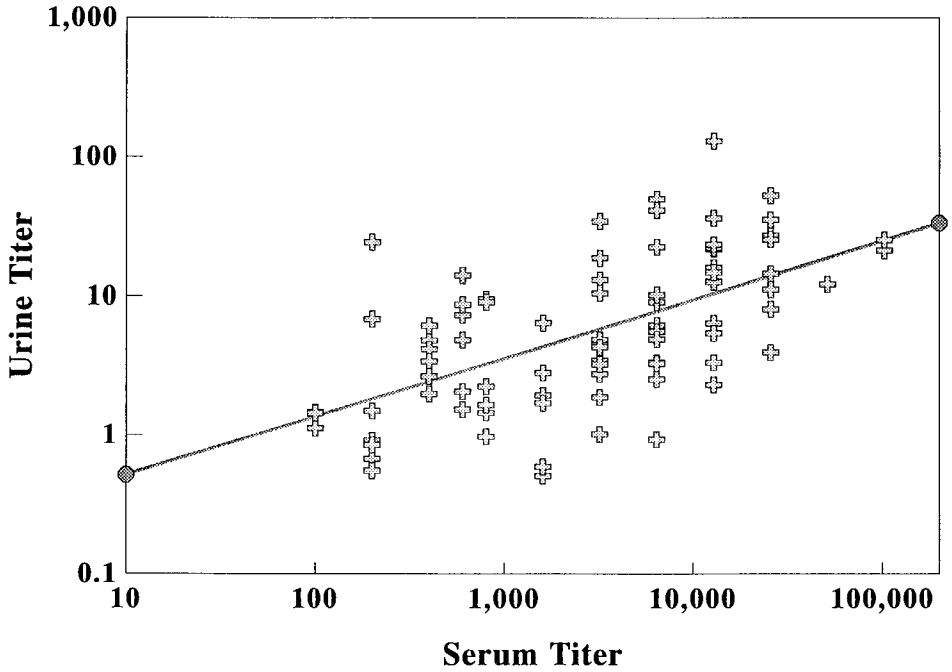

FIG. 2. Correlation between urinary and serum IgG anti-*S. sonnei* LPS titers. $r = 0.59$ ($P < 0.001$).

all and 74% at convalescence or 14 days after vaccination, while the specificity was 100%. Despite the limited sensitivity, the practicability of obtaining urine samples instead of blood samples mainly among infants and young children supports the use of the test to demonstrate exposure to *Shigella* strains. The presence of antibodies to *Shigella* LPS in urine samples will indicate most probably previous *Shigella* infection while a negative finding will still leave a relatively high probability of a false-negative result. We are currently exploring procedures to concentrate the anti-LPS antibodies in urine and in this way to increase the sensitivity of the test.

Significant levels of urinary anti-LPS IgA were induced by both natural infection with *S. sonnei* and parenteral vaccination with the *S. sonnei*-rEPA conjugate vaccine. By showing that the ratio of anti-*S. sonnei* LPS IgA secretory protein titer to anti-*S. sonnei* LPS total IgA titer was 303 times higher in urine than in blood, we demonstrated that the IgA anti-*Shigella* LPS antibodies are of secretory origin. It is probable that the secretory IgA is produced locally in the mucosal epithelium of the urinary tract, reflecting general mucosal stimulation following ingestion of LPS or parenteral delivery of polysaccharide conjugated to rEPA. As the molecular size of secretory IgA is much greater than the upper limit for glomerular filtration through the kidneys, it is unlikely that secretory anti-*S. sonnei* LPS IgA in urine has its origin at a distant mucosal site and is transported into urine via the circulation. Our finding showing the presence of secretory anti-*S. sonnei* LPS in urine following natural *S. sonnei* infection takes a step further previous data indicating that chronic infection of the gastrointestinal tract could elicit a rise in the amount of total secretory IgA in urine (18). The significant levels of anti-*S. sonnei* LPS in urine after vaccination with the *S. sonnei* conjugate vaccine corroborate evidence from animal and human studies of specific secretory immune response at multiple mucosal sites attained by oral, intranasal, and parenteral delivery of bacterial and viral antigens (9–12, 15, 17).

Shigellosis caused by *S. sonnei* and parenteral immunization with the *S. sonnei* conjugate also elicited significant levels of urinary IgG anti-*S. sonnei* LPS. Interestingly, an additional study recently reported detection of specific IgG urinary antibodies in patients with another mucosal infection of the gastrointestinal tract (1). Alemohammad et al. (1) could detect IgG urinary antibodies to *Helicobacter pylori* concurrently with infection of the gastric mucosa with the pathogen, documented by isolation of *H. pylori* in biopsies and detection of specific serum IgG antibodies. Since in normal subjects only 1:500 of the plasma IgG concentration can pass to the Bowman's space of the kidney (16), other routes are probably taken by plasma IgG to reach the urinary tract. Our assumption is that the presence of IgG anti-LPS in urine after natural *Shigella* infection or parenteral vaccination with the *S. sonnei*-rEPA conjugate is a result of transudation of IgG when this immunoglobulin class circulates at high concentrations in blood capillaries all along the infected mucosal site as well as along the mucosal epithelium of the urinary tract. Arrival of serum IgG antibodies in the gastrointestinal mucosa after parenteral delivery of conjugate vaccines is proposed as the main component of the mechanism by which these vaccines may confer protection against enteric infections (19, 21). A similar interpretation was proposed by Kauppi et al. when they showed that, after parenteral immunization with *Haemophilus influenzae* type b conjugate vaccine, they could detect significant levels of secretory IgA and IgG anti-capsular polysaccharide antibodies in saliva (10) and that the presence of these antibodies is probably the reason for a lesser extent of colonization of the respiratory tract by *H. influenzae* type b after vaccination with the *H. influenzae* type b conjugate vaccine. As previously suggested in another study (18), it may also be possible that the urinary IgG is produced by migratory lymphocytes traveling from the lymphoid tissue of the gut through the lymphatic system to the urinary tract.

In summary, in this study we have demonstrated that IgG and IgA antibodies to *Shigella* LPS can be detected in urine after natural *Shigella* infection and vaccination. Although highly specific, the test is of relatively low sensitivity at this stage, which makes it more reliable for documenting the previous exposure to *Shigella* LPS of groups than for documenting that of individuals. A negative result still leaves a relatively high probability of being false negative compared with detection of serum antibodies. Different techniques to increase the sensitivity of the assay in urine are currently being examined in our laboratory. As they are easily measured, urinary anti-*Shigella* LPS antibodies may be used as reliable and direct markers of general mucosal stimulation, replacing cumbersome methods for detection of intestinal secretion of antibodies after vaccination with candidate *Shigella* vaccines.

ACKNOWLEDGMENTS

This study was supported by grant no. DAMD17-93-V-3001 from the U.S. Army Medical Research and Materiel Command, Fort Detrick, Frederick, Md.

We are grateful to Ruhama Ambar, Ita Marcus, and Rachel Heller-Mendelziss for very helpful technical assistance in the laboratory and field work.

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