Presence of Specific Immunoglobulin A-Secreting Cells in Peripheral Blood after Natural Infection with *Shigella sonnei*

NADAV ORR,[1]* GUY ROBIN,[1] GEORGE LOWELL,[2] AND DANI COHEN[1]

Medical Corps, Israel Defence Force, Military Post 02149, Israel,[1] and Walter Reed Army Institute of Research, Washington, D.C. 20307[2]

The appearance of antigen-specific immunoglobulin A (IgA) antibody-secreting cells (ASCs) following natural infection with *Shigella sonnei* during a common-source outbreak caused by this organism was evaluated in a modified enzyme-linked immunosorbent assay (ELISPOT). A mean IgA ASC value of $2,131.6/10^6$ cells against homologous *S. sonnei* lipopolysaccharide (LPS) was detected in blood samples obtained from patients with bacteriologically proven *S. sonnei* shigellosis 5 and 10 days after the onset of disease. In the same blood samples, the level of ASC measured against heterologous antigen (*Shigella flexneri* serotype 2a LPS) was significantly lower than that of the homologous antigen (mean value, $33.12/10^6$ cells). Furthermore, the mean number of activated B cells that secreted anti-*S. sonnei* LPS antibodies was significantly higher among patients with *S. sonnei* shigellosis than it was among patients with non-*Shigella* diarrhea ($2.5/10^6$ cells; standard error, 1.0) and healthy subjects ($5.1/10^6$ cells; standard error, 2.3) ($P < 0.05$). The anti-LPS IgA ASC activity was easily detected within 5 days of the onset of disease, a point at which the levels of anti-*S. sonnei* LPS IgG and even IgA antibodies were hardly detectable in serum.

Immunity against shigellosis is believed to involve local secretory antibodies (10, 11), but both cell-mediated immunity and humoral antibodies may also be important in the mechanism of protection (1–3, 12, 14, 19). The methods available for the direct determination of intestinal antibodies to *Shigella* spp. as well as to other bacterial pathogens in volunteers are cumbersome, time-consuming, and not reliable for use in large-scale studies. It is believed that after antigenic stimulation, activated B lymphocytes from Peyer's patches migrate to local lymph nodes to mature, and they later return to the intestine via the lymphatic system and blood (13). A few studies have demonstrated the possibility of measuring the number of antibody-secreting B cells in peripheral blood following natural infection with enteropathogens or vaccination (6, 8, 9, 16, 17). It has been proposed that the number of the antibody-secreting cells (ASCs) should be a reliable correlate of antigenic stimulation in the gut (8, 9, 17). Since there is evidence that shigellosis confers protection against recurrent disease caused by the homologous *Shigella* group (5, 7), it may be assumed that an efficient *Shigella* vaccine should induce, to a comparable extent, the same immune mechanisms as natural infection does. Furthermore, the number of ASCs appearing after natural infection may serve as a reliable reference criterion for evaluating the ability of a candidate *Shigella* vaccine to stimulate immunocompetent cells in the intestine.

The objective of the present study was to evaluate the extent of appearance of antigen-specific immunoglobulin A (IgA) ASCs in comparison with those of other serological markers following natural infection with *Shigella sonnei* during a common-source outbreak caused by this organism.

MATERIALS AND METHODS

Study population. The study population comprised subjects who were divided into the following three subgroups:

patients with *S. sonnei* shigellosis, patients with non-*Shigella* diarrhea, and healthy control subjects.

(i) **Patients with *S. sonnei* shigellosis.** Nineteen subjects involved in a point-source outbreak of *S. sonnei* shigellosis that occurred in an Israel Defence Force unit were included in the study. The clinical investigation of these patients showed that all of them had diarrhea; two patients complained of abdominal cramps, nausea, and vomiting; and only one patient was febrile. None of the patients reported hematochezia.

Of the subjects with diarrhea, 11 patients with bacteriologically proven *S. sonnei* shigellosis and 8 patients with clinical shigellosis and a negative stool culture for *Shigella* spp. were examined. All the subjects were males aged 18 to 22 years.

(ii) **Patients with non-*Shigella* diarrhea.** The study group comprising patients with non-*Shigella* diarrhea ($n = 11$) included subjects who served in Israel Defence Force units other than that comprising subjects with *Shigella* diarrhea and who presented at their unit clinics with a complaint of diarrhea. Their stool cultures were found to be negative for *Shigella* spp. An episode of diarrhea was defined as more than two liquid stools in 24 h.

(iii) **Healthy control subjects.** The study subjects in the healthy control group ($n = 20$) were males and females between the ages of 18 and 22 years who served in an Israel Defence Force unit other than that affected by the outbreak. None of the subjects experienced any episode of diarrhea or had evidence of any contact with *Shigella* antigens during a period of 2 months prior to the time of the investigation.

Data on the presence or absence of signs or symptoms, date of onset, and a description of the feces were obtained from both symptomatic and asymptomatic subjects at the time of the investigation.

Bacteriology. Stool samples were inoculated onto MacConkey agar and xylose-lysine-deoxycholate (XLD) agar immediately after collection. In parallel, swabs with fecal matter were introduced into Cary Blair transport medium. At a central laboratory, additional culturing of samples from

---

* Corresponding author.

ORR ET AL.

transport medium was done on MacConkey and salmonella-shigella (SS) agars. Isolation and identification of *Shigella* spp. were performed by routine morphological, biochemical, and serological testing, and the strains were submitted to the Reference Laboratory of the Israeli Ministry of Health for serotyping.

LPS preparation. *S. sonnei* (form 1) and *S. flexneri* serotypes 2a and 6 isolated from the patients with shigellosis involved in the outbreaks were used to prepare lipopolysaccharides (LPSs). LPSs from all bacterial strains were prepared by the hot phenol-water method of Westphal and Jann (18).

Serum antibodies. Sera obtained from the subjects in the study were stored at −20°C until they were tested. Serological tests were carried out by an enzyme-linked immunosorbent assay (ELISA). The ELISA was performed in microtitration plates (Costar, Cambridge, Mass.) as described previously (4). LPSs extracted from single strains of *S. sonnei* (form 1) and *S. flexneri* serotype 2a isolated from patients involved in the outbreaks were used as antigens. Goat anti-human IgG or IgA conjugated to alkaline phosphatase (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was used as the secondary antibody. Specific anti-*Shigella* LPS antibodies were calibrated against a gradient of human IgG or IgA as the first antibody on goat anti-human polyvalent antibody (Sigma, St. Louis, Mo.) that was used as the antigen. The anti-LPS antibody levels detected were expressed in micrograms per milliliter.

Isolation of lymphocytes. Venous blood was collected into sterile EDTA-treated tubes, and mononuclear cells were obtained on a Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient. The cells were washed three times in phosphate-buffered saline (PBS) and finally suspended in RPMI 1640 medium supplemented with 10% inactivated newborn calf serum (NBS), both from Biological Industries, Beit-Haemek, Israel.

Detection of ASCs. ASCs were detected by using an ELISPOT assay (16, 17). Plates (96-well, flat-bottom, high-binding; Costar) were precoated with 100 μl of *Shigella* LPS (10 μg/ml in carbonate buffer [pH 9.6]) for 1 h at 37°C, blocked with PBS supplemented with 5% NBS, and washed three times with 0.05% Tween in PBS. The plates were incubated with 100-μl double-dilution gradients of the isolated mononuclear cells in duplicate (2×10^5 to 7×10^5 cells per well as the higher concentration) in RPMI 1640 medium–10% NBS. After 3 h of incubation at 37°C in 7.5% CO_2, the plates were washed three times and 100 μl of alkaline phosphatase-conjugated anti-human IgA diluted 1:1,000 in PBS–5% NBS was added for 2 h at 37°C. After an additional three washes, 100 μl of substrate-agarose was overlaid. The substrate-agarose was prepared as follows. Some 0.14 g of low-electroendosmosis agarose (Sigma) was boiled in 10 ml of barbital buffer until it was completely dissolved. Nitroblue tetrazolium (2 ml; 1 mg/ml in barbital buffer), 40 μl of MgCl (2 M), and 20 μl of 5-bromo-4-chloro-3-indolylphosphate toluidinium (50 mg/ml in dimethylformamide) were added to 8 ml barbital buffer and heated to 56°C in a water bath. The agarose was added to the substrate mixture, and after 15 min at 56°C, the substrate-agarose was ready for use. Specific ASCs were observed after overnight incubation in the dark at room temperature and were visualized as dark blue spots. The specific ASC concentration of each sample was calculated from the dilution which gave between 20 and 50 spots per well and was expressed as the number of ASCs per 10^6 isolated mononuclear cells. The interduplicate variation was
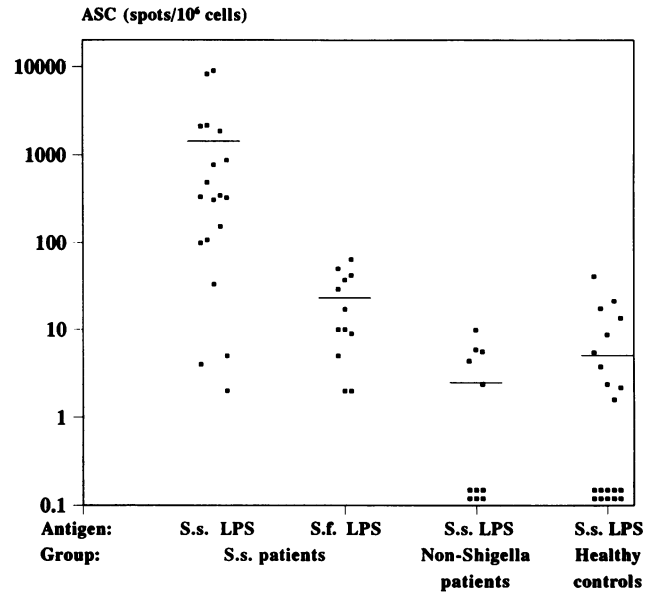

FIG. 1. Number of ASCs observed in blood samples obtained from *S. sonnei*-infected patients and patients suffering from non-*Shigella* diarrhea 5 to 10 days after the onset of disease and from healthy subjects. Each individual is represented by a square. The solid bars represent the mean numbers of ASCs observed in each group. S.s. patients, patients involved in the outbreak with positive or negative stool culture for *S. sonnei*; S.s., *S. sonnei*; S.f., *S. flexneri* serotypes 2a and 6.

28.4% (when more then 36 spots per 10^6 cells were observed).

Statistical analysis. The mean number of ASCs ± the standard error (SE) was calculated for each of the study groups. The statistical significance of the differences in the mean number of ASCs between the patients with *S. sonnei* shigellosis and the two control groups was assessed by one-way analysis of variance and the least-significant-difference procedure (15). The same difference when only two groups were compared was tested by Student's *t* test.

RESULTS

The mean number of activated B cells that secreted anti-*S. sonnei* LPS antibodies was significantly higher in peripheral blood obtained from patients with *S. sonnei* shigellosis 5 to 10 days after the onset of disease ($1,432.3/10^6$ cells; SE, 602.7) than it was in patients with non-*Shigella* diarrhea ($2.5/10^6$ cells; SE, 1.0) and healthy subjects ($5.1/10^6$ cells; SE, 2.3) ($P < 0.05$) (Fig. 1). No significant difference was found in the anti-*S. sonnei* LPS ASC response in culture-positive and culture-negative patients with *S. sonnei* shigellosis involved in the same point-source outbreak ($2,131.6/10^6$ cells [SE, 989.7] and $470.6/10^6$ cells [SE, 258.0], respectively; $P = 0.13$). The ASC response in patients with *S. sonnei* shigellosis (with a positive or negative stool culture) was found to be significantly higher when it was tested against the homologous *S. sonnei* LPS ($1,432.3/10^6$ cells; SE, 602.7) than it was when it was tested against heterologous *S. flexneri* serotype 2a and 6 LPSs ($23.1/10^6$ cells; SE, 6.0) ($P = 0.03$) (Fig. 1). No significant association was found between the specific ASC response and the severity of the disease in the group of *S. sonnei* patients included in this study (data not shown). The sensitivity and specificity of the activation
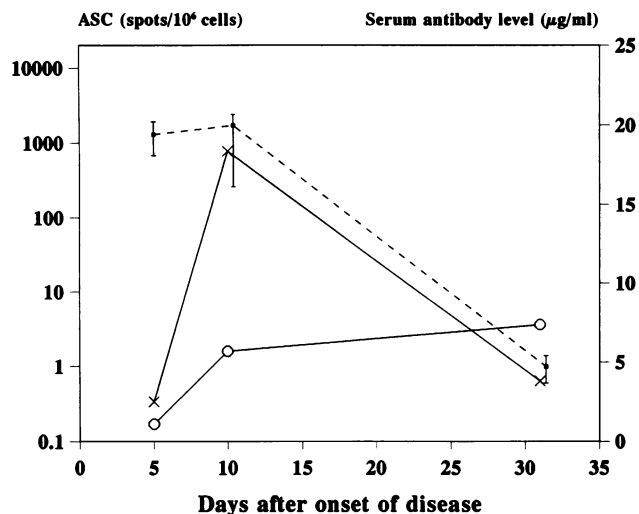
FIG. 2. Kinetics of anti-*S. sonnei* LPS ASC activity (dashed line) and anti-*S. sonnei* LPS IgA (×) and IgG (O) observed in blood samples obtained from *S. sonnei* patients. Bars indicate SEs.

of ASCs following natural infection with *S. sonnei* on the basis of a single, early-convalescent-phase blood sample were calculated. The mean number of ASCs (±3 standard deviations) in peripheral blood samples obtained from healthy subjects without evidence of recent exposure to *Shigella* spp. served as a reference value. The sensitivity of the ASC response, which was defined as the percentage of blood samples obtained from patients with *S. sonnei* shigellosis (positive and negative stool cultures) with a number of ASCs higher than the cutoff value (36.3/10⁶ cells), was 79% (15 of 19 patients). Ten of 11 patients suffering from shigellosis with a positive stool culture for *S. sonnei* had an anti-*S. sonnei* ASC level higher than the cutoff value (sensitivity, 91%). Eleven of 11 blood samples obtained from patients with non-*Shigella* diarrhea had anti-*S. sonnei* ASC levels lower than the cutoff value (specificity, 100%).

The parallel kinetics of humoral IgG and IgA antibody responses and activation of ASCs following natural infection with *S. sonnei* are depicted in Fig. 2. The mean anti-LPS IgG level increased gradually from 1.1 µg/ml 5 days after the onset of disease to 5.7 µg/ml after 10 days and to 7.4 µg/ml on day 31 following exposure. The anti-LPS IgA level increased dramatically, from 2.5 µg/ml on day 5 to 18.3 µg/ml during the second week, and decreased to 3.8 µg/ml on day 31. By contrast, lymphocytes secreting antibodies against *S. sonnei* LPS already appeared at a very high level 5 days after the onset of disease (mean value, 1,301.8/10⁶ cells [SE, 620.5]), reached a peak mean value of 1,714.8/10⁶ cells (SE, 1,454.6) on day 10, and almost disappeared after 31 days (mean, 1/10⁶ cells; SE, 0.4). The humoral antibody response against heterologous *Shigella* LPS (*S. flexneri* serotype 2a) in patients with *S. sonnei* shigellosis was very low all along the immunologic follow-up, ranging from 0.45 to 0.63 µg of IgA per ml and 0.02 to 0.54 µg of IgG per ml (data not shown).

DISCUSSION

Measurement of the ASC response has been used on several occasions as an attempt to assess the degree of stimulation of the intestinal mucosal immune system. It has

been shown that such activity can be demonstrated after peroral vaccination against *S. typhi* (8). In addition, Kantele and colleagues (9) were able to show such an ASC response in patients suffering from diarrhea and infected with *Campylobacter jejuni*, *Salmonella fyris*, *Salmonella enteritidis*, and *Salmonella typhi*. Experimental challenge with *S. sonnei* induced a vigorous ASC response in volunteers who developed disease, and this response was found to be significantly greater than that generated by the oral bivalent *S. typhi-S. sonnei* vaccine (17).

In the present study, we evaluated the extent of appearance of antigen-specific IgA ASCs compared with that of other serological markers following natural infection with *S. sonnei* during a common-source outbreak caused by this organism. The ELISPOT assay that was used to detect the ASC response showed a high level of sensitivity and specificity. The specificity of the assay was documented by the data showing a very low ASC response against *S. flexneri* serotype 2a and *S. flexneri* serotype 6 LPS in patients with *S. sonnei* shigellosis (inter-*Shigella* group specificity) and a similarly low ASC response against *S. sonnei* LPS in patients suffering from non-*Shigella* diarrhea. The level of cells secreting antibody against heterologous antigen in patients with *S. sonnei* shigellosis was, however, slightly higher than the anti-*S. sonnei* LPS ASC level detected in the unexposed control population. This may be explained by a vigorous polyclonal activation which occurred during the primary infection.

Previous data showing that shigellosis confers protection of unknown duration against disease caused by homologous *Shigella* organisms (5, 7) and the assumption that an efficient *Shigella* vaccine must induce an immune response comparable to that induced by natural infection indicate that measurement of the ASC response should be a part of the evaluation of candidate *Shigella* vaccines.

Kantele et al. (8) were impressed by the validity (sensitivity and specificity) and the magnitude of the ASC response following infections caused by several enteropathogens such as *C. jejuni*, *S. fyris*, *S. enteritidis*, and *S. typhi* and indicated the potential use of this assay as a diagnostic tool. This idea was confirmed and broadened by our data showing that the ASC response to *S. sonnei* LPS is highly sensitive and specific and is easily detected within 5 days after the onset of disease, which is much earlier than antibodies in serum are detected. From this point of view, further investigations should focus on a systematic study of the sensitivity and specificity of the assay in patients who are closer to the acute stage of the disease. A simplification of the original procedure of the ASC assay will enable investigators to take advantage of this test's applications in large-scale studies.

REFERENCES

1. **Adamus, G., M. Mulczyk, D. Witkowska, and E. Romanowska.** 1980. Protection against keratoconjuctivitis shigellosa induced by immunization with outer membrane proteins of *Shigella* spp. Infect. Immun. **30:**321–324.
2. **Branham, S.** 1948. Studies with *Shigella dysenteriae* (Shiga). II. The effect of serums on experimental infection and intoxication in mice. J. Infect. Dis. **83:**66–77.
3. **Cohen, D., M. S. Green, C. Block, T. Rouach, and I. Ofek.** 1988. Serum antibodies to lipopolysaccharide and natural immunity to

shigellosis in an Israeli military population. J. Infect. Dis. 157:1068–1071.

4. Cohen, D. I., M. Green, C. Block, 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.

5. DuPont, H. L., R. B. Hornick, M. J. Snyder, J. P. Libonati, S. B. Formal, and E. J. Gangarosa. 1972. Immunity in shigellosis. 2. Protection induced by oral live vaccine or primary infection. J. Infect. Dis. 125:12–16.

6. Forrest, B. D. 1988. Identification of an intestinal immune response using peripheral blood lymphocytes. Lancet i:81–83.

7. 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.

8. Kantele, A. M., H. Arvilommi, and I. Jokinen. 1986. Specific immunoglobulin-secreting human blood cells after peroral vaccination against *Salmonella typhi*. J. Infect. Dis. 153:1126–1131.

9. Kantele, A. M., R. Takanen, and H. Arvilommi. 1988. Immune response to acute diarrhea seen as circulating antibody-secreting cells. J. Infect. Dis. 158:1011–1016.

10. Keren, D. F., H. H. Collins, L. S. Baron, D. J. Kopecko, and S. B. Formal. 1982. Intestinal immunoglobulin A responses in rabbits to a *Salmonella typhi* strain harboring a *Shigella sonnei* plasmid. Infect. Immun. 37:387–389.

11. Keren, D. F., S. E. Kern, D. H. Baver, P. J. Scott, and P. Porter. 1982. Direct demonstration in intestinal secretions of an IgA memory response to orally administered *Shigella flexneri* antigens. J. Immunol. 128:475–479.

12. Lowell, G. H., R. P. MacDermott, P. L. Summers, R. A. Reeder, M. J. Bertovich, and S. B. Formal. 1980. Antibody-dependent cell-mediated antibacterial activity: K lymphocytes, monocytes, and granulocytes are effective against *Shigella*. J. Immunol. 125:2778–2784.

13. Lycke, N. I., and A. Svennerholm. 1990. Presentation of immunogens at the gut and other mucosal surfaces, p. 207–227. *In* G. C. Woodrow and M. M. Levine (ed.), New generation vaccines. Marcel Dekker, Inc., New York.

14. Niesel, D. W., C. B. Hess, Y. J. Cho, K. D. Klimpel, and G. R. Klimpel. 1986. Natural and recombinant interferons inhibit epithelial cell invasion by *Shigella* spp. Infect. Immun. 52:828–833.

15. Rimm, A. A., A. J. Hartz, J. H. Kalbfleisch, A. J. Anderson, and R. G. Hoffmann (ed.). 1980. Basic biostatistics in medicine and epidemiology, p. 225–227. Appleton-Century-Crofts, New York.

16. Sedgwick, J. D., and P. G. Holt. 1983. A solid-phase immunoenzymatic technique for enumeration of specific secreting cells. J. Immunol. Methods 57:301–309.

17. Van De Verg, L., D. A. Herrington, J. R. Murphy, S. S. Wasserman, S. B. Formal, and M. M. Levine. 1990. Specific immunoglobulin A-secreting cells in peripheral blood of humans following oral immunization with a bivalent *Salmonella typhi-Shigella sonnei* vaccine or infection by pathogenic *S. sonnei*. Infect. Immun. 58:2002–2004.

18. Westphal, O., and K. Jann. 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further application of the procedures. Methods Carbohydr. Chem. 5:83–91.

19. Zwillich, S. H., A. A. Duby, and P. E. Lipsky. 1989. T-lymphocyte clones responsive to *Shigella flexneri*. J. Clin. Microbiol. 27:417–421.