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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*

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The ability of bivalent *Salmonella typhi-Shigella sonnei* vaccine strain 5076-1C to stimulate an intestinal immunoglobulin A response in humans was evaluated by detecting gut-derived, trafficking antibody-secreting cells (ASC) in peripheral blood. Following vaccination, an immunoglobulin A-ASC response to O antigens of *S. typhi* and *S. sonnei* was observed in 10 of 13 and 13 of 13 vaccine recipients, respectively. Experimental challenge with pathogenic *S. sonnei* stimulated an ASC response to the *S. sonnei* O antigen in all subjects who developed clinical illness. The magnitude of the ASC response to challenge was significantly greater than that resulting from vaccination. Furthermore, compared with the response of the unimmunized controls, individuals previously immunized with 5076-1C demonstrated a significantly greater ASC response following challenge with *S. sonnei.*

Shigellae are enteric pathogens that colonize and invade the human colonic mucosa, which typically results in febrile illness with dysentery. Because infections due to shigellae pose a serious health threat worldwide, priority has been placed on development of a safe oral vaccine which would elicit an intestinal secretory immunoglobulin A (IgA) response to critical *Shigella* antigens such as lipopolysaccharide (LPS) (4, 17, 20, 25).

Heretofore, for practical reasons, demonstration of an immune response to oral vaccines relied on measurement of serum antibodies (3, 16), which did not necessarily reflect mucosal responses (6, 12, 15, 18, 19, 21). Direct measurement of intestinal secretory IgA antibodies (11, 15, 19, 23) has usually required the collection of intestinal fluids, which is cumbersome. Recently, several investigators reported the transient appearance of antigen-specific antibody-secreting cells (ASC), predominantly of the IgA isotype, in the peripheral blood subsequent to oral vaccination (6, 11, 13) or enteric infection (14). In this context, circulating ASC are considered to be B lymphocytes that originated in the gut-associated lymphoid tissue and entered the blood, following sensitization to antigen, en route to various sites in the common mucosal immune system. By detecting ASC in the circulation following oral immunization, it is possible to quantitate the degree of stimulation of the intestinal mucosal immune system.

In this study we measured the O-antigen-specific IgA-ASC response of volunteers as part of an evaluation of the immunogenicity and efficacy of candidate bivalent vaccine strain *Salmonella typhi-Shigella sonnei* 5076-1C, a derivative of attenuated *S. typhi* Ty21a, which contains the 120-megadalton plasmid of *S. sonnei* and expresses the LPS of both *S. typhi* and *S. sonnei* (10).

Sixteen volunteers took three oral doses of 10^9 viable 5076-1C (lot 87-5-I) vaccine organisms on days 0, 3, and 7 as previously described (2; D. Herrington, Vaccine, in press). Approximately 1 month after vaccination, vaccinees and unimmunized controls were challenged with 5×10^2 CFU of pathogenic *S. sonnei* 53G (2). Blood for serology or ASC studies was drawn prior to and at intervals following the initiation of vaccination or challenge.

LPS O antigens used in the immunological assays were those of *S. typhi* (Difco Laboratories, Detroit, Mich.) and *Plesiomonas shigelloides* (biochemically and immunologically identical to those of *S. sonnei* [1]; kindly provided by A. Lindberg, Huddinge, Sweden). *Vibrio cholerae* O1 Inaba LPS (Sigma Chemical Co., St. Louis, Mo.) was used as a negative control antigen.

Serum IgA response to O antigens was determined by enzyme-linked immunosorbent assay (2). Fourfold or greater rises in titer were considered significant (2, 15, 18, 19, 21).

IgA-ASC were enumerated by using a modification of the enzyme-linked immunospot assay (5, 13, 24); IgG- and IgM-ASC responses were not measured because of limited cell numbers. Peripheral blood mononuclear cells were separated from heparinized blood by a Ficoll (Organon Teknika, Durham, N.C.) gradient. To ensure uniform assay conditions, cells were frozen and stored in vapor-phase nitrogen so that consecutive samples from individual donors could be assayed together. It is possible that freezing had deleterious effects on cell function, but such effects would have been uniform for all samples. Coating, blocking, and washing of microdilution plates (Immunoplate I with flat-bottom wells; Nunc, Roskilde, Denmark) were performed the same way as for the enzyme-linked immunosorbent assay. Wells were coated with 1 μg of LPS or with buffer only. Frozen cells were thawed rapidly in a room temperature water bath and washed twice in RPMI 1640 (Whittaker Bioproducts, Walkersville, Md.). Cell viability (mean, 96.4%; standard deviation, 3.4%; range, 83.3 to 100%) was determined by trypan blue exclusion; viable cells were suspended at 2.5×10^6/ml in RPMI 1640 medium with 10% fetal calf serum, 2 mmol of L-glutamine per liter, and 15 μg of gentamicin per ml and dispensed in 100-μl portions into

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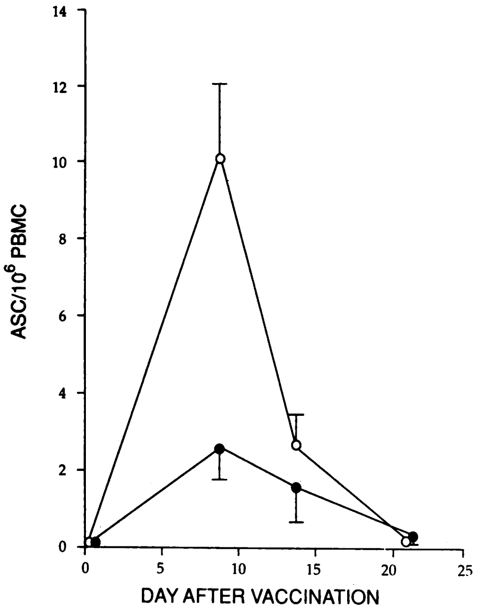

FIG. 1. Course of arithmetic mean ASC responses of volunteers to the *S. sonnei* (○) and *S. typhi* (●) O antigens following vaccination with 5076-1C (n = 13; vertical bars represent 1 standard error). PBMC, Peripheral blood mononuclear cells.

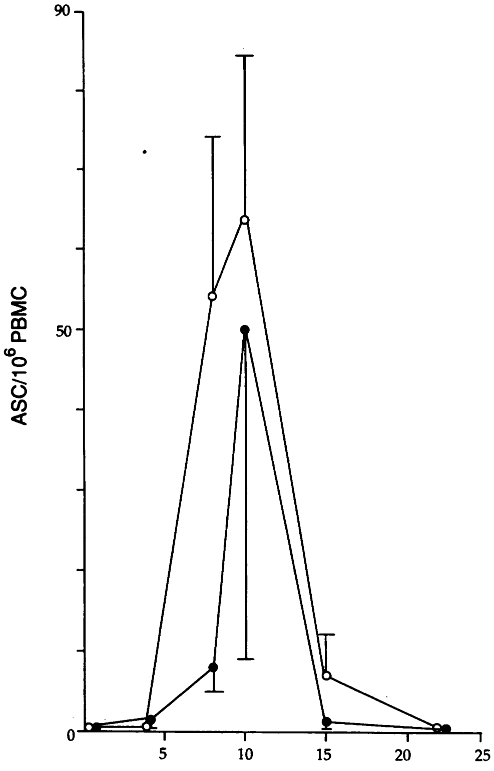

FIG. 2. Comparison of the course of arithmetic mean ASC response to *S. sonnei* O antigen following challenge with *S. sonnei* 53G in vaccinees (○) and controls (●) (n = 13 and 12, respectively; vertical bars represent 1 standard error). PBMC, Peripheral blood mononuclear cells.

four replicate wells of each of the four coating conditions (16 wells in all). Following a 3-h incubation at 37°C in a humidified chamber, wells were washed, and 100 μl of a 1:400 dilution of goat anti-human IgA–alkaline phosphatase conjugate (Kirkegaard-Perry Laboratories, Gaithersburg, Md.) was added to each. Wells were washed again after a 2-h incubation at 37°C, and to each well was added 100 μl of a molten agarose-substrate overlay (0.7% type I: low-EEO agarose [Sigma] with 0.05 mg of 5-bromo-4-chloro-3-indolyl phosphate and 0.1 mg of *p*-Nitro Blue Tetrazolium per ml; U.S. Biochemicals, Cleveland, Ohio). Following a 2-h incubation at room temperature or incubation overnight at 4°C, the presence of O-antigen-specific IgA secreted by individual lymphocytes was visualized as dark-blue spots. Spots were counted by inverting the plates on the stage of a stereomicroscope and were recorded as ASC per 10^6 peripheral blood mononuclear cells.

Blood from 13 of 16 vaccinees was available for immunological studies. Only 8 of 13 and 4 of 13 vaccinees demonstrated a significant rise in serum IgA titer to *S. sonnei* and *S. typhi* O antigens, respectively. In contrast, all 13 vaccinees developed an IgA response to *S. sonnei* O antigen if response was defined as transiently circulating IgA-ASC (compared with serum IgA response, $P = 0.039$ by the Fisher exact test with two-tailed hypothesis), and 10 of 13 developed an ASC response to *S. typhi* LPS as well (compared with serum IgA response, $P = 0.047$). Peak ASC responses occurred on day 9 for most individuals (Fig. 1); higher responses to either antigen were found on day 14 in only one or two instances. The magnitude of ASC response to the *S. sonnei* antigen was independent of ASC response to the *S. typhi* antigen ($r = 0.28$ and $P = 0.356$ by Pearson correlation coefficients). Wells coated with *V. cholerae* LPS or with buffer were consistently negative for ASC.

Experimental challenge with wild-type *S. sonnei* 53G resulted in clinical shigellosis in 5 of 13 vaccinees and 5 of 12 controls (Herrington, in press). Of 13 vaccinees (11 of whom were studied in the preceding vaccination experiments) 10 had detectable ASC responses to the *S. sonnei* O antigen, and 6 of 12 controls showed such a response (Fig. 2). No responses to *S. typhi* or *V. cholerae* LPS were found, demonstrating the specificity of the response. In this small challenge study there was a 100% correlation between the occurrence of clinical shigellosis and development of an ASC response, while serum IgA responses were seen in four of five and three of five ill vaccinees and controls, respectively, and in two of eight and three of seven nonill vaccinees and controls, respectively. Most ASC responses peaked on day 8 or 10 for both groups (one control had a peak value on day 4). Vaccination had a demonstrable priming effect. On day 8 after challenge, the mean vaccinee response increased rapidly; it remained high on day 10 in comparison with the smaller, more transient mean ASC response in controls ($P <$ 0.05 for day 8 or 10 by the Wilcoxon rank sums test with one-tailed hypothesis).

In this study, we showed that Ty21a 5076-1C was an effective vehicle for stimulating an intestinal IgA response to *S. sonnei* O antigen. We also showed that the ASC procedure was a sensitive, specific, and relatively simple method for detecting this response. The lower rate of response to *S. typhi* LPS was not unexpected, since the vaccine strain was cultured under conditions that maximize expression of *S. sonnei* LPS. While parallel measurements of specific IgA in intestinal fluids were not done, evidence from other studies indicates that, following vaccination by the intestinal route and generation of circulating ASC, specific, active IgA-ASC, as well as secretory IgA, can be found in various mucosal sites (6, 7, 11, 22).

The relationship between formation of an IgA-ASC response to *S. sonnei* O antigen and protection against *S. sonnei* disease is unknown. The level of ASC response generated by the vaccine in our study on the whole did not protect vaccinees against challenge, nor was there any correlation between protection and the peak values of individual responses. However, we did note that challenged controls had significantly higher peak ASC values as a result of illness than vaccinees had as a result of vaccination ($P < 0.025$; Wilcoxon rank sums test with two-tailed hypothesis). Dupont et al. (8) showed that the magnitude of the serum antibody response to O antigen following challenge with *Shigella flexneri* correlated with the severity of the clinical illness. The apparently stronger response to illness may be due to the generation of cytokines and T helper cells. The observation that prior illness due to *Shigella* infection can protect against homologous rechallenge (9; Herrington, in press) suggests that the magnitude of the IgA-ASC response may indeed be related to protection and that ASC measurements should be included in future *Shigella* vaccine evaluations.

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