INFECTION AND IMMUNITY, Dec. 1986, p. 920–923
0019-9567/86/120920-04$02.00/0

Vol. 54, No. 3

Secretory Immunoglobulin A Response following Peroral Priming and Challenge with *Shigella flexneri* Lacking the 140-Megadalton Virulence Plasmid

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Received 22 June 1986/Accepted 22 August 1986

This study evaluates the ability of noninvasive *Shigella* spp., lacking the 140-megadalton virulence plasmid, to elicit a mucosal immunoglobulin A immune response in the intestine. For these studies, we used *Shigella flexneri* M4243A1 (which lacks the plasmid and is Sereny test negative) to prime and challenge three groups of rabbits perorally. Both primary and immunoglobulin A memory responses were detectable in these secretions. These findings indicate that a mucosal memory response can be primed by nonpathogenic strains of *Shigella* which lack the virulence plasmid.

Several in vivo and in vitro model systems have been used successfully in the examination of events involved in initiating the local humoral and cellular immune response of the gastrointestinal tract to various microorganisms and their toxic products (2, 5, 8, 12, 15, 16, 21). Our previous work, with a chronically isolated ileal loop system, has documented several features of the local immune response relative to potential *Shigella* vaccine preparations. First, an intestinal secretory immunoglobulin A (IgA) response to *Shigella flexneri* is best elicited by peroral rather than parenteral immunization (10). Second, a local secretory IgA memory response was elicited by peroral immunization with live but not killed *S. flexneri* cells (8, 9). Third, although the *S. flexneri* cells must be alive, relatively nonpathogenic strains such as *Shigella* strain X16 (hybrid of *Escherichia coli* and *S. flexneri*) and *S. flexneri* 2457-0 could elicit local secretory IgA memory responses in intestinal secretions (8, 9). Both of these strains do, however, contain the 140-megadalton virulence plasmid which has been shown to be associated with invasive enteric pathogens (20–22). Recently, the antigens of virulence plasmid-cured strains of enteropathogens have been characterized (4).

Since virulence plasmid-cured strains of enteropathogens have been suggested as candidates for live oral vaccines (14, 21), a key question concerns the role of virlence in the establishment of the mucosal immune responses to these bacteria. In the present study, we examined the primary and secondary mucosal immune responses in intestinal secretions elicited by oral challenge with *S. flexneri* M4243A1. This strain lacks the 140-megadalton virulence plasmid and is negative for invasive properties by the Sereny test (22).

Intestinal secretions were collected daily from chronically isolated rabbit ileal (Thiry-Vella) loops prepared as described previously (7). The intestinal secretions and weekly samples of serum were assayed for their IgA and IgG activity to *S. flexneri* by an enzyme-linked immunosorbent assay (6). The data are presented as geometric means, since others have noted that this better reflects the logarithmic kinetics of the local immune response after immunization (17). The means were calculated by using the $\log_{10}$ of each value for each rabbit to determine the mean, standard deviation, and standard error of the mean. For each daily result, the $\log_{10}$ standard error of the mean was added and subtracted from the mean $\log_{10}$ of the specific immunoglobulin activity to determine the variance; antilogs of these three values were then obtained to give the geometric mean with an upper and lower limit of variance about that mean. Significance was calculated by Student's *t* test.

Three antigen preparations were used in these studies: (i) live *Shigella* strain X16, which invades the intestinal mucosa but does not persist; (ii) *S. flexneri* M4243, which invades the surface epithelium with resultant inflammation and ulceration; and (iii) *S. flexneri* M4243A1, which has been cured of the virulence plasmid and is therefore unable to invade. All strains were tested for invasion by the Sereny test.

Group 1 animals (Table 1) each received a single peroral dose of live, locally invasive *Shigella* strain X16, and yet they had a significantly ($P < 0.01$) weaker local IgA anti-*Shigella* lipopolysaccharide (LPS) response than did the group 2 animals, which received the noninvasive *S. flexneri* 4243A1 strain (Fig. 1). The latter strain proved to be highly immunogenic, despite the lack of the 140-megadalton virulence plasmid and a negative Sereny test. The primary IgA anti-*Shigella* LPS activity reached its peak by day 6 to 8 and was quite consistently present in the secretions from these rabbits. Secretions collected on day 10 and beyond were more variable with regard to the degree of IgA anti-*Shigella* LPS activity, as reflected by the indicated variance. The

TABLE 1. Immunization schedule

Group	Antigen[a]	Day given[b]
1	Live *Shigella* strain X16	0
2	Live *S. flexneri* M4243A1	0
3	Live *S. flexneri* M4243A1	0, 7, 14
4	Live *S. flexneri* M4243A1	−74, −67, −60, 0

[a] Peroral immunization was given via orogastric tube while the animal was lightly anesthetized.
[b] Day −1 is the day of surgical creation of the isolated ileal loop.

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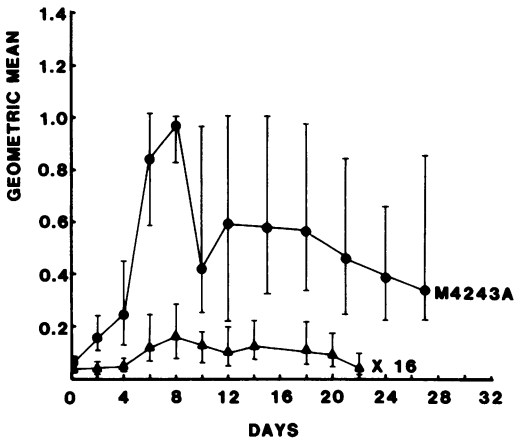

FIG. 1. Comparison of IgA anti-*Shigella* LPS in secretions from animals given a single peroral dose of live *Shigella* strain X16 (triangles) or strain M4243A1 (circles) on day 0. The isolated ileal loops were created on day −1.

local IgG response was trivial, and systemic IgG and IgA against *Shigella* spp. were lacking in both group 1 and 2 animals.

Group 3 animals received three weekly peroral doses of live *S. flexneri* M4243A1 beginning on the day following surgery. The response during week 1 paralleled that of the group 2 animals (Fig. 2). However, following the second and third peroral doses of live *S. flexneri* M4243A1, the IgA anti-*Shigella* LPS activity increased to a peak level on day 20. By the end of the experiment on day 30, the intestinal secretions of the rabbits in group 3 still had strong IgA anti-*Shigella* LPS activity, with a geometric mean more than twice that for the group 1 animals.

For the memory response studies, group 4 rabbits were primed with three peroral doses of live *S. flexneri* M4243A1. The animals were allowed to rest for 2 months following the third peroral dose, and isolated ileal loops were then created. On the day following surgical creation of the loops, a single peroral challenge dose of live *S. flexneri* was given to each rabbit. An impressive secretory IgA memory response was found for the animals given this regimen (Fig. 3). Residual secretory IgA activity to *Shigella* LPS remained even after 60 days following the last peroral dose with this antigen. Further, following the peroral challenge dose on day 0, a striking increase in IgA anti-*Shigella* LPS activity was detected. These responses were significantly greater ($P < 0.01$) than those elicited by a single dose of the invasive *Shigella* strain X16 on all days tested and was greater than the primary response to strain M4243A1 on all days except 2 and 6 through 10.

The role of antigen form in the process by which perorally administered antigens are taken up and prepared by the gut-associated lymphoid tissues is only beginning to be understood. For instance, several studies have documented that cholera toxin, which binds to the GM-1 receptor on epithelial cells and lymphocytes, is an extremely potent mucosal immunogen, while molecules which lack this characteristic are less effective in eliciting a mucosal immune response (16, 17). Peroral administration of live bacteria is much more effective for stimulating a mucosal memory response than are killed preparations of the same bacteria (8). The latter effect is not simply a matter of dose (i.e., replication of live bacteria, thereby increasing the actual dose given (9). It is not clear whether the variable responses seen are due to differences in initial processing of the antigen by the gut-associated lymphoid tissues (15, 19) or to differences in the available repertoire of B-cell (1, 13) and T-cell (11, 23) responses to those forms of the antigen.

In the present study, we examined the role of invasiveness and of the 140-megadalton virulence plasmid in establishing a mucosal immune response to *S. flexneri* antigens. Although previous studies in our laboratory showed that the relatively nonpathogenic *S. flexneri* 2457-0 was able to elicit both vigorous primary and mucosal memory responses (8), it possesses a 140-megadalton virulence plasmid (4). Further, in clinical trials, it has reverted to a pathogenic form and is therefore unsuitable as a mucosal vaccine (3). These findings raised the question of whether expression of the virulence plasmid by a few of the bacteria would account for the mucosal memory results obtained in our previous studies (9).

Results of our Sereny tests on *S. flexneri* M4243A1 were consistent with those of previous studies indicating that this bacterium is unable to invade the epithelium (20, 22). It was therefore an ideal candidate to test for the role of invasion and of the 140-megadalton virulence plasmid in eliciting mucosal immune responses. However, the secretory IgA responses elicited after a single peroral dose were significantly stronger than those of rabbits immunized perorally with the live, locally invasive *Shigella* strain X16. This was somewhat surprising, because it has been assumed that strains with invasive capabilities would be more effective immunogens. These findings may be related to the fact that *Shigella* strain X16 is a hybrid of *E. coli* and *S. flexneri* and, as such, may have relatively less *Shigella* LPS than *S. flexneri* M4243A1 has. When three weekly peroral doses of *S. flexneri* M4243A1 were given, the response continued to

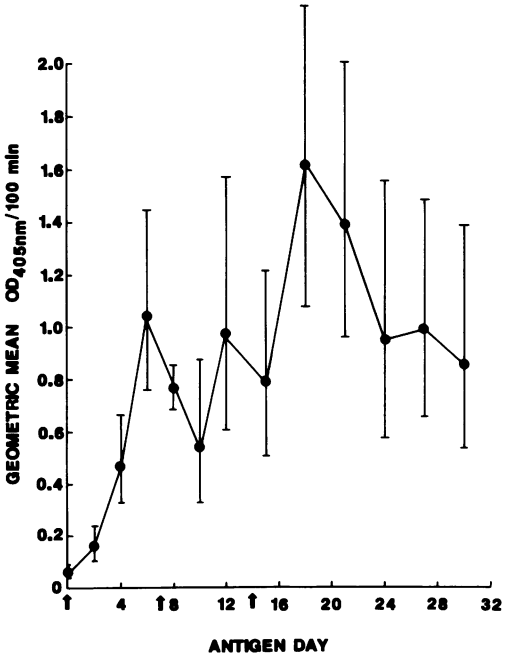

FIG. 2. IgA anti-*Shigella* LPS response in secretions from rabbits given live *S. flexneri* M4243A1 on days 0, 7, and 14. The isolated ileal loops were created on day −1.

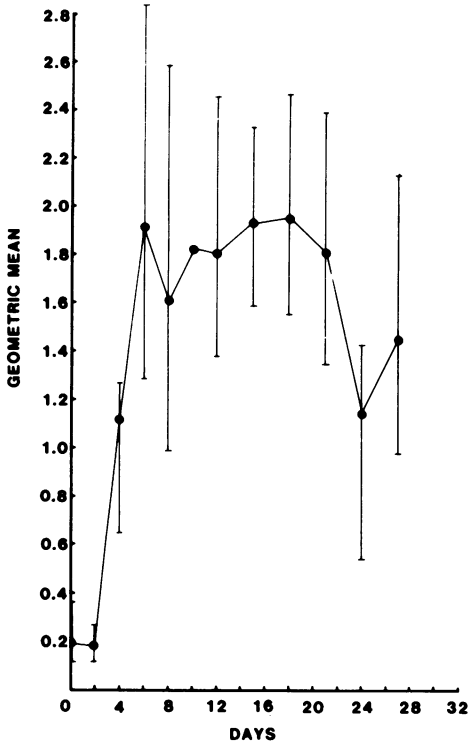

FIG. 3. IgA anti-*Shigella* LPS response in secretions from rabbits primed with three peroral doses of live, noninvasive *Shigella* strains, allowed to rest for 60 days, and challenged on day 0 with a single peroral dose of live *S. flexneri* M4243A1. The isolated ileal loops were created on day −1.

increase to a maximum at day 20 (6 days after the third peroral dose). Further, our results illustrate that this was an effective priming for the mucosal memory response. This is similar to the incremental responses to multiple peroral doses seen previously (8, 9).

These findings document two relevant pieces of information. First, this live, noninvasive *Shigella* strain can prime the mucosal immune system for a memory response. Second, the challenge dose need not be given with a strain with invasive capabilities to elicit the secretory IgA response in primed animals. The most likely explanation for the successful secretory IgA activity to *Shigella* LPS is that initial antigen processing of *Shigella* strains does not depend upon their ability to invade the surface epithelium. Experimental evidence to date indicates that live *Shigella* strains are needed to stimulate a vigorous local immune response to *Shigella* antigens and to prime this system for a mucosal memory response. However, the live *Shigella* strains need not be invasive to elicit a secretory IgA memory response.

This work was supported in part by U.S. Army Medical Research and Development Command contract no. DAMD 17-85-C-5006.

We thank Robin Kunkel, Philip Lewandowsky, and Joseph Wassef for their help with the figures and statistics.

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