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Combined Parenteral and Oral Immunization Results in an Enhanced Mucosal Immunoglobulin A Response to *Shigella flexneri*

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Achieving a vigorous secretory immunoglobulin A (IgA) response in intestinal secretions usually requires multiple doses of antigen given orally, while systemic immunity is more easily attained by parenteral immunization. This study examines the role of combined parenteral and oral immunizations to enhance the early mucosal immune response to an enteropathogen. We have used a chronically isolated intestinal-loop model in rabbits as a probe to monitor kinetically the initial (primary) local immune response to shigella lipopolysaccharide (LPS) following combinations of parenteral immunization intramuscularly (i.m.) and oral stimulation with shigellae. Predictably, effective stimulation of systemic immunity was elicited when heat-killed preparations of *Shigella* sp. strain X16 were given i.m., as shown by strong serum IgG and weak intestinal IgA activity to shigella LPS. A single oral dose of live *Shigella* sp. strain X16 given to unprimed rabbits elicited only a typical weak IgA response in intestinal secretions. However, when an i.m. dose of heat-killed shigellae was followed 1 day later by an oral dose of live *Shigella* sp. strain X16, a hyperstimulation of the early secretory IgA response was elicited, and the response reached levels found previously only after multiple oral administrations of live shigellae. This stimulation did not require the use of an adjuvant. At the same time, the animals receiving this combined oral and i.m. regimen had a lower IgG antishigella LPS activity in serum compared with their response after receiving parenteral antigen in adjuvant alone. These findings indicate that while a dichotomy exists between the systemic and mucosal immune responses, careful orchestration of the stimulatory events can promote a vigorous early local IgA response.

Since the mucosal immune system lies at the portal of entry for enteropathogens, many recent studies have concentrated on methods to prime the intestinal mucosa against these agents. Many approaches have been successful, but they usually require several weeks to achieve strong local immunoglobulin A (IgA) responses. Stimulation of the intestinal mucosal immune response to enteropathogens typically can be achieved by giving multiple oral doses of the antigen preparation or by the use of intraperitoneal priming with adjuvant. These strategies will evoke production of detectable levels of antigen-specific IgA in intestinal secretions or antigen-reactive cells in the lamina propria (3, 4, 8, 12, 30–32, 37, 40).

Recently, several groups using such immunization schedules have documented the existence of a secretory IgA mucosal memory response to enteropathogens and their toxic products in the gastrointestinal tract (1, 13–15, 19, 26). The mucosal memory responses are characterized by a more rapid and vigorous rise in the local IgA activity in response to oral challenge with antigen in primed animals than in unprimed animals. To achieve these high levels of IgA activity in secretions, multiple doses of antigen, often over a period of several weeks, are usually required. Further, in our work with *Shigella flexneri*, live antigen given orally was able to prime the animals for a mucosal memory response (13–15).

Attempts to enhance secretory IgA responses have included altering the form of the antigen, the route and schedule of administration, and the adjuvants used (15, 26). Some workers have employed combinations of parenteral injections of antigen and oral stimulation (6, 20, 29). Most often, priming of animals by parenteral immunization several days to weeks before mucosal stimulation has been ineffective in enhancing mucosal immunity (17, 40). A notable exception is the use of intraperitoneal priming, which probably causes some direct stimulation of mucosal immunity (26, 27). In the present studies, we demonstrate that giving the animals a parenteral priming with heat-killed shigellae 1 day before the oral dose of live shigellae results in an enhanced early IgA antishigella response measurable in intestinal secretions.

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MATERIALS AND METHODS

Preparation of chronically isolated ileal loops in rabbits. The surgical procedure for isolating ileal loops in rabbits has been detailed previously (11). Briefly, while anesthetized, 3-kg New Zealand White rabbits have a 20-cm segment of ileum containing a grossly identifiable Peyer's patch isolated with its vascular supply intact. Silastic tubing (Dow Corning Corp., Midland, Mich.) is sewn into each end of the isolated segment, and the free ends are tunnelled subcutaneously to the nape of the neck, where they are exteriorized and secured. Intestinal continuity is restored by an end-to-end anastomosis.

About 2 ml of secretions and mucus that collect in the ileal loops is expelled daily by injecting 20 ml of air into one of the silastic tubes. The slightly opaque, colorless fluid is stored at −20°C until time of assay.

Immunization. *Shigella* sp. strain X16, a hybrid of *Escherichia coli* and *S. flexneri* which is capable of mucosal invasion but does not persist in tissues and does not cause dysentery, was used for all studies (5). Heat-killed *Shigella* sp. strain X16 was prepared by boiling overnight broth cultures for 10 min. To ensure nonviability, sample cultures were streaked onto MacConkey agar and checked for overnight growth.

Rabbits were immunized by the schedule outlined in Table

TABLE 1. Immunization schedule

Group	n	Antigen[a]	Route	Day
1	7	Heat-killed	Peyer's patch[b]	0
2	10	Live	Oral[c]	0
3	10	Heat-killed	Intramuscular	−1
		Live	Oral[c]	0
4	11	Heat-killed, in CFA	Intramuscular[d]	−1
		Live	Oral[c]	0
5	11	Heat-killed, in CFA	Intramuscular[d]	−1

[a] All doses contained 10^{10} shigellae (*Shigella* sp. strain X16).
[b] Antigen injected into each of five Peyer's patches including the one in the isolated ileal loop.
[c] Given under mild anesthesia via nasogastric cannula.
[d] Given with CFA.

1. As in previous studies, the isolated ileal loops were created on the day prior to oral antigen administration (13–15). Although the isolated segments were not directly exposed to antigen, secretions from these loops accurately reflected the specific IgA content of the intestine due to lymphocyte recirculation (13–15). For oral immunization, the rabbits were lightly anesthetized, an orogastric tube was positioned, and the indicated dose of *Shigella* sp. strain X16 was administered. Parenteral immunization was performed by intramuscular injections at multiple sites over the hips with the heat-killed shigellae, with or without complete Freund adjuvant (CFA) as indicated.

For immunization directly into Peyer's patches, animals were anesthetized, and, under aseptic conditions, a midline abdominal incision was made; the small intestine was gently pulled out, and Peyer's patches were identified from the serosal surface. A 25-gauge needle was used to inject the heat-killed bacteria just beneath the serosal surface covering the Peyer's patches.

Enzyme-linked immunosorbent assay. A previously described enzyme-linked immunosorbent assay for detecting rabbit IgG and IgA antibodies to bacterial products was used to detect specific antibody activity in intestinal-loop secretions and serum (10). Briefly, polystyrene microdilution wells were coated with 0.1 ml of a solution containing 10 μg of *Shigella* sp. strain X16 lipopolysaccharide (LPS) Westphal preparation per ml as previously characterized (10). Immediately before the serum or intestinal secretions were tested, the LPS solution was removed and the wells were washed with phosphate-buffered saline (pH 7.2) containing 0.1% Tween 20. The sample to be assayed was diluted 1/20 in this buffer and incubated in both LPS-coated wells and uncoated wells (to control for nonspecific adsorption) for 4 h. After the wells were washed with the buffer, solutions containing either alkaline phosphatase-conjugated goat anti-rabbit IgA or alkaline phosphatase-conjugated goat anti-rabbit IgG (affinity column purified and shown to be monospecific by enzyme-linked immunosorbent assay [10]) were added to the wells and left overnight at room temperature. After an additional wash with buffer, the substrate reaction was carried out with nitrophenyl phosphate in carbonate buffer (1 mg/ml). The kinetics of the enzyme-substrate reaction were extrapolated to 100 min. The optical density at 405 nm of uncoated wells measured on a Titertek Multiscan MicroELISA Reader (Flow Laboratories, Inc., McLean, Va.) was subtracted from the optical density at 405 nm of the coated wells. Standard solutions of IgG and IgA anti-*Shigella* sp. strain X16 LPS were prepared as described previously (10) and processed daily with the unknown samples. To minimize day-to-day variation, the results of the standards were normalized and the values of the unknown specimens were corrected to these normalized standards (10). By comparing results from quantitative precipitation assays, this assay system detected 1.3 ng of specific antibody per ml and had coefficients of variation of 3.6 and 9% for IgG antishigella LPS and IgA antishigella LPS, respectively (10).

The data are presented as geometric means, since other workers have noted that such a presentation better reflects the logarithmic kinetics of the local immune response after immunization (28). The kinetics 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 day, the $\log_{10}$ of the standard error of the mean was added and subtracted from the mean log of specific immunoglobulin activity; antilogs of these three values were then obtained to give the geometric mean and upper and lower limits of variance about that mean. Data were statistically analyzed with the RS1 interactive data analysis system. Differences between groups on specific days were tested for significance by the Student *t* test.

RESULTS

Immunogenicity of heat-killed shigellae. Previous studies with this model system have demonstrated that oral administration of live shigellae is effective in stimulating both a primary and a memory mucosal response; however, heat-killed shigellae have been totally ineffective in stimulating a memory mucosal response (13–15). To determine whether the heat-treated preparations can be effective stimulants for a mucosal immune response, a single dose of 10^{10} heat-killed cells of *Shigella* sp. strain X16 was injected directly into each of five Peyer's patches (0.2 ml per Peyer's patch) at the time of the surgical procedure to create a chronically isolated ileal loop in the group 1 animals. The Peyer's patch in the isolated loop was one of those injected in each case. By day 4 after surgery, all seven of the rabbits so treated developed significant increases ($P < 0.01$) in the IgA antishigella LPS activity in their loop secretions over day 0 values (Fig. 1). Weak IgG activity in response to shigella LPS was detected in only a few secretions (Fig. 1).

The specific antibody activities in the serum were the opposite of those in the intestinal secretions. The IgG

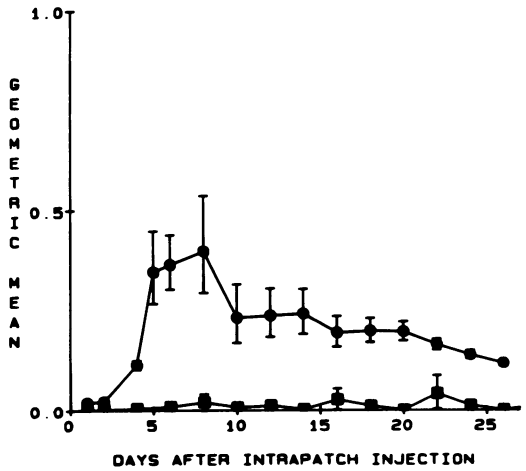

FIG. 1. Geometric mean IgA antishigella LPS (●) and IgG antishigella LPS (■) responses in isolated ileal-loop secretions from rabbits given a single injection of heat-killed shigellae into their Peyer's patches on day 0. Standard errors of the means are indicated.

TABLE 2. Serum IgG and IgA activity to shigella LPS from rabbits given antigen directly into Peyer's patches

Days postimmunization[a]	Anti-LPS activity[b]	
	IgA	IgG
6–7	0.263 (0.228–0.305)	0.479 (0.363–0.630)
8–14	0.268 (0.239–0.299)	1.038 (0.922–1.165)
15–21	0.206 (0.199–0.213)	0.927 (0.814–1.058)
22–28	0.192 (0.184–0.200)	0.824 (0.570–1.193)

[a] Data not available for preimmunization. For comparison, Tables 3 and 4 show values of 0.018 and 0.017 for IgA and 0.015 and 0.026 as geometric means of unimmunized rabbits.

[b] Data expressed as geometric means with variances as described in Materials and Methods.

antishigella LPS activity quickly increased to an overall geometric mean of 1.038, which did not significantly decline by the end of the study on day 28 (Table 2). The serum IgA activity in response to shigella LPS was weak throughout the study (Table 2). This indicates that the heat-killed shigella preparation is immunogenic for both the systemic IgG and local IgA response following immunization directly into Peyer's patches.

Immune responses following oral stimulation with live *Shigella* sp. strain X16. Group 2 rabbits received a single oral dose of 10^{10} live cells of *Shigella* sp. strain X16. This group of rabbits showed the kinetics typical of a primary local IgA response (Fig. 2) (13). The first significant increase in the IgA antishigella LPS activity over preimmunization values was found on day 6, with the response peaking on day 8. These findings are similar to those we have previously reported concerning the response following single immunization with live invasive or even noninvasive shigellae (26, 30). No IgG antishigella LPS activity was found in the intestinal secretions (Fig. 3). Further, no IgG or IgA activity in response to shigellae was detected in the serum.

Immune responses following combined parenteral immunization with heat-killed *Shigella* sp. strain X16 and oral immunization with live *Shigella* sp. strain X16. In group 3, the kinetics of the development of the local IgA response followed those of a primary mucosal immune response (Fig. 2). However, a significantly stronger local IgA response was found on day 10 in these rabbits compared with the response in animals receiving only a single oral dose of live shigellae (Fig. 2, group 2). The IgA antishigella activity reached by day 10 in rabbits given both parenteral and oral immunizations was also stronger than that seen in rabbits which had antigen injected directly into the Peyer's patches (group 1).

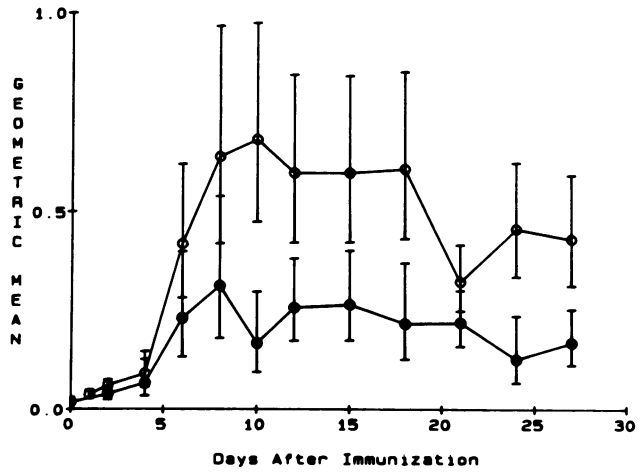

FIG. 2. Geometric mean IgA antishigella LPS responses in isolated ileal-loop secretions from rabbits given a single oral dose of live shigellae on day 0 (●) or a combined parenteral dose of antigen on day −1 and a single oral dose on day 0 (○). Standard errors of the means are indicated.

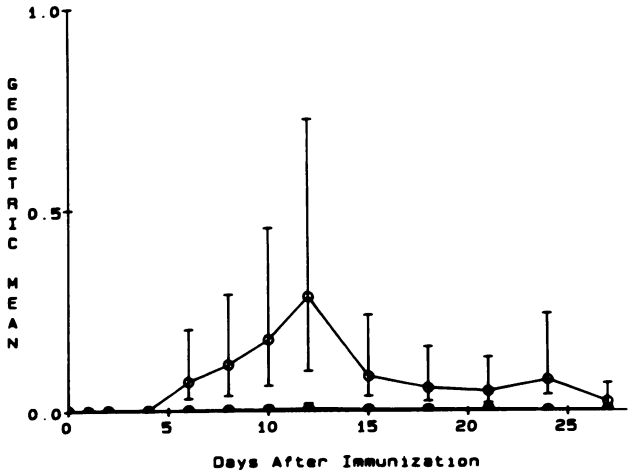

FIG. 3. Geometric mean IgG antishigella LPS responses in isolated ileal-loop secretions from the two groups of rabbits used for Fig. 2. Symbols are defined in the legend to Fig. 2. Standard errors of the means are indicated.

IgG antishigella activity was consistently found in intestinal secretions from these group 3 rabbits (Fig. 3). This response was more variable than the secretory IgA response, but the kinetics paralleled those of the primary secretory IgA response. By day 6 after oral immunization, the IgG antishigella in secretions had increased significantly over day 0 values, and the response peaked on day 12 (Fig. 3).

Immune responses following parenteral stimulation with heat-killed *Shigella* sp. strain X16 in CFA and oral immunization with live *Shigella* X16. Since CFA has been used to enhance and prolong both systemic and mucosal immune responses, the group 4 rabbits were used to determine whether CFA given with the heat-killed antigen would enhance or prolong the secretory IgA response to *Shigella* sp. strain X16 when the combined parenteral and oral immunization schedule was followed. As in the group 3 rabbits, a vigorous secretory IgA response was found in intestinal secretions and was significantly stronger by day 10 than that seen with the single oral dose of live shigellae (Fig. 4). There was, however, no enhancement or extension of the IgA activity compared with that of the group 3 animals. Animals which were given only parenteral heat-killed shigellae in CFA (group 5) gave a weak variable response which lagged behind the primary IgA response seen after a single oral dose of live shigellae (Fig. 4).

Weak IgG antishigella activity was found in secretions of both the group 4 and the group 5 rabbits (Fig. 5), despite the fact that these animals had high serum IgG activity in response to shigellae (Tables 3 and 4). Following a single parenteral immunization with the heat-killed shigellae in CFA, a predictable rise in the serum IgG antishigella LPS activity was seen in all animals within 1 week of immunization (Tables 3 and 4). This serum IgG activity attained maximum levels by week 3 after immunization, when it was significantly greater ($P < 0.01$) than the response by animals

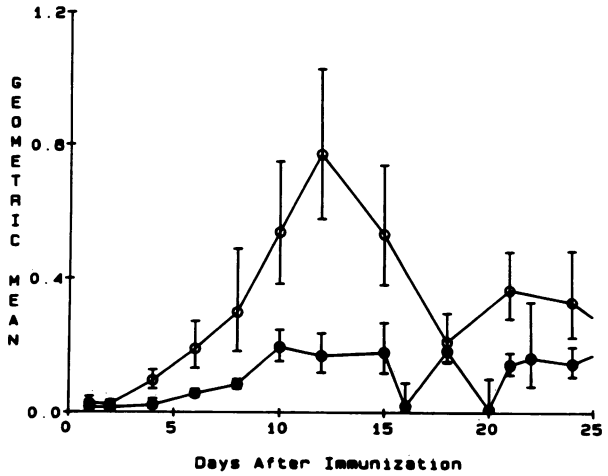

FIG. 4. Geometric mean IgA antishigella LPS responses in isolated ileal-loop secretions from rabbits given a single parenteral dose of killed shigellae in CFA (●) or a combined parenteral dose of antigen in CFA on day −1 and a single oral dose of live antigen on day 0 (○). Standard errors of the means are indicated.

given a direct injection into Peyer's patches (without adjuvant). The IgG antishigella activity in sera from animals receiving combined parenteral antigen in CFA and oral antigen (group 4) was 50% weaker than that of animals receiving parenteral antigen in CFA alone (group 5). No significant decline in this level was seen through the end of the study on day 42. Similarly, the serum IgA antishigella LPS activity quickly increased such that within 1 week all animals had a significant increase over preimmunization values (Tables 3 and 4). By two weeks, this IgA activity had attained its peak and did not decline significantly by the end of the study on day 42. Interestingly, the mean serum IgA activity in the group 5 animals was relatively low compared with that in the group 4 animals. However, these differences were not statistically significant.

DISCUSSION

The vigorous primary IgA response found in intestinal secretions in response to a combined parenteral stimulation with killed *Shigella* sp. strain X16 followed 1 day later by a single oral dose of live shigellae has not been seen previously in studies of mucosal immune responses. Earlier studies of the local IgA response to enteropathogens have shown that animals primed with three oral doses of live invasive or noninvasive shigellae will show a highly significant enhancement of their local IgA antishigella LPS response upon subsequent challenge with the same shigellae (13–15). In contrast, intragastrically administered heat-killed shigellae were totally ineffective in stimulating a mucosal memory response (15).

At least two explanations for the poor results with heat-killed shigellae are possible, and either would have important implications for vaccine preparations against enteropathogens. It is possible that particular epitopes in the killed-antigen preparations are altered in such a manner that they are no longer able to elicit a strong secretory IgA response. Alternatively, the heat-killed preparations may not be taken up effectively by the M cells which are known to exist over lymphoid follicles in the gut and which have been implicated in antigen uptake (21, 24, 34, 38). The former alternative has been examined in the present study, in which the need for M-cell processing was bypassed with the heat-killed preparations of shigellae injected directly into the Peyer's patches. Clearly, the IgA antishigella LPS activity that developed in secretions from all the rabbits in this group demonstrates that this antigen is appropriate to stimulate the mucosal immune system. Interestingly, this response occurred 2 days sooner than when the antigen was administered orally. It is most likely that the earlier inability to stimulate mucosal

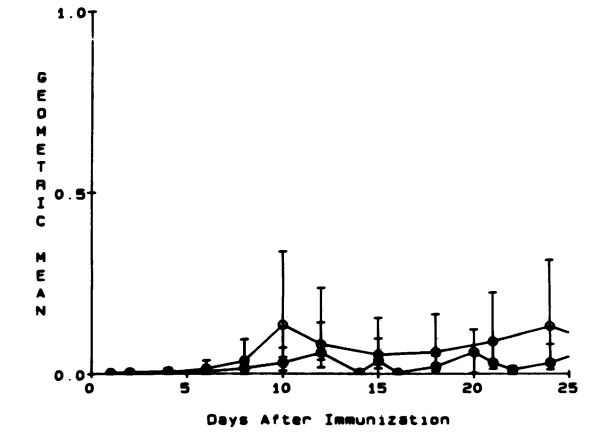

FIG. 5. Geometric mean IgG antishigella LPS responses in isolated ileal-loop secretions from the two groups of rabbits used for Fig. 4. Symbols are defined in the legend to Fig. 2. Standard errors of the means are indicated.

TABLE 3. Serum IgG and IgA antishigella LPS activity in rabbits immunized with antigen via both intramuscular with CFA and oral routes

Time[a] (days)	Activity[b]	
	IgA	IgG
Preimmunization	0.028 (0.018–0.045)	0.018 (0.010–0.033)
Postimmunization		
6–7	0.485 (0.453–0.518)	0.482 (0.445–0.522)
8–14	0.743 (0.669–0.826)	0.803 (0.762–0.846)
15–21	0.784 (0.660–0.932)	0.823 (0.817–0.933)
22–28	0.819 (0.721–0.932)	0.930 (0.862–1.003)
29–35	0.715 (0.583–0.878)	0.924 (0.831–1.027)
36–42	0.889 (0.794–0.996)	0.987 (0.930–1.029)

[a] Intramuscular immunization with heat-killed shigellae in CFA was at day −1. Oral dose of live shigellae was given on day 0.
[b] Results expressed as geometric means with the variances as described in Materials and Methods.

TABLE 4. Serum IgG and IgA activity to shigella LPS in rabbits given heat-killed shigellae in CFA intramuscularly

Time (days)	Activity[a]	
	IgA	IgG
Preimmunization	0.018 (0.011–0.028)	0.015 (0.007–0.032)
Postimmunization		
6–7	0.308 (0.180–0.525)	0.243 (0.120–0.493)
8–14	0.670 (0.494–0.908)	1.294 (1.050–1.596)
15–21	0.380 (0.494–0.908)	1.096 (0.889–1.352)
22–28	0.540 (0.450–0.647)	1.941 (1.607–2.344)
29–35	0.273 (0.166–0.448)	1.728 (1.380–2.168)
36–42	0.565 (0.469–0.681)	1.690 (1.285–2.223)

[a] Expressed as geometric means with the variances as described in Materials and Methods.

immunity with heat-killed-antigen preparations relates to their ineffective processing by the gut mucosa. Although this study provides only indirect evidence to support his contention, Owen et al. have recently reported that M cells were able to take up only viable *Vibrio cholerae* (25). Further, Wolf et al. have shown that adherence of reovirus to M cells is determined at least in part by proteins present on the surface of the virus (39). Such binding proteins may have been altered in our earlier studies by the heat treatment.

When these heat-killed preparations of shigellae in CFA were administered parenterally (groups 4 and 5), predictably strong systemic IgG and moderate systemic IgA antishigella LPS activities were found, while only weak IgG or IgA activity was detected in the loop secretions from these animals. The relatively small amount of IgG activity in intestinal secretions was not surprising. We have shown previously that IgG is not readily transported into the intestinal lumen even when there is a high titer of activity in serum (16). In the present studies, a weak but significant IgG antishigella activity was found in loop secretions from the group 3 animals. Here the combination of parenteral and oral antigen may have served to enhance the local production of IgG. The small but definite IgA antishigella LPS response in secretions of the group 5 animals demonstrates that parenteral immunization with CFA can prime the local immune response to these antigens. This finding has been demonstrated by others with a variety of antigens (6, 20, 29). This may have contributed to the total IgA antishigella activity in the groups with combined oral and parenteral immunizations.

The enhanced stimulation of the early secretory IgA response after a combined parenteral stimulation followed a day later by a single oral dose of the live bacteria could result from a number of cellular interactions. There is strong evidence that existing within gut-associated lymphoid tissues are subpopulations of regulatory T lymphocytes which can help suppress or "switch" the immunoglobulin expression by sensitized B lymphocytes after antigen has been administered orally (2, 9, 18, 22, 23, 33, 36). Further, there is good evidence that the site of antigen challenge can influence the location of the ultimate IgA response (7). In the present studies, the initial systemic antigen administration in groups 3 and 4 may have stimulated B lymphocytes, which were attracted to the antigen present in the gut after the single oral dose of antigen. The importance of the local dose is seen in group 5, in which only a weak local IgA antishigella LPS response was seen when no local antigen was given after the parenteral dose.

In addition to causing a hyperstimulation of the local IgA response, the combination of parenteral and oral doses of antigen resulted in a lower serum IgG response than when a parenteral dose of antigen was given by itself. These findings may relate to the phenomenon of oral tolerance, in which oral immunization with an antigen results in the development of suppressor T cells which inhibit the subsequent systemic response to the same antigen administered parenterally (24, 33, 35).

In the past decade, many studies have documented a dichotomy between the stimulation of the systemic and mucosal immune responses. Oral immunization will usually stimulate secretory IgA responses and can suppress subsequent systemic immunity to protein antigens and haptens. Nonetheless, the present study shows that following parenteral stimulation, the primary mucosal immune response to that antigen can be enhanced if the antigen is administered orally 1 day later. Whether this oral antigen functions to recruit circulating B immunoblasts (stimulated by the parenteral dose) to the intestine or whether it has a role in altering regulatory cells that adjust the isotypic expression and proliferation of the B cells is unclear at the present time. It is clear that this mechanism provides a useful means of rapidly sensitizing the mucosal immune system against infectious agents and their toxic products and offers a model system to better understand the basic immunology of secretory IgA.

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