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Effect of Antigen Form on Local Immunoglobulin A Memory Response of Intestinal Secretions to *Shigella flexneri*

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An enhanced memory response, as shown by increased titers of specific immunoglobulin A (IgA), was seen in intestinal secretions from isolated Thiry-Vella loops in rabbits primed orally with live, locally invasive *Shigella* sp. X16 and challenged 60 days later with a single oral dose of the same antigen. Heat-killed shigella preparations, when used as either the priming or challenge antigen, did not elicit such a memory response in this system. In the present study, the role of antigen form and dosage in eliciting the enhanced local IgA response was investigated. A noninvasive strain, *Shigella flexneri* 2457-0, was capable of significantly enhancing the mucosal IgA memory response, whereas heat-killed *Shigella* sp. X16 was unable to augment the local IgA response, even when the priming dose was increased 100-fold. A proposed mucosal adjuvant, DEAE-dextran, given orally with live *Shigella* sp. X16, did not enhance the local IgA response. Viable, noninvasive shigellae were effective priming agents in enhancing the local IgA memory response. The poor mucosal response to heat-killed shigella preparations is thought to be related to an ineffective delivery of nonviable bacterial antigens into gut-associated lymphoid tissues. The ability of the live, noninvasive strain to elicit a vigorous local IgA memory response when given orally to rabbits was consistent with previous findings that live preparations elicit the best mucosal IgA response.

The most consistent stimulation of a local immune response has been accomplished by administering antigen directly to mucosal surfaces. Bacterial somatic antigens and proteins, such as cholera toxin, with special binding characteristics and physiological activities have elicited the strongest local immunoglobulin (IgA) response in intestinal secretions or have produced large numbers of antibody-containing cells in the lamina propria of the gastrointestinal tract (8, 9, 12, 15, 20, 22, 25, 30, 32).

The existence of the once controversial local IgA memory response has been demonstrated in several recent studies. This response has been elicited by cholera toxin, invasive *Shigella flexneri*, and live virus preparations applied locally (3, 17, 25, 31). Although heat-killed *S. flexneri*, choleragenoid (i.e., the B subunit of cholera toxin which does not produce diarrhea), and inactivated virus are able to produce small local IgA responses, enhanced mucosal IgA memory responses have not been evoked by immunization with these antigens (17, 25, 31). It should be noted, however, that the relatively nontoxic, heat-aggregated form of cholera toxin (procholeragenoid) can elicit a protective IgA response (27).

In the present study, we examined the role of antigen form in establishing a vigorous local IgA memory response to the enteropathogen *S. flexneri* in intestinal secretions. Live, noninvasive *S. flexneri* 2457-0 was capable of eliciting a significant enhancement of the IgA memory response in intestinal secretions, whereas heat-killed preparations were ineffective.

MATERIALS AND METHODS

Preparation of chronically isolated ileal loops. The surgical creation of ileal Thiry-Vella loops in rabbits has been described in detail previously (14). In brief, while 3-kg New Zealand White rabbits were anesthetized, a midline abdominal incision was made, and the terminal ileum was identified. A 20-cm segment of ileum containing a grossly identifiable Peyer's patch was isolated with its vascular supply intact. Silastic tubing (Dow Corning Corp.) was sewn into each end of the isolated segment. This tubing was brought out through the midline incision and tunneled subcutaneously to the nape of the neck, where it was exteriorized and secured. The intestinal continuity was restored by an end-to-end anastomosis, and the midline incision was closed in two layers.

Each day, the secretion and mucus (2 to 4 ml) that collected in the ileal loops were expelled by injecting 20 ml of air into the silastic tubing. The slightly opaque, colorless fluid and mucus expelled from the tubing were studied for specific immunoglobulin content. A subsequent flush with 20 ml of sterile saline helped to remove adherent mucus; the saline was then removed with gentle flushes of air.

Immunization techniques and antigen preparations. The immunization schedule is shown in Table 1. For all rabbits immunized orally, an orogastric tube was inserted (while the animals were anesthetized) immediately before immunization.

Several different antigen and antigen-adjuvant regimens were used. Live *Shigella* sp. X16 is capable of local invasion in the ileum and cecum (4); however, it is not pathogenic in that it does not persist in the tissues and does not produce ulceration or diarrhea. *S. flexneri* 2457-0 is a mutant strain which does not invade the intestine, and therefore, neither ulceration nor diarrhea develops (5). Heat-killed *Shigella* sp. 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.

DEAE-dextran as a 5% solution has been suggested as an adjuvant for the mucosal immune response to some bacteria (1). In the present study, DEAE-dextran was added to live *Shigella* sp. X16 (group VIII, Table 1) to determine whether an enhanced local IgA response would result.

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TABLE 1. Immunization schedule for groups of New Zealand White rabbits[a]

Group	No. of animals tested	Antigen	Dose	Day(s) given[b]
I	8	Live *S. flexneri* 2457-0	10^{10}	0
II	10	Live *S. flexneri* 2457-0	10^{10}	0, 7, 14
III	10	Live *S. flexneri* 2457-0	10^{10}	−75, −68, −61, 0
IV	10	Live *Shigella* sp. ×16	10^{10}	−75, −68, −61
		Heat-killed *Shigella* sp. ×16	10^{10}	0
V	8	Heat-killed *Shigella* sp. ×16	10^{10}	−75, −68, −61
		Live *Shigella* sp. ×16	10^{10}	0
VI	10	Heat-killed *Shigella* sp. ×16	10^{13}	0, 7, 14
VII	10	Heat-killed *Shigella* sp. ×16	10^{13}	−75, −68, −61
		Live *Shigella* sp. ×16	10^{10}	0
VIII	10	DEAE-dextran with live live *Shigella* sp. ×16	10^{10}	0

[a] All shigella strain samples were placed in the stomach via an orogastric feeding tube. The isolated loop was not directly exposed to shigellae.
[b] Day of surgical creation of the isolated loops = day −1 for all groups.

Enzyme-linked immunosorbent assay. As previously described, an enzyme-linked immunosorbent assay for detecting IgG and IgA antibodies to bacterial products was used to detect specific antibody activity in intestinal loop secretions (11, 18). Polystyrene microtiter wells were coated with 0.1 ml of a solution containing 10 μg of *S. flexneri* lipopolysaccharide (LPS) per ml (Westphal preparation). 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 0.05% Tween 20. The sample to be assayed was diluted 1/20 in this buffer and incubated in LPS-coated wells and in uncoated wells (to control for nonspecific adsorption) for 4 h. After the wells were washed with phosphate-buffered saline, solutions containing either alkaline phosphatase-conjugated goat anti-rabbit IgA or alkaline phosphatase-conjugated staphylcoccal protein A were added to the wells and left overnight at room temperature. After an additional wash with buffered saline, 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 (OD_{405}) of uncoated wells, measured on a Titertek Multiscan MicroELISA Reader (Flow Laboratories, Inc., McLean, Va.), was subtracted from the OD_{405} of the coated wells. Standard solutions of IgG and IgA anti-*S. flexneri* LPS were prepared as described previously (11, 18) and processed daily with the unknown samples. To minimize day-to-day variation, the results of the standards were normalized to a fixed number, and the values of the unknown specimens were corrected to these normalized standards. This assay system could detect an amount as small as 1.3 ng of specific antibody per ml and had coefficients of variation of 3.6 and 9% for IgG anti-shigella and IgA anti-shigella antibodies, respectively.

The specific immunoglobulin anti-shigella LPS activity is expressed as the change in OD_{405}/100 min. The data are presented as geometric means; others have noted that this reflects more clearly the logarithmic kinetics of the local immune response after immunization (27). These values were calculated by using the $\log_{10}$ of each value for each rabbit to determine the mean, the standard deviation, and the standard error of the means. For each day the $\log_{10}$ of the standard error 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 with upper and lower limits of variance about that mean.

Statistical analysis. Data were statistically analyzed by the Michigan Interactive Data Analysis System developed by the Statistical Research Laboratory, University of Michigan (6). This package is available on the computer system at the University of Michigan Computing Center. Group distributions were tested for significant differences by using the nonparametric Kruskal-Wallis test (2), which does not require assumptions of normality and equal variances.

RESULTS

Antigen-specific IgA in ileal loop secretions after oral stimulation with live, noninvasive *S. flexneri* 2457-0. The local IgA anti-shigella LPS response in isolated ileal loop secretions after a single oral dose of 10^{10} live, noninvasive *S. flexneri* 2457-0 (group I animals) is shown in Fig. 1. The kinetics of this IgA anti-shigella LPS response are similar to those of our previous study, in which the isolated ileal loops were immunized directly with this same strain of shigella (13). By day 4 after oral immunization, a small but detectable immune response was seen. This local IgA response continued to increase until day 8. Although there was no significant difference in the kinetics of this response compared with that of an earlier study, in which live, locally invasive *Shigella* sp. X16 were given orally on day 0, the height of the present response was greater (17). This difference in the height of the response may relate to a lower density of specific antigen on the *Shigella* sp. X16 strain.

Our previous study with direct immunization of the isolated ileal loops demonstrated that additional, weekly immunizations produced a greater initial IgA response than did a single immunization (16). To determine whether this booster effect would occur in the present system, group II animals were given three oral doses (one per week) of 10^{10} live *S.*

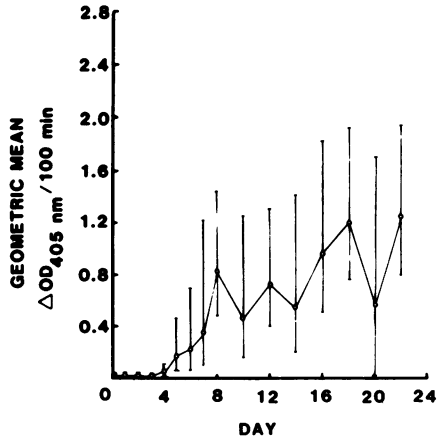

FIG. 1. Geometric mean IgA anti-shigella LPS response in isolated loop secretions from group I rabbits given a single oral dose of 10^{10} live, noninvasive *S. flexneri* 2457-0 on day 0.

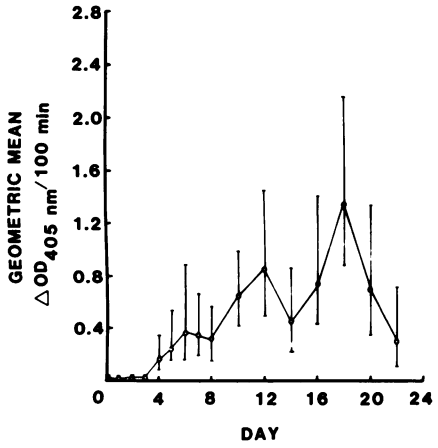

FIG. 2. IgA anti-shigella LPS response in isolated loop secretions from group II rabbits given oral doses of 10^{10} live *S. flexneri* 2457-0 on days 0, 7, and 14.

flexneri 2457-0. The IgA anti-shigella LPS activity from the isolated loop secretions of these rabbits is shown in Fig. 2. Again, a small local IgA response was first detected on day 4 after immunization. By 4 to 5 days after the booster doses on days 7 and 14, increases were seen in the mean values of the IgA anti-shigella LPS response. However, these responses were not significantly greater than the response to the initial immunizing dose of *S. flexneri* (Fig. 1).

To determine whether an enhancement of the local IgA memory response could be elicited by immunization and challenge with noninvasive *S. flexneri*, group III rabbits were primed with three oral doses (one per week) of 10^{10} live *S. flexneri* 2457-0. At 60 days after the third oral dose, a chronically isolated ileal loop was created, and the animals were challenged with a single oral dose of 10^{10} live *S. flexneri* 2457-0. By day 3 after challenge, a significantly greater local IgA response was present in the primed group III versus the nonprimed group I ($P < .001$) (Fig. 3). This difference in IgA response between the primed and nonprimed groups for noninvasive *S. flexneri* was similar to the enhanced IgA memory response achieved in our previous studies, in which live, locally invasive shigellae were used to prime and challenge rabbits. It demonstrated for the first time that, for *S. flexneri*, oral, noninvasive vaccine preparations could significantly enhance the local IgA response.

Antigen-specific IgA response after oral stimulation with heat-killed *Shigella* sp. X16. In our previous studies we demonstrated that when rabbits are immunized orally with three doses (one per week) of killed *Shigella* sp. X16, no local IgA memory response results when the animals are subsequently challenged with a single oral dose of the same heat-killed preparation. It was not clear, however, whether the heat-killed preparation of *Shigella* sp. X16 was a sufficient challenge to evoke an enhanced local IgA memory response. Therefore, in the present study, group IV rabbits were given three oral doses (one per week) of live *Shigella* sp. X16 in a regimen previously shown to consistently elicit an enhanced local IgA memory response. By 60 days after the last dose, the animals were challenged with a single oral dose of 10^{10} heat-killed *Shigella* sp. X16. It is obvious from the data (Fig. 4) that the heat-killed *Shigella* sp. X16 was an inadequate challenge stimulus for the enhanced local IgA response.

Therefore, to determine whether animals could be effectively primed by heat-killed *Shigella* sp. X16 for challenge with a live agent, a group of eight rabbits (group V) was given three oral doses (one per week) of 10^{10} heat-killed *Shigella* sp. X16. By 60 days after the last dose, a chronically isolated ileal loop was created in each rabbit. The next day, the animals were given a single challenge dose orally of 10^{10} live *Shigella* sp. X16. This was the same dose that elicited a vigorous enhancement of the local IgA memory response in our previous studies, where animals were primed effectively with three oral doses (one per week) of 10^{10} live *Shigella* sp. X16 (17). The local IgA response in intestinal secretions from these rabbits after their challenge dose is shown in Fig. 5. Only a weak local IgA response was seen in these animals. This indicated that, at a dose of 10^{10}, the heat-killed *Shigella* sp. X16 preparation was ineffective in priming the mucosal immune system.

To determine whether an inadequate dose of heat-killed bacteria was given, an oral priming dose of 10^{13} heat-killed *Shigella* sp. X16 was given to group VI rabbits on days 0, 7, and 14. Only a weak primary local IgA anti-shigella LPS response in isolated loop secretions resulted from this immunization schedule (data not shown). Even so, it was possible that these animals were primed to develop an

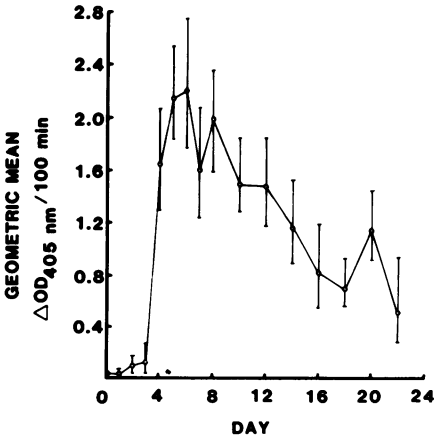

FIG. 3. IgA anti-shigella LPS response in isolated loop secretions from group III rabbits primed with three weekly oral doses of 10^{10} live *S. flexneri* 2457-0 60 days prior to the creation of the isolated ileal loop on day −1 and the oral challenge on day 0.

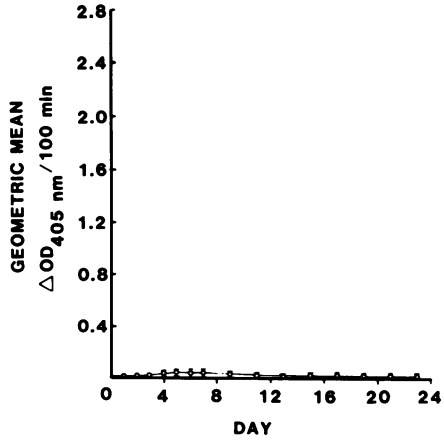

FIG. 4. IgA anti-shigella LPS response in isolated loop secretions from group IV rabbits primed with live, locally invasive *Shigella* sp. X16 (Table 1) and challenged on day 0 with killed *Shigella* sp. X16.

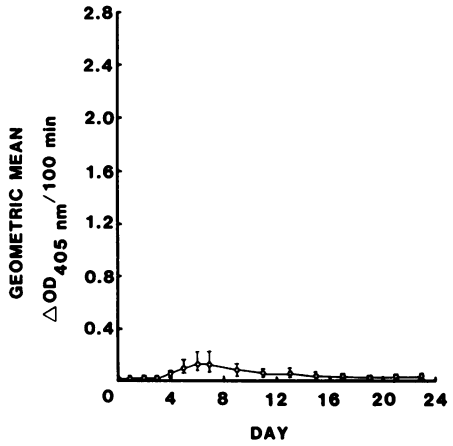

FIG. 5. IgA anti-shigella LPS response in isolated loop secretions from group V rabbits primed with killed *Shigella* sp. X16 (Table 1) and challenged on day 0 with a single oral dose of live *Shigella* sp. X16.

enhanced local IgA memory response upon challenge with the live, locally invasive bacteria. Therefore, group VII rabbits were primed with three oral doses (one per week) of 10^{13} heat-killed *Shigella* sp. X16; 60 days after the third dose, a chronically isolated ileal loop was created in each animal. The next day, the animals were challenged with a single dose of live *Shigella* sp. X16. Once again, only a weak local IgA anti-shigella response resulted (Fig. 6).

Effect of DEAE-dextran adjuvant on the local immune response to *Shigella* sp. X16. To determine whether the proposed mucosal adjuvant DEAE-dextran (1) would be able to enhance the lcoal IgA response, a dose of 10^{10} live *Shigella* sp. X16 was given via an orogastric tube in a 5% DEAE-dextran solution. The local immune response was determined for 4 weeks after this stimulation. Only a weak local IgA response resulted from the inclusion of the DEAE-dextran adjuvant (data not shown).

IgG anti-*Shigella* sp. X16 response. In all of the above groups, no or trivial amounts of IgG anti-shigella LPS were found in either serum or intestinal secretions. This lack of IgG in intestinal secretions after oral immunization was consistent with our previous studies (15, 16) and is not due to the instability of IgG in the isolated ileal loops (19).

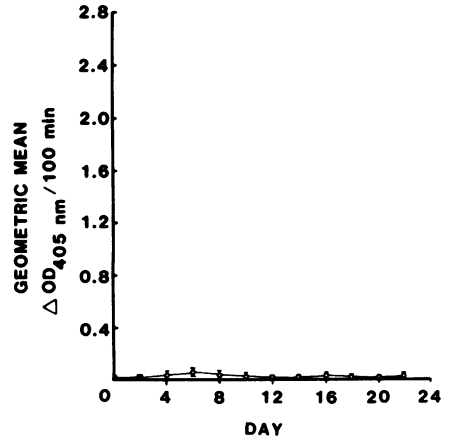

FIG. 6. IgA anti-shigella LPS response in isolated loop secretions from group VII rabbits given three oral doses of 10^{13} heat-killed *Shigella* sp. X16 and challenged after 60 days with a single oral dose of 10^{10} live *shigella* sp. X16 on day 0.

Shedding of live bacteria. Loop fluids and stool samples were cultured on MacConkey agar plates to detect shedding of shigellae as described previously (17). Shigellae were found in stool samples as early as 1 day and for as long as 17 days after oral challenge. Although no shigellae were detected in some rabbit stool samples, most of the animals shed for at least 2 or 3 days. Overall, there was somewhat more shedding of live shigellae in the present study with *S. flexneri* 2457-0 than in our previous work (17) with *Shigella* sp. X16.

DISCUSSION

In the present series of experiments, we demonstrate that invasion per se was not essential for priming an animal for an enhanced local IgA memory response to shigella antigens in intestinal secretions. Although our previous studies established that oral administration of live, locally invasive *Shigella* sp. X16 significantly enhance the mucosal memory response, we demonstrate here that noninvasive *S. flexneri* 2457-0 could cause an equally vigorous mucosal memory response when used to prime and challenge test animals. This finding advanced our understanding of the role that antigen form plays in enhancing the mucosal IgA response to enteropathogens.

In our system live bacteria, although noninvasive, could be attracted to the surface epithelium via a chemoattractant mechanism such as that demonstrated by Freter et al. (7). The uptake of such viable noninvasive bacteria has been demonstrated for *Vibrio cholerae* by Owen et al. (24). They found that live, noninvasive *V. cholerae* were taken up by the specialized "M" cells overlying Peyer's patches, but killed vibrios were not. The persistence of such bacterial antigens in gut-associated lymphoid tissues (GALT) would allow a continuous recruitment of memory cells. This would explain the persistence of the antigen-specific IgA in intestinal secretions 60 days after the last administration of live, noninvasive shigellae in the present study.

The inability of GALT to take up the killed vibrios (24) would have resulted in a poor local immune response to such killed bacterial antigens. Indeed, we have already demonstrated that heat-killed *Shigella* sp. X16 administered orally does not prime rabbits for an enhanced local IgA response to *S. flexneri* antigens when the animals are challenged with the same heat-killed antigens orally (17). In the present study, we were concerned that the heat-killed shigella preparation may not have been an adequate challenge stimulus. The results from group IV animals, which were given an optimal priming dose of live, locally invasive *Shigella* sp. X16, showed that no enhanced local IgA response was elicited when heat-killed shigellae were given orally as the challenge dose. Our results from group V rabbits, however, indicated that even when an appropriate challenge was given with 10^{10} live *Shigella* sp. X16, rabbits primed with 10^{10} heat-killed shigellae did not demonstrate enhanced local IgA response compared with nonprimed rabbits.

Since total dosage may be an important factor (live shigellae multiply within the gut), three oral priming doses (one per week) of 10^{13} heat-killed *Shigella* sp. X16 were given to group VI animals. There was no significant difference between the local IgA response of this group and that of animals given only 10^{10} heat-killed *Shigella* sp. X16 orally. Further, no enhancement of the local IgA memory response was seen in animals primed with this higher dose of heat-killed *Shigella* sp. X16 and later challenged orally with 10^{10} live *Shigella* sp. X16. Clearly, the dose alone was not the critical factor.

The failure of heat-killed *Shigella* sp. X16 administered orally to prime for an enhanced mucosal IgA memory response could be due to an inadequate delivery of nonviable antigen into GALT. Such a mechanism would explain why effective, mucosally applied vaccines have been live and attenuated (rather than killed) bacteria or viruses (17, 31). It would also be consistent with the demonstration that live vibrios are taken up by GALT more efficiently than are killed vibrios (24).

These findings indicated that if killed vaccines are to be effective against musocal infections they must either be applied with some adjuvant or artificially introduced into GALT. Although Beh found the DEAE-dextran conjugate to be an effective enhancer of local immunity to live bacteria (1), the present study with this proposed mucosal adjuvant and live *Shigella* sp. X16 failed to show an enhancement of the local IgA activity. However, this study only examined the role of this adjuvant on the local IgA response to the initial administration of shigella preparations and so did not rule out a possible enhanced memory effect.

The details of the cellular mechanism(s) for eliciting a vigorous local immune response to enteropathogenic bacteria are only beginning to be understood. The stimulation of the mucosal surface by an appropriate antigen triggers T and IgA-B memory cells which persist in GALT (8, 28). Pierce and Cray have hypothsized that two types of memory cells are generated in priming for a mucosal memory response (26). One group of cells would circulate only briefly before homing to the GALT while the other group of cells would circulate continuously until recruited by antigens. Because the rabbit ileal loops in this study were separated from the intestinal stream just before the challenge dose was given, the results here suggest that homing of antigen-committed IgA plasmablasts could occur even in the absence of antigen restimulation. This mechanism would be consistent with the finding of Ottaway and Parrott that the homing of lymphoblasts is dependent on the blood supply in general and not on a specific local antigen event (23).

The nature of the antigen itself is critically important in determining whether a local IgA response will be elicited. Whereas biologically active molecules like cholera toxin are potent local immunogens, soluble proteins like bovine serum albumin are often ineffective mucosal immunogens, although their administration orally may evoke a systemic immune response (29; C. O. Elson and W. Ealding, Fed. Proc. **42**:645, 1983). With bacterial vaccines, the macromolecular structures on the surface of the particular bacterial strains used as immunogens are important factors. For example, Ivanoff et al. found that the ability to elicit a local IgA memory response to *Salmonella typhimurium* in mice is dependent on the length of the outer membrane polysaccharide chains (10). Those strains with longer chains are better able to evoke a local IgA memory response.

A previous study from our laboratory has indicated that to achieve an enhanced local IgA memory response to *Shigella flexneri*, oral immunization is effective, but parenteral immunization (without adjuvant) is not (19). Others have found that parenteral immunization may actually suppress the subsequent local immune response to the same antigen (20, 21). The present work extended our findings by showing that viable, noninvasive preparations were effective priming agents in enhancing the local IgA memory response. Further, we suggest that the ineffective delivery of nonviable, bacterial somatic antigens into GALT could be responsible for the poor immunogenicity of heat-killed preparations. Future studies will examine whether altering the variables of polysaccharide structure and encouraging antigen delivery into GALT, either with adjuvants or by attaching the antigen to cholera toxin or liposomes, will elicit a more effective IgA memory response with nonviable vaccine preparations.

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