INFECTION AND IMMUNITY, Mar. 1981, p. 1193-1202
0019-9567/81/031193-10$2.00/0

Vol. 31, No. 3

# Role of Antigen Form in Development of Mucosal Immunoglobulin A Response to *Shigella flexneri* Antigens

DAVID F. KEREN,[1]* HUGH H. COLLINS,[2] PETER GEMSKI,[2] PETER S. HOLT,[2] AND SAMUEL B. FORMAL[2]

*Department of Pathology, The University of Michigan, Ann Arbor, Michigan 48109,[1] and Department of Bacterial Diseases, The Walter Reed Army Institute of Research, Washington, D.C. 20012[2]*

One major stumbling block in the development of an effective means to immunize against shigellosis and other enteric diseases has been the lack of a means to assess sequential mucosal immune reponses to different potential immunogens. In the present study, we compared the abilities of live invasive organisms, noninvasive organisms, and nonviable antigen preparations of shigella to elicit mucosal immune responses. Whereas previous studies have found that effective immunity was produced best by vaccination with live invasive strains of shigella, in the present study, live noninvasive strains that did not produce any histopathological damage were consistently able to produce local (immunoglobulin A) immune responses as vigorous as those of the invasive strains. Further, acetone-killed shigella antigen was also an effective mucosal immunogen, whereas hot phenol-water-extracted shigella lipopolysaccharide was ineffective, possibly due to the method of preparation. A single oral or parenteral priming was ineffective in enhancing the mucosal immune response when restimulated 1 month later with the same antigen. However, a mucosal memory response was found to be present several months after a triple mucosal stimulation with a locally invasive vaccine strain.

Bacillary dysentery results primarily from invasion of the colonic epithelium by shigella with subsequent multiplication and persistence of the shigella in the tissue (27, 28, 45, 46). Focal ulcerations at these sites of invasion result in the blood-tinged, mucus-containing frequent stools that characterize dysentery. A watery diarrhea in shigellosis that may accompany, but often occurs apart from, dysentery may be due to the production of a toxin by the shigella (24, 35, 43). Protection against dysentery by vaccination has been a long-sought goal, but has not been attained, at least partly due to the lack of experimental models to aid in understanding the mucosal immune response to shigella antigens.

Although it is well known that parenteral immunization with killed shigella vaccines results in high levels of circulating antibodies to this antigen, these vaccines have serious local and occasionally systemic side effects; moreover, there is little evidence that vaccinated individuals have any protection against the natural disease (11, 16, 17). In fact, the use of parenteral vaccination against dysentery has been abandoned at the present time (48).

In contrast, orally administered shigella have been shown to confer some protection in both experimental animals and humans, but this protection against subsequent challenge with the immunizing strain did not correlate with serum antibody titer. Most of the techniques used for assay did not address the question of specific mucosal antibodies (4, 7, 8, 10, 14, 31, 33, 34). This immunity could have reflected a local immune response. As a consequence, we have investigated the local immune response of rabbits to a variety of shigella vaccine preparations.

The present work uses our previously described chronically isolated (Thiry-Vella) intestinal loop model in rabbits (15, 19, 20, 21, 49) to compare invasive, noninvasive, acetone-killed whole shigella, and purified shigella lipopolysaccharide (LPS) with regard to their immunogenicity. These studies demonstrate that killed shigella antigens can be effective local immunogens and provide evidence for the existence of a mucosal memory response to shigella antigens.

## MATERIALS AND METHODS

**Preparation and care of Thiry-Vella loops.** Our previously described method (19) was used to construct chronically isolated segments of ileum, 20 cm long, in 32 2- to 3-kg New Zealand white rabbits. Briefly, while the animals were anesthetized with a combination of xylazine and ketamine, a midline abdominal incision was made, the terminal ileum was identified, and a 20-cm segment containing a single Peyer's patch was isolated with its vascular supply intact. An end-to-end anastomosis restored continuity to the ileum. Silastic tubing was sewn into each end of the 20-cm segment of ileum. The silastic tube was

brought out through the midline abdominal incision and tunneled subcutaneously to an opening in the nape of the neck where it was secured.

Secretions from the isolated loops were collected daily by injecting 20 ml of air into one end of the silastic tubes, thereby expelling loop fluid from the other silastic tube. Approximately 2 to 4 ml of fluid was obtained daily from each loop. This fluid was centrifuged at 3,500 rpm for 10 min on a Sorvall GLC-3 centrifuge to separate mucus and cell debris. The clear-to-translucent supernatant was stored at −20°C. After the collection of specimens, the loops were flushed with saline to prevent mucus from obstructing the silastic tubes. This saline was removed from the loop by repeated injections of air.

**Bacterial preparations.** Three live shigella strains, one acetone-killed strain, and one Westphal LPS preparation containing *Shigella flexneri* 2a somatic antigens were used. Strain M4243 has been shown to invade the epithelium of guinea pig intestine and to persist with the formation of focal ulcerations (7). Shigella X16 (a *S. flexneri-Escherichia coli* hybrid) invades locally, may form ulcers, but does not thrive (persist) in guinea pig intestine (9). Strain 2457-0 is noninvasive (10). The preparation of acetone-killed strain M4243 (AK-M4243) and the shigella LPS preparations have been described previously (10, 11, 20).

To determine the effect of the live shigella strains on the rabbit Thiry-Vella loops, $10^8$ live bacteria were administered on the first day after surgery. Histological sections of the isolated loop were prepared from samples taken at 12 and 24 h. The sections were stained with hematoxylin and eosin and Giemsa (to enhance bacterial morphology).

**Immunization schedule.** The isolated Thiry-Vella loops were given $10^8$ live bacteria, 2.5 mg of acetone-killed bacteria per ml, or 100 μg of shigella LPS per ml in 4 ml of phosphate-buffered saline on days 1, 8, and 15 after surgery as shown in Table 1. The dose of AK-M4243 was the equivalent of $4 \times 10^{10}$ bacteria (6). One hundred micrograms of purified LPS represented the equivalent of $5 \times 10^9$ bacteria (dry weight) and were used because equivalent doses of protein antigens (cholera toxin, cholera toxoid, and keyhole limpet hemocyanin) have been shown to be effective immunogens in Thiry-Vella loops (15, 41, 49). Live invasive strains were tested by guinea pig conjunctivitis assay before use (29). Daily intestinal secretions and weekly sera were stored at −20°C for assay.

**Enzyme-linked immunosorbent assay.** Enzyme-linked immunosorbent assay for immunoglobulin A (IgA) and IgG activity to shigella LPS Was performed as described previously (18). All reactions were performed in duplicate. This technique allows detection of as little as 1.3 ng of specific antibody per ml (18). Coefficients of variation of replicate samples of IgG and IgA anti-shigella LPS are 3.6 and 9.0%, respectively. Significance was assessed by Student's *t* test.

## RESULTS

**Histopathological lesions produced by live antigens.** *S. flexneri* strain M4243 previously has been shown both to invade and persist when administered orally to starved guinea pigs (9). Similarly, M4243 created significant focal ulcerations in the Thiry-Vella loops within 24 h of administration intraloop (Table 2). The ulcerations were predominantly present over the Peyer's patches, although we noted occasional ulcerations overlying villi. Giemsa staining demonstrated bacterial invasion in the mucosa by 12 h after intraloop immunization (Fig. 1). *S. flexneri-E. coli* hybrid strain X16 previously has been shown to invade but not to persist when injected orally into starved guinea pigs (9). When the X16 was administered into the rabbit Thiry-Vella loops, focal accumulations of granulocytes were seen by 12 h, and focal ulcerations were present 24 h after bacterial challenge. In addition, bacterial invasion of the surface epithelium was demonstrated by Giemsa stain (Table 2). *S. flexneri* strain 2457-0 previously has been shown to be incapable of invading the surface epithelium when injected orally into

TABLE 1. *Immunization schedule*

| Group | No. of animals | Antigen | Dose[a] | Route | Schedule[b] |
|---|---|---|---|---|---|
| M4243 | 7 | Live 4243 | $10^8$ | Intraloop | 1, 8, 15 |
| X16 | 6 | Live X16 | $10^8$ | Intraloop | 1, 8, 15 |
| 2457-0 | 6 | Live 2457-0 | $10^8$ | Intraloop | 1, 8, 15 |
| AK-M4243 | 4 | Acetone-killed M4243 | 2.5 mg/ml | Intraloop | 1, 8, 15 |
| LPS | 6 | Shigella X16 LPS | 100 μg/ml | Intraloop | 1, 8, 15 |
| Oral primed | 7 | Live X16 | $10^{10}$ | Oral[c] | −30 |
| | | Live X16 | $10^8$ | Intraloop | 1, 8, 15 |
| Parenteral primed | 6 | Heat-killed X16 | $10^{10}$ | Intravenous | −30 |
| | | Live X16 | $10^8$ | Intraloop | 1, 8, 15 |

[a] Dose given in 4 ml of phosphate-buffered saline.
[b] Day 0 = day of surgical creation of Thiry-Vella loop.
[c] Oral dose given via orogastric tube.

starved guinea pigs (10, 27). In a like manner, no ulceration or evidence of bacterial invasion of the mucosa was found when 2457-0 was administered into the Thiry-Vella loops (Table 2).

**Local immune response to live shigella antigens.** By day 7 after intraloop administration of $10^8$ live shigella, most of the animals in each group (M4243, X16, 2457-0) had a measurable IgA anti-shigella LPS response (Fig. 2). The response reached a plateau during the second week, although some animals (especially those immunized with 2457-0) demonstrated a further increase after the third intraloop stimulation with antigen.

TABLE 2. *Histopathology produced by live shigella antigens*

| Shigella strain | Guinea pig response[a] | | Rabbit Thiry-Vella loop | |
|---|---|---|---|---|
| | Invasion | Persistence | Invasion | Histopathology |
| M4243[b] | + | + | + | Focal ulceration mainly over Peyer's patches |
| X16 | + | − | + | Focal ulceration mainly over Peyer's patches |
| 2457-0 | − | − | − | No ulceration |

[a] Guinea pig response from Formal et al. (9) (see text).

[b] Live bacteria ($10^8$) were administered.

Whereas the mean IgA anti-shigella LPS levels differ, sometimes considerably, on the days tested, there was no statistically significant difference ($P < 0.05$) among these three groups for any day. We consider this variation in response to be a reflection of the use of outbred New Zealand white rabbits. Although all rabbits in any one group had similar temporal response to the antigens, the actual strength of the response varied considerably in each group as shown by the standard error of the mean in Fig. 2. By day 25 after the first intraloop antigenic stimulation (and 11 days after the third antigenic challenge), the mean IgA anti-shigella LPS response in all three groups decreased although none had returned to base-line values.

IgG anti-shigella LPS was not detectable in loop secretions from most animals in the three groups. Notable exceptions were two rabbits, one in the M4243 group and one in the 2457-0 group, that had weak (less than 0.5 optical den-

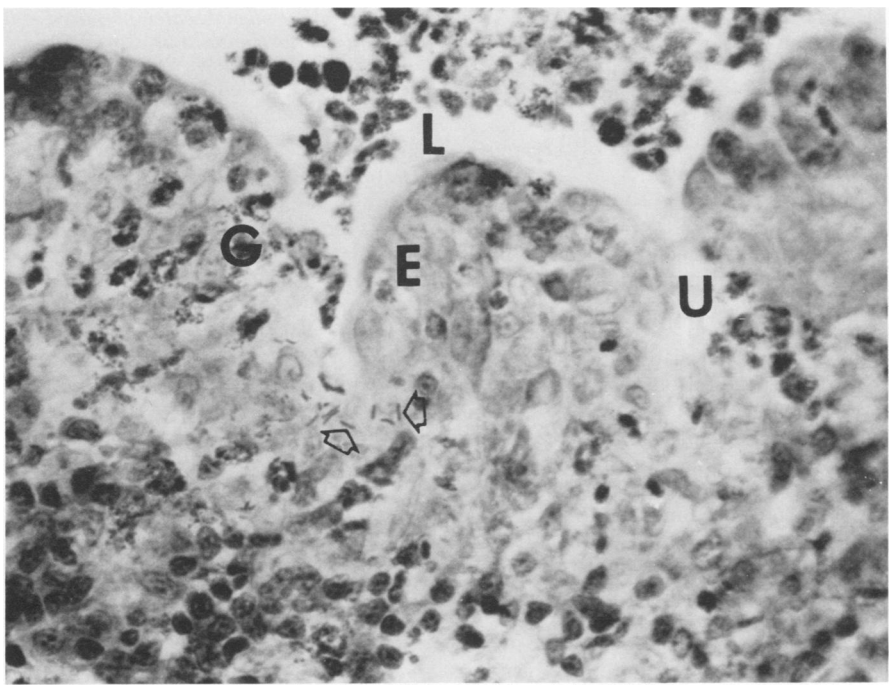


FIG. 1. *Brightfield photomicrograph of epithelium overlying a Peyer's patch dome of rabbit given S. flexneri M4243 12 h previously. Numerous bacilli (arrows) have invaded the surface epithelium (E), two ulcerations (U) are present with pus in the lumen (L), and granulocytes (G) are passing through the damaged epithelium. (Giemsa, 750×).*

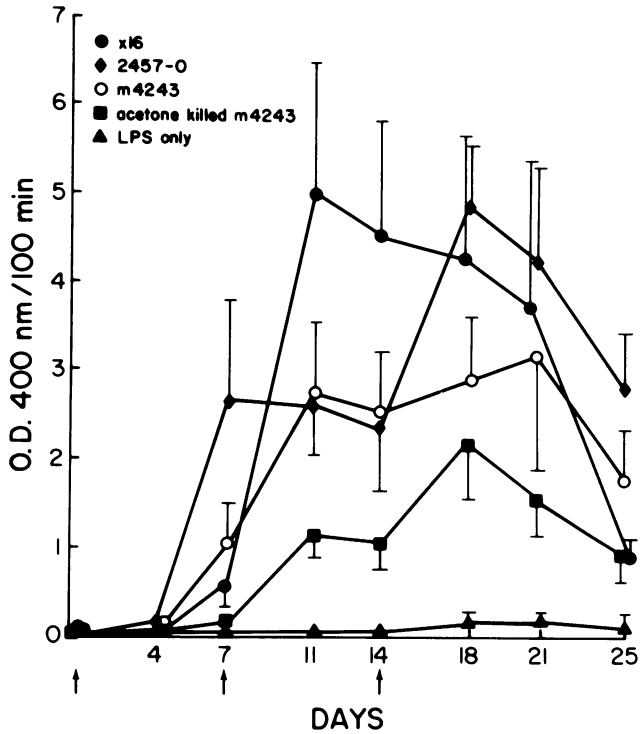


FIG. 2. *Mean IgA anti-shigella LPS in Thiry-Vella loop fluid from animals immunized intraloop with $10^8$ live M4243, X16, 2457-0, 2.5 mg of AK-M4243 per ml, or 100 μg of hot phenol-water-prepared shigella LPS. The vertical axis expresses the net OD at 400 nm/100 min, and the horizontal axis expresses days after first intraloop stimulation. Arrows indicate days when intraloop antigens were given. Standard error of the means is indicated.*

sity [OD] units) IgG anti-shigella LPS responses in secretions on several days.

**Local immune response to nonviable shigella antigens.** The kinetics of the IgA anti-shigella LPS response to acetone-killed shigella administered intraloop paralleled those of the live shigella antigens tested (Fig. 2). The mean IgA anti-shigella level on most days was lower than the response elicited by the live shigella antigens. Again, these differences were not significant ($P < 0.05$).

The shigella LPS elicited a weak IgA anti-shigella LPS response in only three of six animals, but no IgG anti-shigella LPS was detected in intestinal secretions from any rabbit in either group stimulated by nonviable shigella antigens.

**Effect of a single oral or parenteral priming dose of shigella on subsequent local immune response.** To determine whether a single oral or parenteral dose of shigella antigen would "prime" the bowel and enhance the secretory immune response to subsequent local administration of that same antigen, seven rabbits were given $10^{10}$ live shigella X16 via an orogastric tube (orally primed), and six other rabbits were given $10^{10}$ heat-killed shigella X16 intravenously (parenterally primed). At 30 days after this priming, a chronically isolated Thiry-Vella loop was created in each animal. The loops were stimulated with $10^8$ live shigella X16 on days 1, 8, and 15 after surgery.

Although a vigorous local IgA-shigella LPS response was found in all animals of each group (Fig. 3A and B), there was neither significant enhancement nor suppression of the local IgA anti-shigella response as compared to the non-primed X16-stimulated animals.

**Local memory response to shigella.** Secretions from four of the rabbits, immunized intraloop with $10^8$ live shigella X16 on days 1, 8, and 15 after creation of the Thiry-Vella loops, were studied. At a time when the level of local antibody to shigella LPS was thought to have returned to background levels, the animals were restimulated with a single dose of $10^8$ live shigella X16 intraloop (Fig. 4A–D). Antibody profiles for these animals had local IgA anti-shigella LPS responses (greater than 0.3 OD units) considerably sooner after rechallenge than after the original intraloop dose of shigella X16 (Table 3).

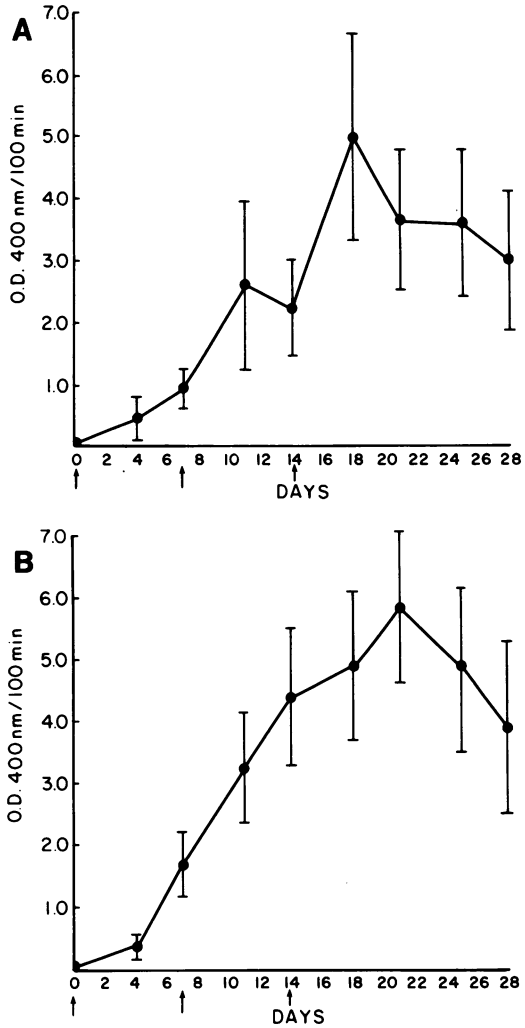


FIG. 3. *Mean IgA anti-shigella LPS in Thiry-Vella loop fluids from animals primed orally (A) or parenterally (B) with shigella X16 1 month before receiving the intraluminal dose of shigella X16. Arrows indicate days when intraloop antigens were given. Standard error of the mean is indicated.*

IgA anti-shigella LPS levels in secretions from the fourth animal (rabbit 364) had not returned to base-line levels at the time of restimulation. Therefore, although IgA anti-shigella LPS levels were detectable within 3 days after restimulation, this response cannot be compared with the response after the original intraloop dose of shigella X16 (Table 3). Interestingly, rabbit F was inadvertently given a fourth intraloop stimulation on day 23, which was followed on day 28 by a very high IgA anti-shigella LPS response (Fig. 4C).

**Serum antibody responses.** Only trivial IgA or IgG anti-shigella LPS levels were detected occasionally in sera of animals given intraluminal antigen. Notable exceptions, however, were the animals immunized parenterally with shigella X16. Predictably, five of the six animals developed serum IgG anti-shigella antibodies. Interestingly, the highest levels in the parenterally primed group consistently appeared in the serum after intraloop rechallenge (Fig. 5) with shigella, whereas when the Thiry-Vella loops were challenged without prior parenteral priming, no or trivial IgG anti-shigella LPS resulted in the sera.

## DISCUSSION

The concept of using a mucosal route to immunize against dysentery was first championed over half a century ago by Besredka (1). That mucosal immunity was involved in the natural response to shigella was suggested by Davies' demonstration in 1922 that agglutinating antibodies to shigella appear in the stool before their appearance in the serum of patients with active dysentery (2). Recent increased awareness of the role of local immunity in protecting mucosal surfaces from infections has restimulated interest in mucosal vaccines against shigellosis.

Several different mutant and hybrid strains of shigella with varying abilities to invade and multiply in tissue have been developed in attempts to establish a vaccine to shigellosis (9, 10, 30, 32). When these live vaccines are given orally, some are effective in eliciting protection in experimental animals and in humans (4, 7, 8, 10, 14, 31, 33, 34). For example, it was previously found that local protection in monkeys could be achieved best with a live invasive shigella (10). We now describe a vigorous local secretory IgA response against shigella LPS not only to live invasive strains M4243 and X16, but also in live noninvasive 2457-0 and, somewhat surprisingly, in AK-M4243. Yet these bacteria behave similarly in the isolated rabbit intestinal loops as in previous studies using oral injection into starved guinea pigs with regard to invasiveness and histopathology produced (7, 9, 10, 27). However, AK-M4243 was judged ineffective in protecting monkeys from experimental shigellosis when fed doses of 30 mg at 3-day intervals (10). The difference may be due to a species characteristic response to the shigella LPS; however, it more likely reflects degradation of the AK-M4243 antigen in the gut lumen or perhaps inadequate dosage in the monkey. Studies are in progress to assess the effect of multiple oral immunizations with both live and killed shigella on the subsequent local immune response.

It is of further interest that the ulcerations

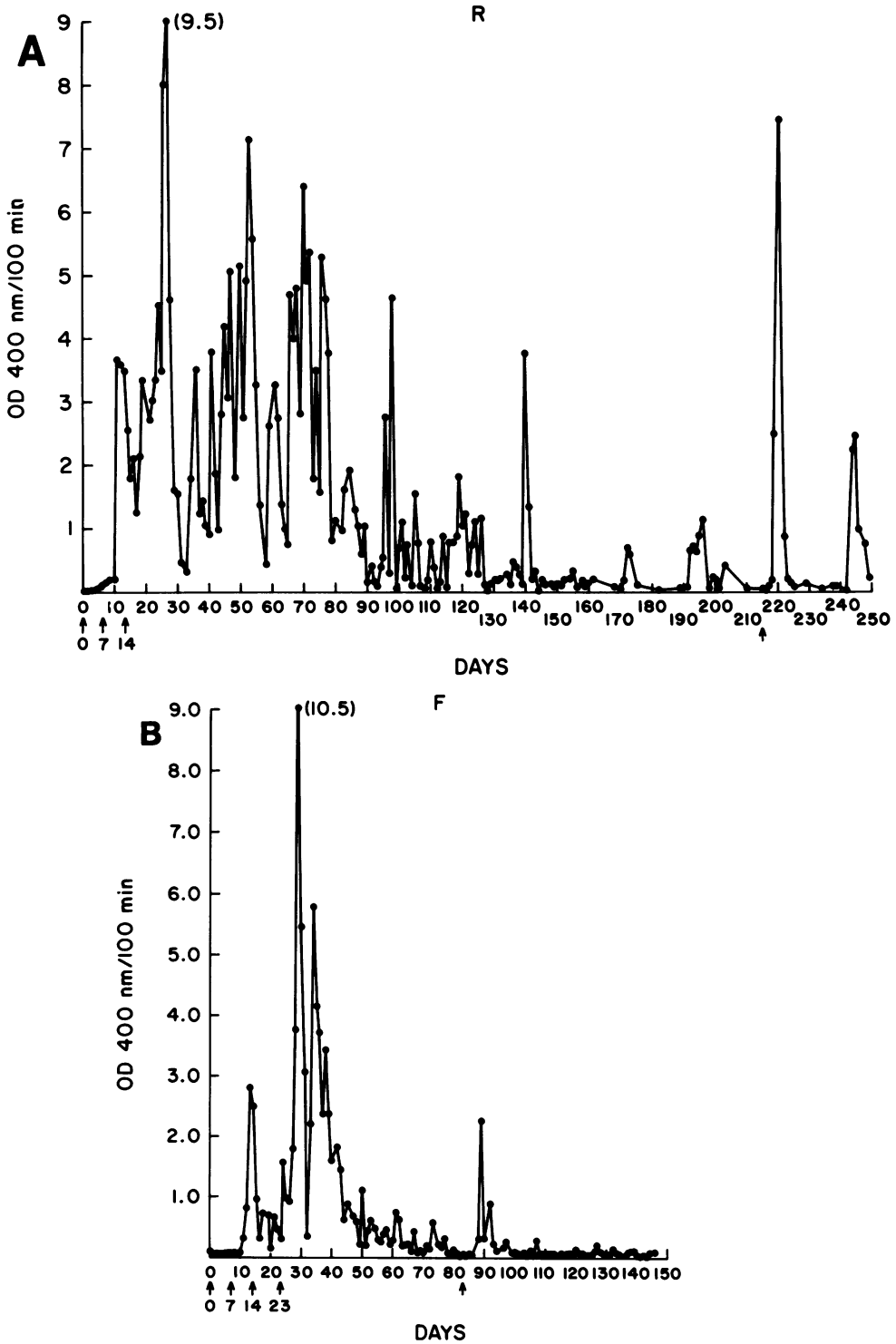


FIG. 4. *IgA anti-shigella LPS in Thiry-Vella loop fluids from animals stimulated with shigella X16 intraloop as indicated by the arrows. (A) rabbit R, (B) rabbit F, (C) rabbit 364, (D) rabbit 372.*

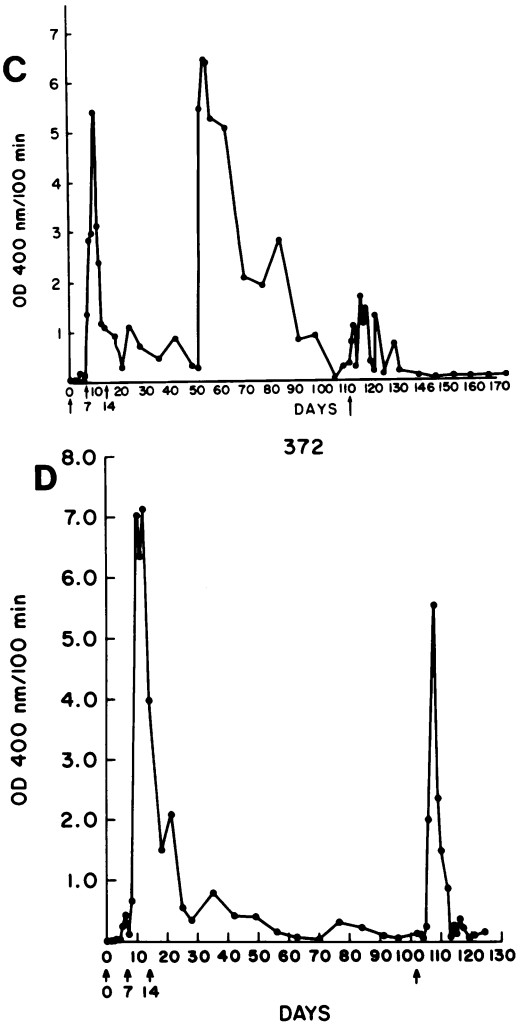


FIG. 4—*Continued.*

produced by the invasive shigella occurred primarily in the epithelium overlying Peyer's patches. This phenomenon has been previously described by LaBrec and Formal in guinea pig intestine (26). It may relate to the physical prominence of these structures, which would increase the chance that the nonmotile shigella would attach and invade this surface. Alternatively, the epithelium overlying Peyer's patches, which is known to contain specialized surface epithelial cells (M-cells) that are especially adept at sampling macromolecules and passing them to nearby lymphocytes (36), may also be able to engulf the bacteria or may be more susceptible to invasion by shigella. Lastly, a chemotactic gradient has been shown by Freter et al. to be important in the attraction of *Vibrio cholerae* to surface epithelium (12); a similar mechanism may be important in the attraction of shigella to the Peyer's patches.

Our demonstration of a local immune response to noninvasive or killed shigella antigens is not, however, without precedent. At least two field trials have indicated that oral immunization to shigella can be achieved with killed preparations (14, 23), and experimental studies have shown immunity to keratoconjunctivitis shigellosis in rabbits after oral immunization with shigella endotoxin (25). Multiple weekly doses may be important in optimizing the response to the killed shigella antigens as we have previously demonstrated for live, invasive shigella antigens (21).

The fact that purified LPS alone was relatively ineffective in eliciting a local immune response remains to be further investigated. The dose (100 μg/ml in 4 ml of phosphate-buffered saline) was the same dose that has been shown to be effective in stimulating a local immune response to several antigens: cholera toxin, cholera toxoid, keyhole limpet hemocyanin, and 2,4-dinitrophenol-keyhole limpet hemocyanin in our Thiry-Vella loop model (15, 41, 49). This dose of LPS is about 30 times greater than the amount of LPS presented to the loops by $10^8$ live cells (B. Sied, personal communication). Since the live bacteria multiply, however, it is difficult to compare actual dosage.

LPS preparations may differ considerably with regard to their immunogenic capabilities; these depend on the method used to prepare the LPS (44), as well as on the genetic capabilities of the animal to respond (5, 44, 47). A destructive effect of phenol on LPS has been blamed for the poorer mitogenic responses elicited from mouse spleen cells (44). Further, recent studies indicate that an endotoxin protein that is separated from LPS by phenol extraction is an effective B cell activator (13). Since a hot phenol-water method was used to prepare the LPS for the present studies (20), it is possible that B cell-activating

TABLE 3. *Comparison of initial and memory response in secretions from Thiry-Vella loops*

| Rabbit | Initial response | Memory response[a] |
|---|---|---|
| R | 9[b] | 3 |
| 364 | 8 | –[c] |
| F | 11 | 5 |
| 372 | 5 | 3 |

[a] Animals were not restimulated for at least 2 months before restimulation with the same antigen.

[b] Day after immunization when IgA anti-X16 was 0.3 OD units.

[c] Values had not returned to base line at time of restimulation.

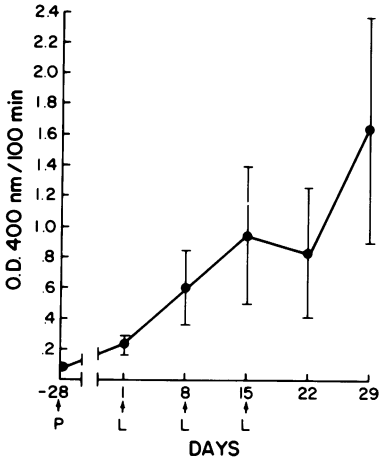


FIG. 5. *Mean IgG anti-shigella LPS in sera from animals primed parenterally (P) with shigella X16 before intraloop (L) stimulation. Arrows indicate days of antigen stimulation related to surgical creation of the Thiry-Vella loop (Day 0). Standard error of the mean is indicated.*

components were removed or destroyed, accounting for the poor response seen. Alternatively, or in addition, a higher dose of LPS may be necessary to achieve a good local immune response. That the form of antigen is important in eliciting mucosal immunity has recently been demonstrated by Pierce for cholera toxin preparations (37). Similarly, in the present system although we are measuring antibody to the purified shigella LPS preparation, perhaps some constituent on the cell wall of the intact or only partially degraded cell wall is important in the initial processing of the antigen.

Since we were most familar with the X16 strain, we attempted to prime the local immune response by giving a single oral or intravenous dose of X16, followed a month later by the three weekly $10^8$ intraloop stimulations. We were not able to demonstrate any enhancement of the local immune response that followed. The parenteral dose did, however, succeed in priming the systemic immune response, so that after the subsequent intraloop stimulations, serum IgG against shigella X16 was detectable in five of the six animals in this group. This is in contrast to the other rabbits that received live invasive shigella wherein no serum IgG response is usually seen. Stimulation of serum antibody responses by mucosal antigen has been shown repeatedly by Rothberg et al. (42), and in the present situation it was clearly enhanced by prior parenteral priming.

Whether production of systemic IgG antibodies to the shigella would be desirable for protection is conjectural at the present time. No local IgG against shigella LPS was detectable in the intestinal secretions from these parenterally primed rabbits. Our recent studies on the stability of IgG and IgA in Thiry-Vella loop secretions support the concept that IgG against shigella is not produced locally and is not merely degraded rapidly in the lumen of the isolated loops (22). Interestingly, other workers using a similar rabbit model with mucosal stimulation by shigella did find detectable hemagglutination titers against shigella in the sera of their rabbits (3). However, the doses of live, invasive shigella used in their studies were from two to more than three logs greater than in the present studies, which could account for this difference. They also found coproantibodies, using a passive hemagglutination technique, which appeared in approximately the same sequence as the local IgA responses described in the present study (3).

It is also of interest that there was no evidence of suppression of the local immune response after prior parenteral priming with shigella X16. Recent studies by Yardley et al., Hamilton et al., and Pierce and Koster (15, 39, 49) have found suppression of the local immune response to cholera toxin after prior parenteral priming with cholera toxin or cholera toxoid. In contrast, others have indicated that parenteral priming enhances the subsequent oral immunization with cholera toxin (38, 40). This is obviously a complex phenomenon, and whether suppression or enhancement occurs may depend on dose, antigen form, route, and temporal sequence of immunization. These complexities serve to emphasize the importance of using carefully controlled experimental models of local immunity to explore these variables in depth before extensive field trials in humans.

Lastly, for several months we collected the daily secretions of four rabbits stimulated by three weekly doses of X16. At a time when their IgA against shigella was thought to have fallen to base-line levels, three of the four animals produced a more rapid response to a single local injection of X16 than they did after their first exposure to the antigens. The fourth animal also responded, but since its response was not yet at a base-line level when stimulated, it is not possible to determine its significance. Nonetheless, these findings provide evidence that at least some mucosal memory response to shigella antigens can be elicited. By studying the various parameters of mucosal immunization in the isolated loop model, it will be possible to optimize the mucosal memory response, which is the key to development of effective vaccines against dysentery and other mucosal infectious diseases.

## ACKNOWLEDGMENTS

The authors are indebted to Bob Sied at the Walter Reed Army Institute of Research for determining the level of LPS in X16 preparations.

This work was supported in part by University of Michigan Biomedical Research Council grant no. 016153 and by Horace H. Rackham grant no. 387455.

## LITERATURE CITED

1. **Besredka, A.** 1930. P. 268. *In* Immunity in infectious disease. A series of studies. Bailliere, Tindell and Cox, London.
2. **Davies, A.** 1922. An investigation into the serological properties of dysenteric stool. Lancet **ii**:1009–1012.
3. **Denchev, V., A. Trifonova, M. Jeleva, S. Duncheva, V. Bratanova, Z. Dimitrova, and P. Kovachev.** 1974. Immunity against dysentery after enteral immunization with live vaccine. Acta Microbiol. Acad. Sci. Hung. **21**:71–74.
4. **DuPont, H. L., R. B. Hornick, M. J. Snyder, J. L. Libonati, S. B. Formal, and E. J. Gangarosa.** 1972. Immunity in shigellosis. II. Protection induced by oral live vaccine or primary infection. J. Infect. Dis. **125**:12–16.
5. **Eisenstein, T. K., and C. R. Angerman.** 1978. Immunity to experimental salmonella infection: studies on the protective capacity and immunogenicity of lipopolysaccharide, acetone-killed cells, and ribosome-rich extracts of *Salmonella typhimurium* in C3H/HeJ and CD-1 mice. J. Immunol. **121**:1010–1014.
6. **Formal, S. B., L. S. Baron, and W. Spilman.** 1956. The virulence and immunogenicity of *Salmonella typhosa* grown in continuous culture. J. Bacteriol. **72**:168–172.
7. **Formal, S. B., T. H. Kent, S. Austin, and E. H. LaBrec.** 1966. Fluorescent antibody and histological study of vaccinated and control monkeys challenged with *Shigella flexneri*. J. Bacteriol. **19**:2368–2376.
8. **Formal, S. B., T. H. Kent, H. C. May, A. Palmer, S. Falkow, and E. H. LaBrec.** 1966. Protection of monkeys against experimental shigellosis with a living attenuated oral polyvalent dysentery vaccine. J. Bacteriol. **92**:17–22.
9. **Formal, S. B., E. H. LaBrec, T. H. Kent, and S. Falkow.** 1965. Abortive intestinal infection with an *Escherichia coli-Shigella flexneri* hybrid strain. J. Bacteriol. **89**:1374–1382.
10. **Formal, S. B., E. H. LaBrec, A. Palmer, and S. Falkow.** 1965. Protection of monkeys against experimental shigellosis with attenuated vaccines. J. Bacteriol. **90**:63–68.
11. **Formal, S. B., R. M. Maenza, S. Austin, and E. H. LaBrec.** 1967. Failure of parenteral vaccines to protect monkeys against experimental shigellosis. Proc. Soc. Biol. **125**:347–349.
12. **Freter, R., P. C. M. O'Brien, and M. S. Macsai.** 1979. Effect of chemotaxis on the interaction of cholera vibrios with intestinal mucosa. Am. J. Clin. Nutr. **32**:128–132.
13. **Goodman, G. W., and B. M. Sultzer.** 1979. Characterization of the chemical and physical properties of a novel B-lymphocyte activator, endotoxin protein. Infect. Immun. **24**:685–696.
14. **Grahneis, H.** 1974. Successful field trials with oral killed *Shigella sonnei* vaccine. Acta Microbiol. Acad. Sci. Hung. **21**:75–80.
15. **Hamilton, S. R., J. H. Yardley, and G. D. Brown.** 1979. Suppression of local intestinal IgA response to cholera toxin by subcutaneous administration of cholera toxoids. Infect. Immun. **24**:422–426.
16. **Hardy, A. V., T. Decapito, and S. P. Halbert.** 1948. Studies of the acute diarrheal diseases. XIX. Immunition in shigellosis. Public Health. Rep. **64**:685–688.
17. **Higgins, A. R., T. M. Floyd, and M. A. Kader.** 1955. Studies in shigellosis. III. A controlled evaluation of a monovalent shigella vaccine in a highly endemic environment. Am. J. Trop. Med. Hyg. **4**:281–288.
18. **Keren, D. F.** 1979. Enzyme-linked immunosorbent assay for immunoglobulin G and immunoglobulin A antibodies to *Shigella flexneri* antigens. Infect. Immun. **24**:441–448.
19. **Keren, D. F., H. L. Elliott, G. D. Brown, and J. H. Yardley.** 1975. Atrophy of villi with hypertrophy and hyperplasia of Paneth cells in isolated (Thiry-Vella) ileal loops in rabbits. Gastroenterol. **68**:83–93.
20. **Keren, D. F., P. S. Holt, H. H. Collins, P. Gemski, and S. B. Formal.** 1978. The role of Peyer's patches in the local immune response of rabbit ileum to live bacteria. J. Immunol. **120**:1892–1896.
21. **Keren, D. F., P. S. Holt, H. H. Collins, P. Gemski, and S. B. Formal.** 1980. Variables affecting the local immune response in Thiry-Vella loops. I. Role of immunization schedule, bacterial flora, and postsurgical inflammation. Infect. Immun. **28**:950–956.
22. **Keren, D. F., P. J. Scott, and D. Bauer.** 1980. Variables affecting the local immune response in Thiry-Vella loops. II. Stability of antigen-specific IgG and secretory IgA in acute and chronic Thiry-Vella loops. J. Immunol. **124**:2620–2624.
23. **Ketyi, I., K. Rauss, and A. Vertenyi.** 1974. Oral immunization against dysentery. Acta Microbiol. Acad. Sci. Hung. **21**:81–85.
24. **Keusch, G. R., G. F. Grady, L. J. Mata, and J. McIver.** 1972. Pathogenesis of shigella diarrhea. I. Enterotoxin production by *Shigella dysenteriae* 1. J. Clin. Invest. **5**:1212–1218.
25. **Kowalewska, D., and M. Malczyk.** 1974. Immunity to keratoconjunctivitis shigellosa in rabbits after oral immunization with *Shigella sonnei* free endotoxin. Acta Microbiol. Acad. Sci. Hung. **21**:99–102.
26. **LaBrec, E. H., and S. B. Formal.** 1961. Experimental shigella infections. IV. Fluorescent antibody studies of an infection in guinea pigs. J. Immunol. **87**:562–572.
27. **LaBrec, E. H., H. Schneider, T. J. Magnani, and S. B. Formal.** 1964. Epithelial cell penetration as an essential step in the pathogeneis of bacillary dysentery. J. Bacteriol. **88**:1503–1518.
28. **Levin, M. M., H. L. DuPont, S. B. Formal, R. B. Hornick, A. Takeuchi, E. J. Gangarosa, M. J. Snyder, and J. P. Libonati.** 1973. Pathogenesis of *S. dysenteriae* (Shiga) dysentery. J. Infect. Dis. **127**:261–270.
29. **Mackel, D. C., L. F. Langley, and L. A. Venice.** 1961. The use of the guinea pig conjunctivae as an experimental model for the study of virulence of *Shigella* organisms. Am. J. Hyg. **73**:219–223.
30. **Meitart, T., I. T. Sulea, L. Gogulescu, E. Baron, C. Tempea, and G. Istrati.** 1974. Immunogenic value of live, inactivated, and cell-free dysentery vaccines. Acta Microbiol. Acad. Sci. Hung. **21**:103–107.
31. **Mel, D., E. J. Gangarosa, M. L. Radovanovic, B. L. Arsic, and S. Litoinjenko.** 1971. Studies on bacillary dysentery. Bull. W.H.O. **45**:457–464.
32. **Mel, D. M., R. G. Papo, A. L. Terzin, and L. Vuksic.** 1965. Studies on vaccination against bacillary dysentery. 2. Safety tests and reactiogenicity studies on a line dysentery vaccine intended for use in field trials. Bull. W.H.O. **32**:637–645.
33. **Mel, D. M., A. L. Terzin, and L. Vuksic.** 1965. Studies on vaccination against bacillary dysentery. I. Immunization of mice against experimental shigella infection. Bull. W.H.O. **32**:633–636.
34. **Mel, D. M., A. L. Terzin, and L. Vuksic.** 1965. Studies

on vaccination against bacillary dysentery. 3. Effective oral immunization against *Shigella flexneri* 2a in a field trial. Bull. W.H.O. **32**:647–655.

35. **O'Brien, A. D., M. K. Genry, M. R. Thompson, B. P. Doctor, P. Gemski, and S. B. Formal.** 1979. Shigellosis and *Escherichia coli* diarrhea: relative importance of invasion and toxigenic mechanisms. Am. J. Clin. Nutr. **32**:229–233.
36. **Owen, R. L.** 1977. Sequential uptake of horseradish peroxidase by lymphoid follicles epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study. Gastroenterol. **72**:440–451.
37. **Pierce, N. F.** 1978. The role of antigen form and function in the primary and secondary immune responses to cholera toxin and toxoid in rats. J. Exp. Med. **148**:195–206.
38. **Pierce, N. F., and J. L. Gowans.** 1975. Cellular kinetics of the intestinal immune response to cholera toxoid in rats. J. Exp. Med. **142**:1550–1563.
39. **Pierce, N. F., and F. T. Koster.** 1980. Priming and suppression of the intestinal immune response to cholera toxoid/toxin by parenteral toxoid in rats. J. Immunol. **124**:307–311.
40. **Pierce, N. F., R. B. Sack, and B. K. Sicar.** 1977. Immunity to experimental cholera. III. Enhanced duration of protection after sequential parenteral-oral administration of toxoid to dogs. J. Infect. Dis. **135**:888–896.
41. **Robertson, S. M., and J. J. Cebra.** 1976. A model for local immunity. Ric. Clin. Lab. **6(Suppl. 3)**:105–119.
42. **Rothberg, R. M., S. C. Kraft, and S. M. Michalek.** 1973. Systemic immunity after local antigenic stimulation of the lymphoid tissue of the gastrointestinal tract. J. Immunol. **111**:1906–1913.
43. **Rout, W. R., S. B. Formal, R. A. Giannella, and G. J. Dammin.** 1975. Pathophysiology of shigella diarrhea in the rhesus monkey: intestinal transport, morphological and bacteriological studies. Gastroenterol. **68**:270–278.
44. **Skidmore, B. J., D. C. Morrison, J. M. Chiller, and W. O. Weigle.** 1975. Immunologic properties of bacterial lipopolysaccharide (LPS). J. Exp. Med. **142**:1488–1508.
45. **Swift, H., and Z. Hruban.** 1964. Focal degradation as a biological process. Fed. Proc. **23**:1026–1037.
46. **Takeuchi, A., H. Sprintz, and E. H. LaBrec, and S. B. Formal.** 1965. Experimental bacillary dysentery. An electron microscopic study of the response of the intestinal mucosa to bacterial invasion. Am. J. Pathol. **47**: 1011–1044.
47. **Watson, J., M. Largen, and K. P. W. J. McAdam.** 1978. Genetic control of endotoxic responses in mice. J. Exp. Med. **147**:39–49.
48. **World Health Organization.** 1972. Oral enteric bacterial vaccines, p. 19–33. *In* World Health Organization technical report service no. 500. World Health Organization, New York.
49. **Yardley, J. H., D. F. Keren, S. R. Hamilton, and G. D. Brown.** 1978. Local (IgA) immune response by the intestine to cholera toxin and its partial suppression with combined systemic and intra-intestinal immunization. Infect. Immun. **19**:589–597.