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Variables Affecting Local Immune Response in Ileal Loops: Role of Immunization Schedule, Bacterial Flora, and Postsurgical Inflammation

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Several variables inherent in chronically isolated ileal (Thiry-Vella) loops in rabbits were studied for their effect on the local immune response of the intestine to live, locally invasive bacteria (*Shigella* X16). A much more vigorous local immunoglobulin A response to *Shigella* X16 was elicited when rabbits were immunized in their Thiry-Vella loops shortly after surgical creation of the loop than if a week were allowed to pass before they were immunized. Three major differences existed in Thiry-Vella loops on the day after surgery and a week later: (i) their microbial flora, (ii) nonspecific acute inflammation due to the surgery itself, and (iii) the histological appearance of the intestine. On day 1 after surgical creation of the Thiry-Vella loop, there were few bacteria in the loop, and the histology was that of normal small bowel except for mild acute inflammation due to the surgery. By day 6 after surgery, all loops contained large numbers of *Pseudomonas aeruginosa* and other aerobes, an atrophy of intestinal epithelium occurred, and the acute inflammation due to surgical trauma had subsided. By artificially colonizing Thiry-Vella loops with 10^8 or 10^{10} live *P. aeruginosa* on the day of surgery, we found that the presence of these bacteria alone did not greatly diminish local immune responses to live *Shigella*. Furthermore, when the acute inflammation due to surgical trauma was recreated in loops 6 days old, no enhancement of the immune response was seen as compared to nontraumatized 6-day-old Thiry-Vella loops. The difference between immunization soon after surgery and a week later related to changes that occur in the loop itself with increased isolation. Finally, multiple immunizations of Thiry-Vella loops resulted in a more vigorous local immunoglobulin A response than a single immunization. These studies demonstrated that Thiry-Vella loop models can be useful in studying the kinetics of local immune responses by the intestine only if careful attention is paid to key variables inherent in the Thiry-Vella loop models themselves.

The mucosa of the gastrointestinal tract contains a wealth of immunoglobulin-secreting cells and their precursors (3, 5). After oral immunization, many plasma cells with specific antibody (predominantly immunoglobulin A [IgA]) to the immunizing antigen can be found in the lamina propria of the gut, and secretory IgA can be detected in intestinal secretions (4, 9, 24). However, the mechanism for optimally stimulating the local immune response is still poorly understood.

In an attempt to define the parameters involved in eliciting mucosal immunity, several groups have developed chronically isolated intestinal (Thiry-Vella) loops in a variety of species, namely, swine, sheep, rabbits, calves, dogs, and rats (13, 14, 16, 18, 25, 29). By challenging these loops directly with various antigens, a remarkable homogeneity has been observed in the kinetics of the initial local immune response to bacteria, viruses, toxins, and relatively inert molecules such as dinitrophenol-keyhole limpet hemocyanin or keyhole limpet hemocyanin only (11, 13, 14, 25, 28, 31; S. R. Hamilton, J. H. Yardley, and D. F. Keren, Fed. Proc. **37**:1681, 1978). Despite the use of different animals, with various antigen doses, a distinctive local immune response is usually detected by day 7, and often as early as day 4, after local antigenic stimulation (11, 17, 18, 25, 28, 31; Hamilton et al., Fed. Proc., **37**:1681, 1978). The local immune responses described thus far could be maintained only by repeated local immunizations (11, 14, 17, 25, 31; Hamilton et al., Fed. Proc. **37**:1681, 1978), as has been the case in most other enquiries into local immune mechanisms (4, 7, 19, 21, 23, 26).

However, no other groups have looked at the relative strengths of specific antibodies to see whether the age of the isolated loop itself is a factor in the mucosal immune response obtained.

Although these Thiry-Vella loop models have been valuable for studying local immunity in the intestine, the data generated by their use have not yet taken into consideration how variables inherent in the model itself affect the results obtained. For instance, the fact that the bacterial flora and histology of these loops change after isolation (16; P. S. Holt, D. F. Keren, and H. H. Collins, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, B26, p. 18) has not been noted by most other groups, yet these changes may have a significant effect on the development of the local immune response in this model. The present investigation examines how the local and systemic immune responses to a locally invasive *Shigella* strain are affected by: (i) different immunization schedules, (ii) nonspecific inflammation after surgical creation of the Thiry-Vella loops, and (iii) bacterial flora that colonize these loops.

MATERIALS AND METHODS

Construction of isolated ileal segments. A single 20-cm ileal loop was constructed in each of 56 3- to 4-kg New Zealand White rabbits as previously described (16). Briefly, after anesthetization by phenobarbital, a midline abdominal incision was made and a 20-cm segment of ileum containing a grossly identifiable Peyer's patch was isolated. After silastic tubing (Dow-Corning) was sewn into each end of this segment of ileum, the ends of the tubing were brought out through the midline abdominal incision and tunnelled subcutaneously to the nape of the neck, where they were exteriorized and secured. Continuity was restored to the intestine by an end-to-end anastomosis of the remaining ileum.

Care of ileal loops and collection of ileal secretions. Each day, secretions that accumulated in the ileal loops were expelled by injecting 20 ml of air into one silastic tube. The slightly opaque, colorless fluid was centrifuged at 2,000 rpm for 10 min, and clear supernatant was separated and stored at −20°C for future assay. The loops were then flushed with 20 ml of sterile saline, which was subsequently removed by 20- and 40-ml flushes with air. The latter two flushes prevent mucus from obstructing the silastic tubing.

Immunization. The ileal loops were immunized with 10^8 live *Shigella* X16 (a hybrid of *Shigella flexneri* and *Escherichia coli* which can invade the surface epithelium of experimental animals but has limited ability to multiply once within the tissue [8]). These organisms were suspended in 4 ml of saline and given intraloop by the various schedules listed in Table 1.

Quantitation of bacterial flora of Thiry-Vella loops. Fresh daily loop secretions from 25 rabbits (15 received live shigellae, 10 did not; no difference in bacterial flora was found between these two groups except for the artificially administered shigellae in the former) were studied for the type of bacterial flora present. Samples of the loop secretions were plated onto the listed media and incubated at 37°C for 24 h. Media used to detect aerobic bacteria were: blood agar (5% sheep erythrocytes in Trypticase soy agar), MacConkey's lactose agar, and blood agar with 0.25% phenyl ethyl alcohol. To detect the more facultative anaerobes, samples were plated on blood agar, blood agar with 0.25% phenyl ethyl alcohol, and Rogosa agar (for lactobacilli) and placed in a bell jar with anaerobic conditions produced by the GasPak Anaerobic system (BBL Microbiology Systems, Cockeysville, Md.) for 48 h. These conditions, however, did not allow detection of strict anaerobes. Isolates were identified by using the API 20E system (Analytab Products, Inc., Plainview, N.Y.), by oxidation or fementation of various carbohydrates, and by indophenol oxidase production.

Bacterial density in loop fluids from the 10 rabbits not immunized with shigellae was determined by serial dilutions on the above media. Colony counts were reported as colonies per milliliter (of loop fluid).

Enzyme-linked immunosorbent assay for specific IgG and IgA. The details of the enzyme-linked immunosorbent assay used to determine the specific antibody content of both serum and ileal loop secretions have been described previously (15). Briefly, polystyrene test tubes (Falcon Plastic 2052 tube) were coated with a solution containing 10 μg of *Shigella* X16 lipopolysaccharide per ml or with a solution containing 10 μg of *Pseudomonas aeruginosa* lipopolysaccharide (Westphal preparations from the same organisms used to immunize the loops) per ml in 0.05 M carbonate buffer (pH 9.6). Immediately before testing, the antigen solution was removed and the tubes were washed with 0.01 M phosphate-buffered saline (pH 7.3) containing 0.05% Tween 20 (Fisher Scientific Co., Pittsburgh, Pa.) and 0.02% sodium azide (PTA). The rabbit sera or intestinal secretions to be tested were diluted 1:20 in PTA, and 0.5 ml was added both to tubes coated with antigen and to uncoated tubes (as

TABLE 1. *Immunizing schedule*

Group	Immunization day[a]	Comment
I	6, 13	Animals followed for 20 days after surgery
II	1, 8, 15	
III	1	
IV	1, 8, 15	Loops colonized with 10^8 *P. aeruginosa* on day 0 and day 1 after surgery
V	1, 8, 15	Loops colonized with 10^{10} *P. aeruginosa* on day 0 and day 1 after surgery
VI	6, 13, 20	Loops reoperated on day 5 after initial surgery
VII	6, 13, 20	Animals followed for 27 days after surgery

[a] Intraloop; day of surgery = day 0.

a control for nonspecific adsorption). After the tubes were incubated for 4 h on a horizontal rotary shaker, they were washed again four times with PTA, and 0.5 ml of alkaline phosphatase-conjugated goat anti-rabbit IgG or alkaline phosphatase-conjugated goat anti-rabbit IgA (prepared as described previously [15]) was added to the appropriate tubes. The reaction mixtures were incubated for 18 h at room temperature with agitation. After four more PTA washes, 1 mg of the substrate, *p*-nitrophenyl phosphate disodium (Sigma Chemical Co., St. Louis, Mo.), in 1 ml of 0.05 M carbonate buffer (pH 9.8) containing 0.001 M magnesium chloride, was added to each tube. The reaction was developed at room temperature until absorbance at 400 nm was approximately 1.0 (the enzyme-substrate reaction is linear to this level [6]), being stopped at 12.5, 25, 50, or 100 min by the addition of 1 ml of 0.2 N NaOH. All results were extrapolated to 100 min. The optical density of 400 nm of uncoated tubes was subtracted from the optical density of 400 nm of the antigen-coated tubes containing the same dilution.

IgG against *Shigella* X16 lipopolysaccharide (IgG anti-X16), IgA anti-X16, and IgA anti-*P. aeruginosa* (anti-PA) standards (prepared as described previously [15]) were processed daily with the unknown loop fluids and sera. To minimize day-to-day variation, results of the standards were normalized to a fixed number, and the values of the unknown specimens were corrected to these normalized standards. This enzyme-linked immunosorbent assay system can detect as little as 1.3 ng of specific antibody per ml and has coefficients of variation of 3.6 and 9.0% for the IgG anti-X16 and IgA anti-X16 standards, respectively (15, 17).

Results for each group of rabbits are expressed as the mean ± standard error of the mean. Significance was assessed by Student's *t* test.

RESULTS

Bacterial flora in Thiry-Vella loops. Bacteria were not usually detected in the Thiry-Vella loops for the first 2 days after surgery. By day 4 after surgical creation of the isolated loops, more than half of them were colonized, and by day 12 all loop fluids contained bacteria (Fig. 1). *P. aeruginosa* were overwhelmingly the most prevalent bacteria, having a density of from 10^6 to 10^8 colonies per ml. *P. aeruginosa* often coexisted with other *Pseudomonas* species (10^2 to 10^4 colonies per ml), *Achromobacter xylosoxidans* (10^2 to 10^4 colonies per ml), *Acinetobacter calcoaceticus* (10^2 to 10^3 colonies per ml), *E. coli* (10^1 to 10^3 colonies per ml), and, rarely, *Bacteroides* species or *Staphylococcus aureus*.

Although anaerobic bacteria were not identified, this may have been due to the insensitivity of the method. Ongoing studies with this model, however, suggest that anaerobes are not prevalent in these Thiry-Vella loops even when more rigorous isolation techniques are used (McClatchey et al., personal communication).

Effect of immunization schedule. Seven rabbits were immunized with 10^8 live *Shigella* X16 intraloop on days 6 and 13 after surgery (group I). The daily secretions were collected until day 20 after surgery. Only a weak local antibody response was detected in these rabbits (Fig. 2), even after the second local immunization.

To follow the secretions for a longer period of time and give a third intraloop injection, six rabbits were immunized with 10^8 live *Shigella* X16 intraloop on days 1, 8, and 15 after surgery (group II). Secretions from these animals were collected until day 22 after surgery. As depicted in Fig. 3, the mean IgA anti-X16 reaction was considerably stronger in this group than in the group I animals. This increased IgA anti-X16 response was not merely due to the third injection, since the mean IgA anti-X16 was significantly greater in group II animals than in group I animals on days 11 ($P < 0.01$) and 14 ($P < 0.05$) after the first antigen administration, yet only

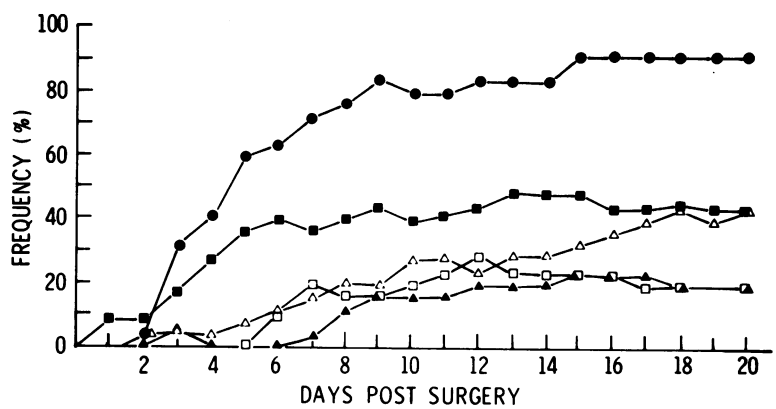

FIG. 1. *Percent of ileal loop fluids from 25 rabbits that contain P. aeruginosa* (●); *E. coli* (■); *Pseudomonas sp.* (△); *A. xylosoxidans* (▲); *A. calcoaceticus* (□).

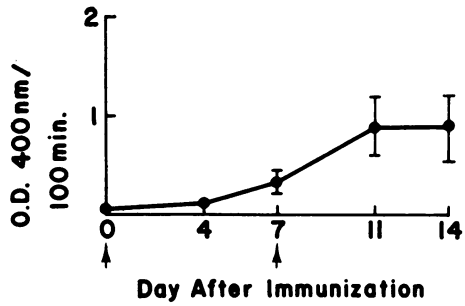

FIG. 2. *Mean IgA anti-X16 in fluids from ileal loops first immunized on day 6 after surgery (group I). Vertical axis expresses the mean net optical density at 400 nm per 100 min for the isolated loop fluid enzyme-linked immunoassay results (see the text). Standard error of the mean is indicated. Arrows indicate days when loops received 10^8 live Shigella X16.*

two doses of antigen had been given to each group. After the third dose of antigen to the group II animals on day 14, no further increase in mean IgA anti-X16 was demonstrated (Fig. 3).

Six animals were immunized only on day 1 after surgery (group III) to see whether a second dose was necessary to achieve a maximal response. As shown in Fig. 3, the mean IgA anti-X16 responses of group III were considerably less than the group II responses on days 11 ($P < 0.02$) and 14 ($P < 0.05$). Clearly, the intraloop boost with 10^8 *Shigella* X16 on day 7 enhanced the subsequent local immune response in the group II animals. Overall, the responses of the group III animals were similar to those of group I.

Effect of ileal loop flora. We have demonstrated that our rabbit loops contained relatively few bacteria for the first 2 days after surgery, but thereafter they are quickly colonized, mainly with *P. aeruginosa* and other aerobes. To determine whether the *P. aeruginosa* was interfering with the local immune response to *Shigella* X16 in group I, the isolated loops of seven rabbits were colonized with 10^8 live *P. aeruginosa* (group IV), and the isolated loops of five other rabbits were colonized with 10^{10} live *P. aeruginosa* (group V) on both the day of surgery and day 1 after surgery. These rabbits were immunized with *Shigella* X16 according to the schedule that gave the best local immune responses, i.e., days 1, 8, and 15 after surgery. The mean IgA anti-X16 responses in both group IV and V were somewhat decreased as compared to the group II animals (Fig. 4). However, the decrease observed was not significant for any day for either group IV or group V.

Lack of correlation of IgA activity against *Shigella* X16 with IgA activity against *P. aeruginosa*. Since Thiry-Vella loops in this study were colonized with *P. aeruginosa*, we wanted to determine whether local antibodies were formed to that microorganism, and whether the formation of such antibodies had any effect on the local immune response to *Shigella* X16. Therefore, both IgA anti-PA and IgA anti-X16 activity were measured by enzyme-linked immunosorbent assay in loop fluids from animals artificially colonized with 10^8 *P. aeruginosa* (group IV) or with 10^{10} *P. aeruginosa* (group V) before local immunization with *Shigella* X16. A single strain of *P. aeruginosa* isolated from the chronic ileal loop flora was used for all colonizations.

The IgA anti-PA and IgA anti-X16 values in loop secretions from these animals for days 14 and 18 after the first X16 intraloop stimulations are shown in Table 2. Data from days 14 and 18 are presented because a strong local IgA anti-X16 response was usually present by these days, which allowed comparison with the IgA anti-PA response. There was no correlation between the presence or absence of IgA anti-PA and the amount of IgA anti-X16 detected in these loop

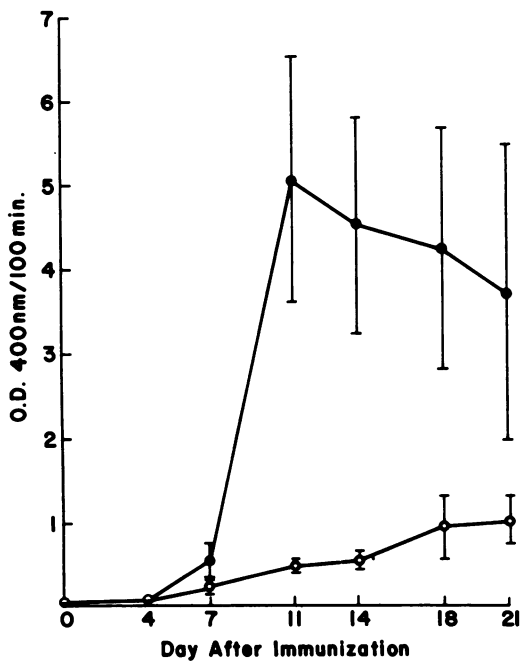

FIG. 3. *Mean IgA anti-X16 in fluids from ileal loops immunized on days 1, 8, and 15 after surgery (●) or only on day 1 after surgery (○) with 10^8 live Shigella X16. Bars indicate standard error of the mean.*

secretions. For instance, strong IgA responses against both *P. aeruginosa* and *Shigella* X16 were detected in secretions from rabbits 16 and 182 on days 14 and 18. Secretions from animals such as no. 973 had only trivial activity against *P. aeruginosa* but strong activity to *Shigella* X16 on day 14, whereas the reverse was true for the day 18 response of rabbit 185.

Effect of postsurgery inflammation. To determine whether postsurgery inflammation was responsible for the enhanced immune response seen in the group II animals, a second operation was performed. On day 5 after surgical construction of isolated ileal loops in eight rabbits, the animals were reoperated on. During this second operation, the cuffs of intestine sewn to the silastic tubes were removed (thereby recreating the original surgical trauma to these segments) (Fig. 5). The newly traumatized ileal loop ends were sewn into silastic tubing, and the abdomen was closed. These loops were stimulated on days 1, 8, and 15 after this second surgical procedure (days 6, 13, and 20 after the original creation of the loop) (group VI). They were, however, already colonized by bacteria and had the morphological changes (atrophy of villi with hypertrophy of crypts) that loops would have on day 6 after surgery (17).

The mean IgA anti-X16 values in these loop secretions were compared with those of rabbits immunized on days 6, 13, and 20 (but not reoperated) that were followed for 27 days after surgery (group VII). The group VI and VII rabbits had decreased immune response as compared to group II ($P < 0.05$ for days 11 and 14) (Fig. 6).

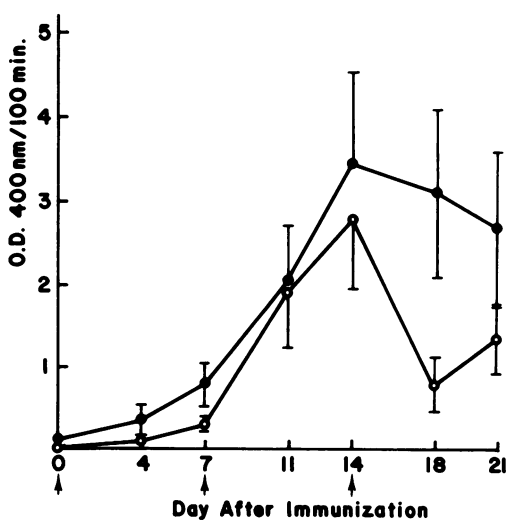

FIG. 4. *Mean IgA anti-X16 in fluids from ileal loops colonized with 10^8 (●) or 10^{10} (○) live P. aeruginosa before being immunized with 10^8 Shigella X16 on days 1, 8, and 15 after surgery. Arrows indicate days when loops received shigellae.*

TABLE 2. *IgA against Shigella X16 and P. aeruginosa in the same loop fluids*

Group	Rabbit no.	Optical density at 400 nm			
		Day 14[a]		Day 18[a]	
		IgA anti-X16	IgA anti-PA	IgA anti-X16	IgA anti-PA
IV[b]	70	1.565	1.264	3.106	0.914
	75	2.990	—[c]	1.923	—
	182	8.354	2.701	5.758	4.548
	185	0.413	0.277	0.303	2.515
	16	2.228	1.912	6.822	2.734
	01	0.700	3.090	0.697	1.497
	129	0.026	0.237	0.207	0.568
V[d]	49	0.476	1.828	0.228	0.274
	55	4.310	0.951	1.358	0.260
	973	5.636	0.168	2.078	0.884
	699	3.273	—	0.198	—
	774-2	0.293	3.788	0.090	3.488

[a] Day after first intraloop administration of *Shigella* X16.

[b] Group IV received 10^{10} *P. aeruginosa* on days 0 and 1 and 10^8 *Shigella* X16 on days 1, 8, and 15 after surgery.

[c] —, Not done.

[d] Group V received 10^8 *P. aeruginosa* on days 0 and 1 and 10^8 *Shigella* X16 on days 1, 8, and 15 after surgery.

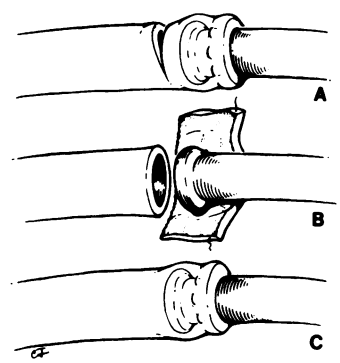

FIG. 5. *Procedure to recreate acute surgical trauma to isolated loops. On day 5 after initial surgery, the cuff of intestine attached to the silastic tubing was incised (A) and removed (B). The freshly traumatized loop was pulled over the end of the silastic tubing and sewn in place (C) as during the original surgery.*

DISCUSSION

Although Thiry-Vella loop models are useful for probing the kinetics of local immune responses, variables inherent in these models must

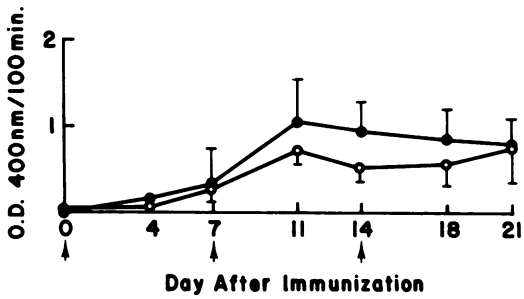

FIG. 6. *Mean IgA anti-X16 in fluids from ileal loops immunized days 6, 13, and 20 after initial surgical creation of the loop.* (●) *Rabbits that had a second surgical procedure on day 5 after initial preparation of the isolated loops;* (○) *undisturbed loops. Bars indicate standard error of the mean. Arrows indicate days when loops were given* 10^8 *live Shigella X16.*

be taken into consideration when interpreting results. For instance, a strong mucosal immune response was stimulated best by three weekly doses of bacteria beginning on day 1 after surgery. Animals given only one dose developed weak though sustained responses. Furthermore, animals whose immunizations did not begin until 6 days after surgery gave inferior local IgA responses. Most animals, regardless of dosage schedule, gave no or trivial systemic IgG anti-X16 or IgA anti-X16 responses, and only trivial IgG anti-X16 was detected, rarely, in ileal loop secretions (data not shown).

The most likely explanations for the inferior local immune responses produced by animals immunized on days 6 and 13 after surgery related to changes that occur in the bacterial flora and morphology of isolated Thiry-Vella loops as they age (16; Holt et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, B26, p.18). In the present study, the Thiry-Vella loop secretions had few bacteria for the first 2 days after surgery; however, by day 6 after surgery, most of the loop secretions contained at least 10^8 *P. aeruginosa*. Other aerobes, including *E. coli*, *A. xylosoxidans*, *A. calcoaceticus*, and other *Pseudomonas* species, also colonized these loops to a lesser extent. Since *P. aeruginosa* was by far the most predominant species, we were concerned that it might interfere with the local immune response to the live *Shigella* X16. However, results of our studies whereby the loops were artificially colonized with 10^8 or 10^{10} live *P. aeruginosa* on day 1 after surgery indicate that colonization had little effect on the local immune response to *Shigella* X16.

Several studies have shown that segments of small intestine isolated for longer than 6 days undergo an atrophy of the villi that is usually accompanied by a hypertrophy of the crypts (and, at least in the rabbit, a hyperplasia of Paneth cells) (1, 10, 12, 16, 20). The etiology of the villus atrophy is unclear, but has been attributed to: (i) loss of intraluminal nutrients (10, 27), (ii) loss of other intraluminal substances such as bile (16, 30), or (iii) the decreased functional absorptive work-load in such segments (2). Also unclear is whether specialized surface epithelial cells (M cells) that overlie Peyer's patches and take up macromolecules (22) also undergo change in Thiry-Vella loops. Atrophied M cells could interfere with initial processing of antigen and would explain the weak local immune responses obtained in animals immunized on days 6 and 13 after initial surgery (groups I, VI, and VII).

Since the best local immune responses were obtained when immunization began the day after surgery, we were concerned that nonspecific inflammation after surgical creation of the Thiry-Vella loops may have artificially enhanced the local immune response. However, the data generated from animals whose loops were retraumatized on day 5 after surgery indicate that no significant enhancement of the local immune response results from nonspecific inflammation after surgery.

Finally, the amount of lymphoid tissue included in these loops will affect the local immune response elicited. Inclusion of a Peyer's patch in a Thiry-Vella loop enhances the strength of the early local immune response, although comparable immune responses can be seen after longer periods in loops lacking Peyer's patches (17). The latter response may relate to the presence throughout the intestine of large numbers of isolated lymphoid follicles which morphologically resemble Peyer's patches. Other workers using Thiry-Vella loops have also noted an enhanced immune response with a Peyer's patch present in the loop (13), and some have observed almost no local immune response when a Peyer's patch was not included (28).

Although Thiry-Vella loops are useful models for studying local immunity, future work must take into account more of the variables inherent within this model system. The present study has demonstrated that dosage schedule, bacterial flora, and mucosal atrophy may be important factors in using this model system to probe local immunity. Future studies must deal with these and other factors such as the integrity of follicle-associated epithelium with chronic isolation, type and function of the inflammatory cells that egress into these loops, and the stability of immunoglobulin secretions in a Thiry-Vella loop.

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