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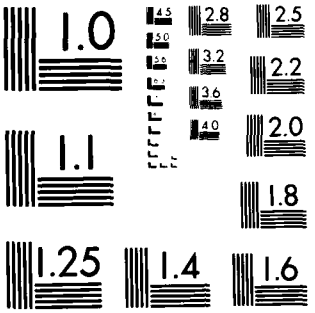

AN INVESTIGATION OF THE MEMORY RESPONSE OF THE LOCAL IMMUNE SYSTEM TO SHIGELLA ANTIGENS

ANNUAL REPORT

JANUARY 1984

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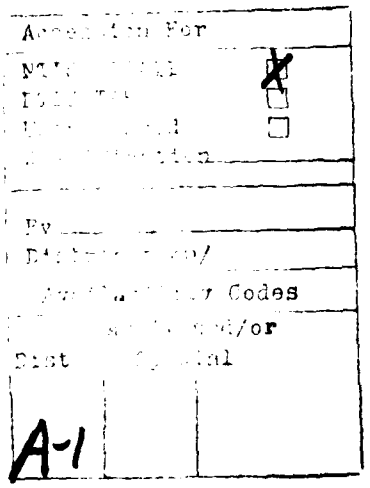

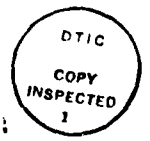

FOREWARD

During the course of this work, the author was greatly assisted by Roderick McDonald, Liz Struble, Patricia Scott, Scott Kern, and Arthur Rosner. In addition, the excellent Laboratory Animal Medicine Department at The University of Michigan continues to provide excellent care for our animals. The help of thse individuals expecially Drs. Ringler and Carey is deeply appreciated.

In conducting the research described in this report, the investigator followed the "Guide for Care and Use of Laboratory Animals" prepared by The Committee on Care and Use of Laboratory Animals of The Institute of Laboratory Animal Resources, National Research Council (DHEW Publication #(NIH) 78-23, 1978).

INTRODUCTION

This fourth annual report includes work completed from 1 February, 1983 to the present (31 January, 1984).

In the past few years, the focus of work from this and other laboratories has been to explore the role of both systemic and mucosal immunity to alter the natural course of enteropathogenic infections. Due to its relative inaccessibility, it has been difficult to examine, in a sequential fashion, the development of the mucosal IgA response to various vaccines. The main approach to this problem that our laboratory has used is the chronically isolated ileal loop model in rabbits (1). We have used these loops as a probe to follow the kinetics of the local IgA response to S. flexneri antigens in the gut lumen. The feasibility of using this approach to study local immunity evolves from the information produced in many laboratories defining the route of antigen stimulation and lymphocyte trafficking throughout the body after mucosal presentation of antigen. After uptake of antigen by specialized "M" cells over Peyer's patches or isolated lymphoid follicles (2,3), IgA precursor B lymphocytes and regulatory T lymphocytes are stimulated (4-7). These cells migrate to the mesenteric lymph nodes, the thoracic duct, mature in the spleen (probably in other undefined locations as well), and eventually travel back to the gut mucosa as well as to other mucosal surfaces (8-11). In addition, a substantial amount of IgA is transported into the bile and other mucosal structures from the serum (12,13).

Recently, our laboratory has shown that by immunizing animals orally with live, locally invasive or noninvasive shigella significant enhancement in the local IgA response is consistently achieved to subsequent oral challenges with live invasive shigella (simulating the time course of a natural infection) (14-16). Therefore, we have hypothesized that the mucosal route of vaccination against dysentery and other primary enteropathogenic infections can be accomplished by using an oral vaccine with the appropriate antigen. In the past year and a half, we have explored the key variables in the stimulation of such mucosal immune responses and most recently are determining the functional significance of having achieved a vigorous antigen-specific local immune response.

The presently reported studies include exploration of the initial processing of macromolecules in the lumen by "M" cells overlying isolated lymphoid follicles, the immunogenic capabilities of killed shigella antigen preparations when presented directly to the gut-associated lymphoid tissues, the effect of parenteral adjuvants on shigella-specific local IgA responses and their enhancement by concurrent local antigen administration, and functional in vivo and in vitro studies demonstrating that shigella-specific secretory IgA can collaborate with intra-epithelial lymphocytes to protect against this enteropathogen.

METHODS

Preparation of Chronically Isolated Ileal Loops. The surgical creation of ileal Thiry-Vella loops in rabbits has been described in detail previously (1). In brief, while 3 kg New Zealand white rabbits are anesthetized with Rompun and Ketamine, a midline abdominal incision is made and the terminal ileum is identified. A 20 cm segment of ileum containing a grossly identifiable Peyer's patch is isolated with its vascular supply intact. Silastic tubing (Dow-Corning) is sewn into each end of the isolated segment. This tubing is brought out through the midline incision and tunnelled subcutaneously to the nape of the neck where it is exteriorized and secured. Intestinal continuity is restored by an end-to-end anastomosis and the midline incision is closed in two layers.

Each day about 2-4 ml of secretions and mucus that collect in the ileal loops are expelled by injecting 20 ml of air into one of the silastic tubes. The slightly opaque, colorless fluid and mucus expelled from the tubing is studied for specific immunoglobulin content. A subsequent flush with 20 ml of sterile saline helps to remove adherent mucus. This saline is then removed by repeated gentle flushes with air. In the present studies over 90% of animals were able to complete the experiments. These superior results are directly attributable to the excellent surgical techniques and care mentioned in the Forward.

Enzyme-linked Immunosorbent Assay (ELISA). The ELISA procedure was used to express the immunoglobulin titer of loop fluids and serum in terms of OD units. For the assay, microtiter plates are coated with a solution containing Shigella flexneri lipopolysaccharide (Westphal preparation). Immediately prior to testing serum samples or loop secretions, the antigen solution is removed and the wells are washed with a phosphate-buffer containing Tween 20 (PT). The fluid to be assayed is diluted in the PT buffer and incubated in the coated wells and in uncoated wells (to control for nonspecific adsorption) for four hours on a horizontal rotary shaker. The plates are washed with PT and incubated overnight with either alkaline phosphatase-conjugated Staph protein A (to detect IgG) or anti-rabbit IgA. Following another PT wash, substrate reaction is carried out with nitrophenyl phosphate in carbonate buffer. The OD 405 nm of the substrate reaction is read on a TiterTek MicroELISA reader. Kinetics of the enzyme-substrate reaction are extrapolated to 100 minutes. The OD 405 nm of uncoated wells are subtracted from those of the coated wells. Specific IgG and IgA standards are processed daily with unknown fluids as previously described (17).

Microtiter Lowry Protein Determination. For expressing the antigen-specific IgA in terms of mg of total protein in intestinal secretions, a Microtiter Lowry procedure was used. The following reagents were prepared: Reagent A: 2% Na_2CO_3 in 0.1 N NaOH; Reagent B: 2% NaK tartrate; Reagent C: 1% $CuSO_4$; Reagent D: 1 ml Reagent B + 1 ml Reagent C brought to 100 ml with Reagent A (prepared just prior to each assay). Reagent E is 2 N phenol Folin-Ciocalteau solution diluted to 0.5 N with distilled water. Bovine serum albumin (Pentex, fraction V) was diluted serially in double distilled deionized water for the standard solutions of 10, 50, 100, 250, 500, and 1000ug/ml. For the procedure, in each well of a microtiter plate was added 50ul of the standard

or a loop fluid diluted in water. One hundred and fifty ul of Reagent D was added and incubated at room temperature for 10 minutes on a shaker at rapid speed. Fifty ul of Reagent E was then added by repeat pipetting, and O.D. was read at 600nm on the TiterTek reader after 30 minutes incubation at room temperature. One well was used per sample. For each plate, one standard series was run. With this technique, standard deviation as a percentage of the mean value for 15 repetitions of a loop fluid pool standard was 6.4% (2.152 mg/ml + 139ug/ml).

In Vitro Assay of IgA Function With Intestinal Lymphocytes Against Shigella. Shigella-specific secretory IgA was purified as described previously (17,18). The studies of the functional in vitro activity were performed in collaboration with Dr. George Lowell and Dr. Aldo Tagliabue. Single cell suspensions were obtained by teasing spleen, Peyer's patches, mesenteric lymph nodes and peripheral lymph nodes and centrifuging the cell populations through gradients. The bactericidal activity was performed in Dr. Tagliabue's laboratory using the leukocyte-mediated interference with bacterial growth procedure originally described by Lowell et al. (19). Briefly, 10^4 shigella are put into 15 ml conical tubes together with media with or without specific antibodies and are centrifuged at 1300 x G for 10 minutes at 4° C. Specific lymphoid suspensions are added at various target:effector ratios. Following a second centrifugation, experimental and control tubes are incubated at 37° C for 2 hours. Thereafter, the pellets are resuspended to a standard volume of 1 ml and cultured on tryptose agar overnight. The percentage of antibacterial activity is:

$$100 - 100 \times \frac{\text{Colonies in experimental tubes}}{\text{Colonies in control tubes}}$$

In Vivo Assay of IgA Function to Prevent Shigella Invasion. Rabbits were fasted for 24 hours allowing intake only of water. Following anesthesia as described above, a midline abdominal incision was made and 10 cm segments of small bowel were ligated, leaving the vascular supply to the segment intact. Fresh overnight broth cultures of S. flexneri were mixed with buffer, specific IgA anti-shigella LPS or with nonspecific IgA as indicated. Several doses of the shigella were used as indicated in the text. These mixtures were injected into the isolated segments and left overnight in vivo. Damage to the intestine was measured by quantifying the amount of fluid accumulation, the gross changes, histologic changes and an invasion index.

In Vitro Assay for Adherence of Bacteria to Suspensions of Epithelial Cells. These studies attempt to provide a quantitative estimate of the number of bacteria adhering per intestinal epithelial cell. Rabbit colon is washed thoroughly with a prewarmed (37° C) solution of saline with 1mM dithiothreitol. The colon ends are ligated and the colon is filled with a 50mM phosphate buffer containing sodium citrate (27mM) and incubated for 20 minutes at 37° C. The intestine is then washed with PBS containing EDTA and dithiothreitol for half an hour at 37° C. This solution contains epithelial cells and is centrifuged at 500 X G for 10 minutes. The cells in the precipitate are washed in PBS and viability is determined with trypan blue. Overnight broth cultures of S. flexneri M4243 were incubated with these cells for varying periods of time and attachment was assessed with Giemsa staining.

Electron Microscopic Studies of "M" Cells over Isolated Lymphoid Follicles. Guinea pigs were given India ink mixed into their drinking water for 1-3 months. When the intestine was removed, the location of the isolated follicles could be seen with a hand lens because of the presence of carbon-laden macrophages. Tissue samples for electron microscopy were removed, minced with a scalpel blade, and fixed in 3% glutaraldehyde and PBS for 2 hours. Postfixation was carried out in 2% S-collidine-buffered osmium tetraoxide. The tissue was stained with 2% uranyl acetate and embedded in polybed. Sections were made on a Sorvall MT2 ultramicrotome and stained with Reynold's lead acetate. Electron micrographs were taken on a Zeiss 109 fiberoptic transmission electron microscope.

RESULTS

ANTIGEN-UPTAKE BY INTESTINAL EPITHELIAL CELLS

The initial processing of macromolecular antigens by the gut is performed at least by the "M" cells which overlie Peyer's patches (2). However, it was unclear whether antigenic material could be processed by other structures along the gut lumen. Since we and others demonstrated that local IgA responses could be achieved in the absence of Peyer's patches, another route (or routes) for antigen processing must exist (20-22). We had previously suggested that the isolated lymphoid follicles, which are present throughout the gastrointestinal tract may serve a similar antigen-processing role. This, however, was difficult to prove since these isolated follicles were too small to be seen with a hand lens, and therefore, were difficult to isolate for study by electron microscopy. We hypothesized that if these structures were covered by specialized epithelium that could take up macromolecules and present them to the underlying lymphocytes, we could take advantage of this by using a label that would be grossly visible such as India ink. After guinea pigs had ingested India ink mixed in their drinking water for 1-3 months, their isolated follicles were readily visible both from the serosal and mucosal surfaces using a hand lens (figures 1 and 2). These areas were excised and processed for electron microscopy as described in the Methods section. Unequivocal demonstration of "M" cells were made in all of the animals studied (figure 3). These findings provide the first explanation for the ability to elicit mucosal immune responses in the absence of Peyer's patches. The role of these "M" cells in the processing of shigella for a mucosal immune response will be the subject of future studies.

We have begun investigations on another cell in the epithelium of the intestine which has been shown to be capable of phagocytizing luminal microorganisms and which contains intracytoplasmic apparatus such as lysozyme for digesting bacteria. The Paneth cell normally lies at the base of the crypts of Lieberkuhn, but under conditions of bacterial overgrowth such as the blind loop syndrome, or in our isolated ileal loop model (23), they can undergo a striking hypertrophy and hyperplasia. Dr. Scott Kern, working in our laboratory has taken advantage of the hyperplastic state of Paneth cells in the isolated loop model system to separate Paneth cells and for the first time, grow them in tissue culture (figure 4). The ability of these cells to process shigella antigens and to deal with these and other enteropathogens with lysozyme or an antibody-dependent cell-mediated cytotoxicity system will be studied in the next two years.

Figure 1. Disecting microscope view of Peyer's patch from guinea pig fed India ink for 3 months. The dome regions which contain an abundance of "M" cells are evident by their dense staining (arrows).

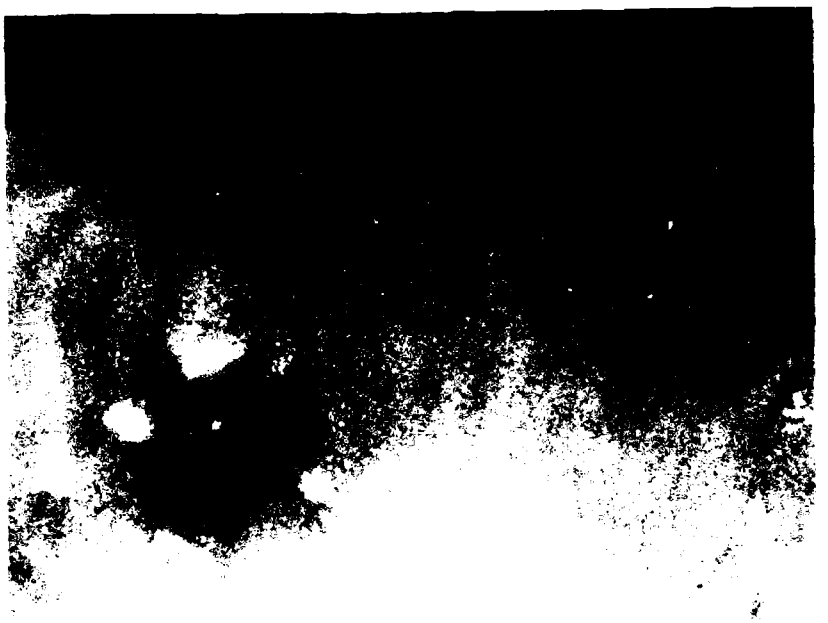

Figure 2. Disecting microscope view of the mucosal surface of an isolated lymphoid follicle from a guinea pig fed India ink for 3 months. The India ink is gathered-up by the "M" cells and collected in the underlying lymphoid follicles. By making these structures grossly visible, we have been able to isolate them for ultrastructural studies.

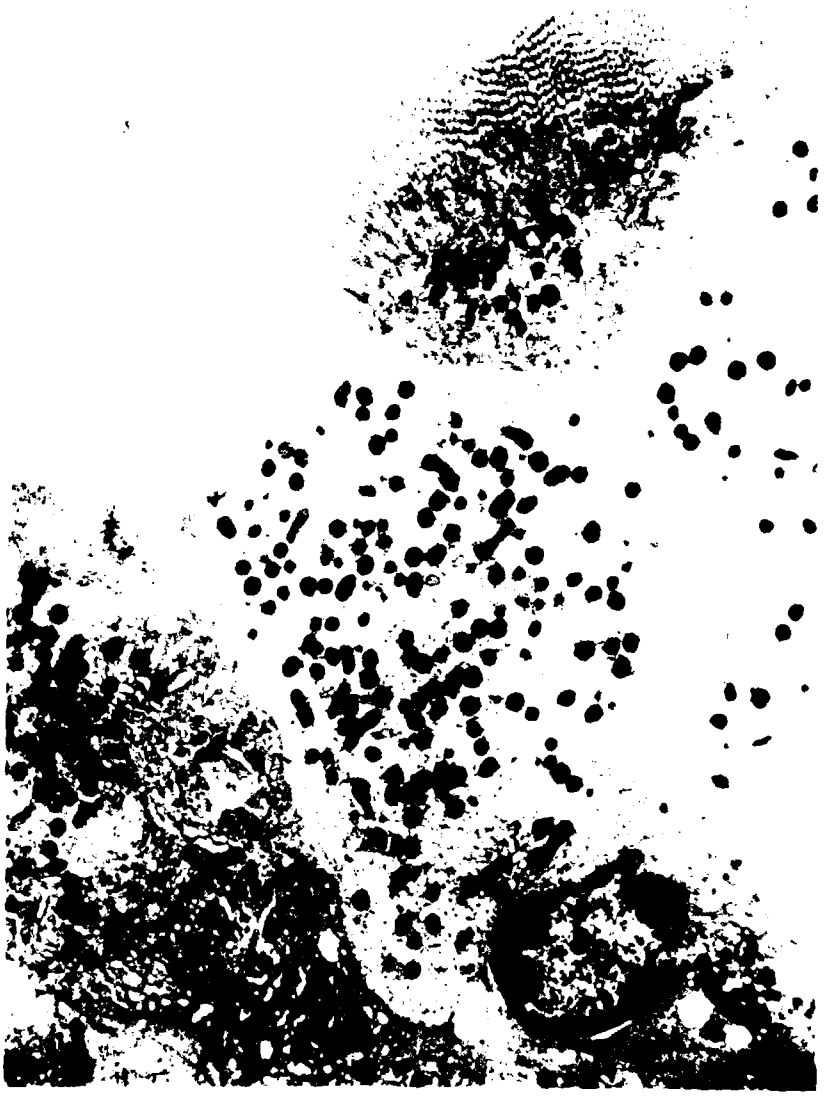

Figure 3. Electron photomicrograph of "M" cell overlying the isolated lymphoid follicles from a guinea pig fed India ink for 3 months. These cells play a major role in processing antigen from the gut lumen. Our future studies on these cells will concentrate on the differential processing of live versus killed vaccine preparations of shigella (see text and 1984 proposals).

Figure 4. This electron micrograph shows a Paneth cell in tissue culture at 48 hours after isolation. Dr. Scott Kern working in our laboratory has achieved the first <u>in vitro</u> isolation of Paneth cells for functional studies. These epithelial cells along with "M" cells represent the major phagocytic epithelial cells along the gut lumen. In addition, Paneth have unique microbicial capabilities that will be the subject of our future studies (see text and 1984 proposals).

STIMULATION OF THE MUCOSAL IgA RESPONSE TO SHIGELLA

Our earlier studies of the local IgA response to shigella have shown that animals primed with three oral doses of live invasive or noninvasive S. flexneri will show a highly significant ($p<.001$) enhancement of their local IgA anti-shigella LPS response upon subsequent challenge with the same live shigella (14-16). Although various killed preparations of shigella (heat-killed, acetone-killed and purified LPS) can elicit a local IgA response, the response is always much weaker than that from live shigella and we have not been able to prime the animals for the markedly enhanced local IgA response by oral priming with the killed preparations. Since the live bacteria are able to multiply within the gut lumen, it was likely that the resultant final concentration of bacteria that reached the gut-associated lymphoid tissues was considerably greater when live rather than killed preparations were given. To test the possibility that the lower response to killed preparations merely reflected a lower final concentration, we primed animals with massive doses of 10^{13} heat-killed shigella orally. Only a weak primary local IgA anti-shigella LPS response in isolated loop secretions resulted from this immunization schedule (preliminary data given in last year's annual report). Even so, it was possible that these animals were primed to develop a local IgA enhanced response upon challenge with the live, locally invasive shigella (recapitulating the natural infection). Therefore, a group of 10 rabbits was primed with three weekly oral doses of 10^{13} heat-killed Shigella X16; sixty days after the third dose a chronically isolated ileal loop was created in each animal. The day after surgery, each animal was challenged with a single dose of live Shigella X16. As shown in figure 5, only a weak primary IgA anti-shigella LPS response resulted.

At least two explanations for the above findings 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 T cell help for a memory response. Alternatively, the altered killed preparations may not be taken-up efficiently by the "M" cells. On the latter point, Owen et al. have recently reported that the "M" cells were only able to take-up viable Vibrio cholerae but not killed vibrios (24). Therefore, in the present studies we sought to determine the immunogenicity of the killed preparations bypassing the need for "M" cells by giving a single dose of 10^{10} heat-killed Shigella X16 directly in each of five Peyer's patches (0.2ml/Peyer's patch). These were given during the surgical procedure to create a chronically isolated ileal loop in each animal. The Peyer's patch in the isolated loop was one of those injected in each case. All seven of the rabbits so treated developed significant ($p< .01$) increases in the IgA anti-shigella LPS activity in their loop secretions by the sixth day after surgery (figure 6). Although the mean IgA anti-shigella LPS activity declined slowly over the next three weeks, the activity was still detectable at four weeks (figure 6).

Although the heat-killed Shigella X16 directly injected into Peyer's patches could elicit an IgA response locally, it was not clear whether such a treatment would prime the animals for an enchanced local IgA response to an oral challenge with the live invasive bacteria. To study this, a group of 10 rabbits have been immunized with a single dose of 10^{10} heat-killed Shigella X16 directly into 3-5 Peyer's patches as above. Thirty days later, a

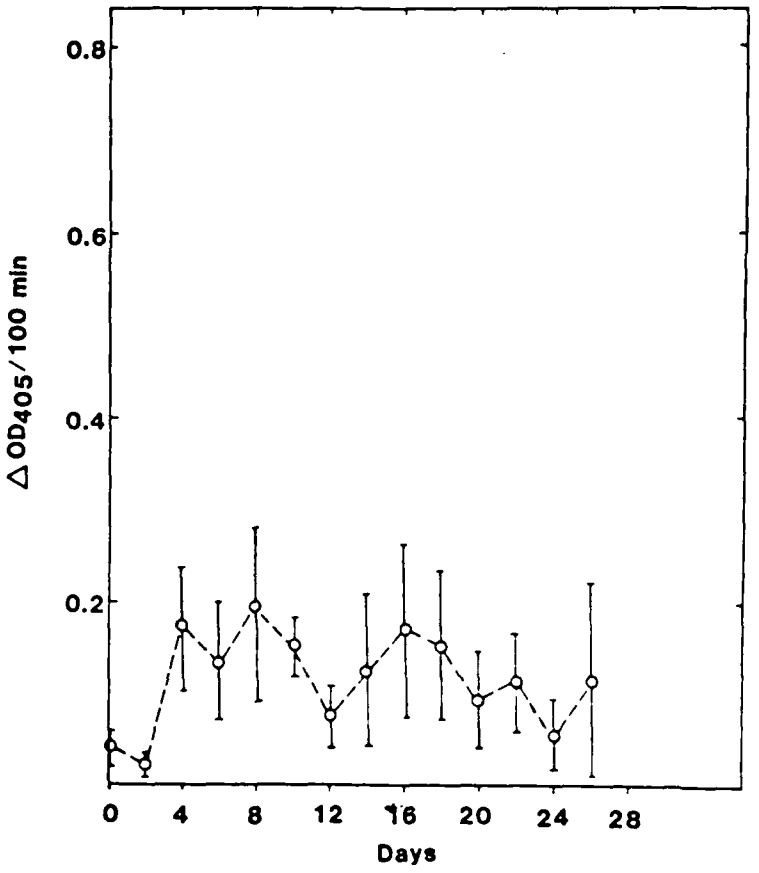

Figure 5. Mean IgA anti-shigella LPS response in intestinal secretions from 10 rabbits primed with three weekly oral doses of 10^{13} heat-killed Shigella X16. After resting for 60 days, a chronically isolated ileal loop was created in each rabbit and the next day (day 0), a single oral challenge dose of 10^{10} live Shigella X16 was given. Only a typical weak primary IgA response resulted in the loop secretions indicating that oral priming even with extraordinarily large doses of killed bacteria lacking adjuvant is ineffective in stimulating an enhanced local IgA response.

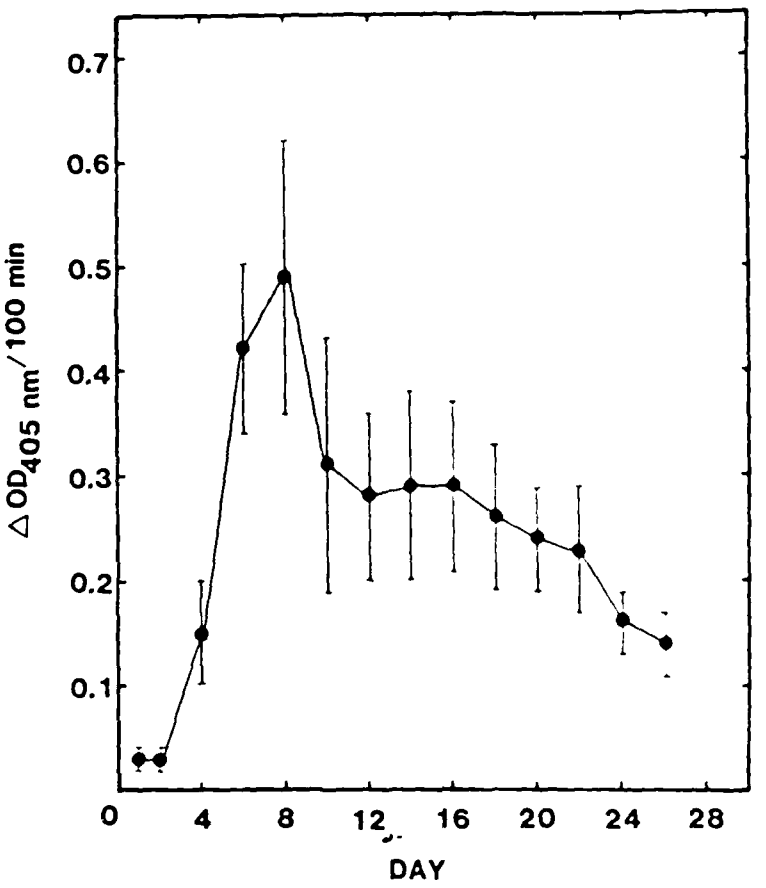

Figure 6. Mean IgA anti-shigella LPS response in intestinal secretions from rabbits given heat-killed Shigella X16 directly into their Peyer's patches. The vigorous IgA anti-shigella response that was always present by day 6 in these animals demonstrates that the heat-killed preparation is antigenic and can stimulate a local IgA response when presented directly to the gut-associated lymphoid tissues. These findings indicate that the processing of live versus killed bacteria by either "M" cells or Paneth cells may be an important determinant of the eventual local IgA response.

chronically isolated ileal loop was created in each of two animals. The next day, a single oral challenge dose of 10^{10} live Shigella X16 was given. The local IgA activity to shigella LPS for these two rabbits showed a surprisingly high residual IgA anti-shigella LPS activity on the day after surgical creation of the isolated loop (Table 1). Although the IgA anti-shigella LPS activity increased in both animals, it was difficult to judge the significance of their responses over the high residual activity. Interestingly, there was virtually no IgG activity in these isolated loop secretions. Therefore, in the next 8 animals, we are waiting for two months after the initial intrapatch injection before administering the challenge dose of live shigella. We anticipate a significant enhancement will be seen in these isolated loop secretions which will indicate that it is the initial uptake of the antigenic material which needs to be improved in order to achieve an effective IgA memory response with nonviable antigen vaccines. In the accompanying proposal we suggest the use of liposomal preparations to accomplish this goal.

Our past studies have tried to recapitulate potential vaccine protocols that could be extrapolated to human use. As such, we have not used routes such as intraperitoneal or complete Freund's adjuvant. However, our attempts to prime animals for an intraluminal IgA response with parenteral regimens using heat-killed S. flexneri have been unsuccessful. We noted that others using such routes and adjuvants have been able to create heightened mucosal immune responses to a variety of antigens (25-27). Therefore, in the present studies, we have primed rabbits with a single parenteral dose of 10^{10} heat-killed S. flexneri in complete Freund's adjuvant (given at multiple sites). A chronically isolated ileal loop is created in each animal on the day of the parenteral immunization. The animals studied to date have shown a small increase in the local IgA anti-shigella LPS activity (Table 2 shows these early results). By contrast, the assays performed to date on the serum from these animals indicate a consistent vigorous systemic IgG anti-shigella LPS response by these animals. This, of course, would be expected from a potent systemic adjuvant like complete Freund's. The parenteral administration of heat-killed shigella in complete Freund's adjuvant is effectively stimulating the appropriate B and T immunoblasts to produce the vigorous IgG response observed.

Recent work from Strober's laboratory indicates that antigen-specific IgA helper T cells and IgG suppressor T cells are elicited in murine Peyer's patches after protein feeding (28). This being the case, we reasoned that a concomitant oral administration of live Shigella X16 could induce suppression of this IgG response in the serum and enhance the local IgA response. Therefore in a series of animals presently under study, we have given the same parenteral dose of heat-killed shigella in complete Freund's adjuvant as described above. In addition, however, following creation of their isolated ileal loops they are given 10^{10} live Shigella X16 orally. The exciting findings in the first three animals studied to date are shown in Tables 2 and 3 to allow comparison with the parenterally-only primed group. All three animals with combined parenteral adjuvant and oral immunization (M-238,239 and 240) displayed extraordinary local IgA anti-shigella activity as compared to M-235,236 and 237 (two more rabbits assayed since creation of the table have the same results). Furthermore, the few sera assayed to date suggest a marked suppression in the systemic IgG responses in line with the data reported for protein antigen feeding (28). Obviously, these preliminary findings need to be further documented, but they are the most consistent and strongest local

IgA responses ever seen in our laboratory following a single oral administration of antigen with or without parenteral priming!

FUNCTIONAL ROLE OF ANTIGEN-SPECIFIC IgA

Both *in vivo* and *in vitro* techniques have been explored to examine the functional significance of the IgA response to enteropathogens. The best evidence to date supports the original concept of Freter that the role of IgA is to prevent the attachment of potentially harmful intraluminal microorganisms and their toxic products (29). In the present studies, we have begun to examine the effects of IgA anti-shigella LPS on invasion and survival of *S. flexneri*.

Our first attempts used *in vivo* acutely ligated segments of rabbit intestine to examine the effects of invasive shigella. Acutely ligated segments of intestine were created in 16 rabbits as described in the Methods section. Three dilutions of overnight broth cultures of *S. flexneri* M4243 (invasive) were used; 1:5, 1:50 and 1:500. They were premixed with an equal volume of buffer only, IgA anti-shigella LPS or nonspecific IgA. Four acutely ligated loops were created in each animal. The above three mixtures and a fourth consisting of sterile broth as a negative control were placed into these segments of bowel. After an overnight incubation, the volume in the segments was measured, and the mucosal surface was graded as to the degree of erythema, purulent exudate (pseudomembrane) or frank necrosis present. Areas over Peyer's patches were graded separately from the rest of the intestine due to the previously published tendency of shigella to invade this epithelium (30). Histologic sections of all the segments were examined and two grading scales were used. Degree of inflammation and tissue damaged was graded from "0" for none to "3" for large mucosal ulcerations with massive purulent exudates. The extent of invasion of the epithelium was graded separately from "0" for none to "3" for invasion extending into the underlying lamina propria. Unfortunately, no consistent results were found with this system. Results of the fluid accumulation studies shown in Table 4 are typical of these findings. Although some animals such as 1,2 and 9 gave evidence of protection with antigen-specific IgA, most other animals did not. Indeed, the antigen-specific IgA loop occasionally had more fluid accumulation than the loop containing bacteria alone (rabbits 4,5,8,13 and 16). Similar inconsistent results were found in the gross and histologic studies of invasion and inflammation (data not shown).

We believe that the difficulty of these *in vivo* studies relates to the inability to control the large number of variables inherent in that system. Therefore, our present and future studies of the functional significance of the specific IgA anti-shigella LPS response will concentrate on *in vitro* systems which allow careful control of these variables. During the past year, our collaborative studies with Drs. Tagliabue and Lowell on the activity of IgA anti-shigella LPS in an antibody-dependent cell-mediated cytotoxicity system have been most successful. Using the antibacterial index defined in the Methods section, we have shown that our IgA anti-shigella LPS could collaborate with intestinal lymphocytes to interfere with the survival and growth of *Shigella* X16 (31,32). Our future studies will extend these findings and explore the capabilities of mononuclear and Paneth cells to collaborate with IgA anti-shigella LPS in preventing shigella infections.

ELISA METHODOLOGY AND DATA EXPRESSION

In the past, we have expressed the data from our ELISA system in terms of change in O.D. per 100 minutes. Some workers have preferred to express data on serum activity against a particular microorganism as a titer while others have corrected data for the volume or total protein in the secretion. Titering is done by selecting an O.D. reading for the particular ELISA system as the minimum reproducible value. Then, twofold dilutions are performed on the sample to be tested. The last dilution giving an O.D. reading above the selected value is considered the titer of the antiserum or secretion. Another concern in our laboratory is that the protein content of intestinal secretion varies from day to day, unlike total protein in serum which is relatively constant from day to day in a single animal. Therefore, to correct for day to day variation in protein concentration, we have performed total protein concentrations using the microLowry procedure outlined in the methods section and expressed the change in O.D. per mg of protein in the loop secretion. Two examples of the data generated using these three options are shown in Table 5. Clearly, all three methods are able to give an account of the development of the IgA response in these animals. However, we believe that the expression of the information as a titer will not allow us to distinguish real measurable differences which our ELISA method can achieve. By adding the total protein determination, a correction is made for a net increase or decrease in total protein secretion not related to the IgA response. Since IgA is only a minor component of intestinal secretion protein, if there is an actual increase in the amount of IgA secreted due to the immunization, this will not cause a significant reduction in final result. Therefore, the future work from this laboratory will correct all secretion data to the total protein in the sample.

OTHER COLLABORATIVE INVESTIGATIONS

In addition to the above studies, we have collaborated with other investigators concerning the systemic and mucosal immune responses to pathogenic bacteria. With Tramont *et al.* (33) we used a modification of our previously described ELISA system (see Methods section) to detect IgG, IgA and IgM antibody activity to LPS of *S. typhi* and the 5076-1C transconjugate strain wherein the Form I antigen of *S. sonnei* is transferred (via its plasmid) to *S. typhi* strain 21a. This transconjugate expresses the somatic antigens *S. typhi* 9 and 12 and *S. sonnei* Form I and our previous studies have shown that these elicit specific antibodies detectable by the ELISA system (34). In the present studies, assays were performed on serum samples from human volunteers given the vaccine strain orally. The results shown in Table 6 demonstrate the responses in the two individuals out of 10 tested that developed an antibody response.

Studies on the serologic relationships of five *P. multocida* strains representing the five most commoly isolated somatic serotypes from rabbit infections were performed. Pasteurella infections are one of the more frequent problems that occur in our rabbit colony and Carey *et al.* are in the process of developing vaccines to these pathogens. In the present studies, we have characterized the major serologic groupings for which vaccines need to be created (35). Again, a modification of the ELISA technique in the methods section was used. Specificities and cross-reactivities of the ELISA system are evident from the data in Table 7. These studies will provide a basis for future work using epidemiologic investigations on infections and for the prevention of pastuerella infections using specific vaccinations in our rabbit colonies.

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Table 1. IgA Anti-Shigella LPS Activity After Direct Priming in Peyer's Patches and an Oral Challenge

Oral Antigen Day	Loop Secretion IgA Response M-233	M-234
0*	.588+	.581
2	.489	.418
4	.933	.365
6	.744	.688
8	.781	.928
10	.870	.882
12	.694	1.093

*Rabbits primed with 10^{10} heat-killed shigella given directly into five Peyer's patches on day -30. On day -1 an isolated loop was created and on day 0 animals were given a single oral challenge with 10^{10} live Shigella X16.

+Results expressed as OD 400 nm/100 min.

Table 2. IgA Anti-Shigella LPS in Loop Secretions

After IM Priming in Freund's Complete Adjuvant

	Parenteral Only[1]			Parenteral + Oral[2]		
Ag Day	M-235	M-236	M-237	M-238	M-239	M-240
1	.090	.073	.002	.104	.017	.026
6	.060	.047	.014	.615	.245	.190
12	.092	.129	.091	1.010	2.032	1.828
18	.156	.410	.066	.320	.520	1.645
24	.540	.171	.040	.384	.270	1.482
30	.487	.070	.084	.556	.092	.697
36	----	.169	.134	.428	.041	.125

[1]Parenteral only rabbits primed with 10^{10} heat-killed shigella IM in CFA on day 0.

[2]Parenteral + oral rabbits given same priming with additional dose of 10^{10} live oral Shigella X16 on day 1.

Table 3. IgG Anti-Shigella LPS in Serum

After IM Priming in Freund's Complete Adjuvant

	Parenteral Only			Parenteral + Oral		
Ag Day	M-235	M-236	M-237	M-238	M-239	M-240
-1	.028	-----	-----	.031	.003	.002
7	.307	.540	-----	-----	.007	.058
13	1.047	1.089	1.173	.139	.266	.536
21	-----	1.566	-----	-----	-----	-----
30	-----	1.930	-----	-----	-----	-----
36	-----	1.211	-----	-----	-----	-----

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[1]Parenteral only rabbits primed with 10^{10} heat-killed shigella IM in CFA on day 0.

[2]Parenteral + oral rabbits given same priming with additional dose of 10^{10} live oral Shigella X16 on day 1.

Table 4. Volume Data

M4243 Only		M4243 + Specific IgA	M4243 + Nonspecific IgA	Sterile Broth
1	20[1]	5	1	0
2	16	1	7	2
3	7.2	9	13	1
4	5	7	11	0
5	7	20	4	2
6	0	0	0	0
7	1	1	1	0
8	1	5	16	1
9	11	2	10	0
10	1	1	6	0
11	11	10	7	0
12	2	1	20	2
13	14	15	12	0
14	4	1	0	0
15	2	0	20	6
16	5	6	11	11

[1]Volume of fluid in ml from acute loop challenge studies (see text).

Table 5. Expression of ELISA Data

	M-232			M-221		
Day	Titer	O.D.*	O.D.*/mg Protein	Titer	O.D.*	O.D.*/mg
0	< 10	.010	.045	< 10	.030	.250
7	≥ 40	.683	3.148	≥ 40	.763	3.376
13	20	.812	3.760	≥ 40	.669	1.325
22	≥ 40	.662	5.520	10	.084	1.270

*Assays performed at 1:20 dilution unless otherwise stated.

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Table 6. IgG and IgA Antibody Response to LPS of 5076-1C Hybrid and the LPS of S. typhi of the Two Volunteers who Developed Such a Response (both vol. immunized with 1 x 10^{10} cells)[5]

			IgG		IgA	
Donor	Day	Ag	1:100[1]	1:10[1]	1:100[1]	1:10[1]
RC[2]	1[3]	5076-1C	1.002[4]	2.213	0.244	1.653
		S. ty.	0.900	2.487	0.345	1.326
	8	5076-1C	2.729	3.045	1.774	3.556
		S. ty.	2.679	3.433	1.373	2.650
	22	5076-1C	3.732	3.716	0.576	2.837
		S. ty.	3.434	3.846	0.534	1.943
SL	1	5076-1C	0.115	0.547	0.048	0.167
		S. ty.	0.194	0.854	0.043	0.148
	8	5076-1C	0.267	1.047	0.048	0.241
		S. ty.	0.288	0.985	0.047	0.189
	22	5076-1C	0.262	1.029	0.051	0.157
		S. ty.	0.252	1.047	0.061	0.204

1 serum dilution

2 RC had received parenteral typhoid vaccine in 1959, 1963, 1964, 1969, 1978

3 day 1 = day vaccine given

4 OD readings, ELISA (see methods)

5 Data from Tramont, et al. (33).

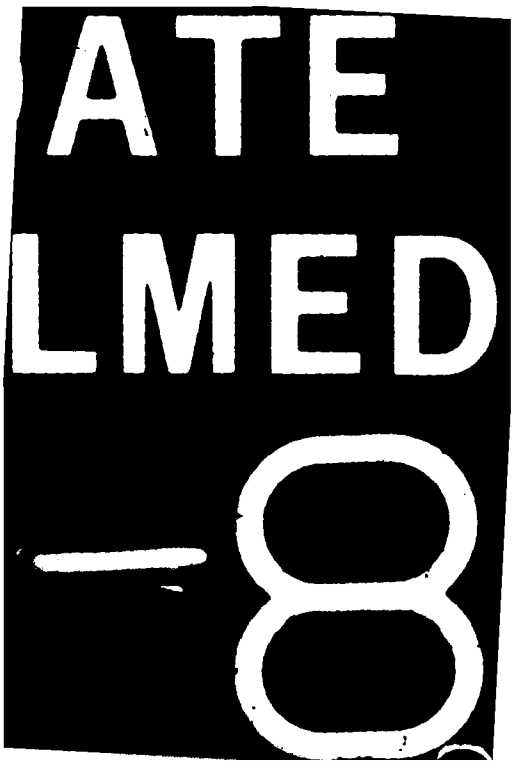
Table 7. Specificity and Cross-reactivity of antisera and lipopolysaccharide in an ELISA[c]

P. multocida Antisera Somatic Serotype	Rabbit No.	Optical density[a] (405 nm at 100 min)				
		Serotype of P. multocida LPS (ELISA antigen)				
		1	3	4	12	15
1	1	4.06+1.27	0.1	0.1	0.1	0.1
1	2	4.65+1.17	0.1	0.1	0.1	0.1
3	7	0.1	0.1	0.1	0.29+0.34	0.1
3	8	0.1	0.21+0.03	0.1	0.1	0.1
4	3	0.1	0.1	1.96+1.05	1.24+0.97	0.1
4	4	0.10+0.03	0.1	1.25+0.66	1.76+1.48	0.32+0.28
12	5	0.1	0.1	0.10+0.03	4.31+1.91	0.1
12	6	0.33+0.16	0.27+0.27	0.39+0.13	2.97+1.35	0.45+0.16
15	11	0.1	0.1	0.1	0.1	5.25-1.88
15	12	0.1	0.1	0.1	0.1	3.75+2.32
-[b]	9	0.1	0.1	0.1	0.1	0.1
-[b]	10	0.1	0.1	0.1	0.1	0.1

[a]Expressed as mean + SD, 3 replicates on different days, values corrected to 100 min.

[b]Immunized with *B. bronchiseptica*, *Pasteurella*-free.

[c]From Carey, *et al.* (35)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