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

FINAL REPORT

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An Investigation of the Memory Response of the Local Immune System to Shigella Antigens

In the past seven years, our laboratory has established the chronically isolated ileal loop model in rabbits as a probe for following the mucosal immune response to enteropathogens and their toxic products. We have studied several modes of immunization and several potential vaccine preparations with this model system. Our emphasis has been to use routes of antigen administration which would be feasible in human immunization attempts. With this model system, we have been able to follow the kinetics of the secretory immune response to Shigella flexneri. We have found that oral administration of nonpathogenic strains of Shigella flexneri are able to elicit a secretory IgA memory response in gastrointestinal secretions. Parenteral immunization alone is ineffective in achieving this result. Heat-killed preparations of Shigella are also ineffective in eliciting a secretory IgA memory response. This likely reflects the poor uptake of this material by the gut-associated lymphoid tissues. Traditional parenteral adjuvants such as complete Freund's adjuvant are not...
19. ABSTRACT (continued)

Effective in priming the mucosal immune response by an oral route. The secretory IgA created to Shiga toxin can prevent the toxic effects of this molecule in vitro. This suggests that the production of a secretory IgA memory response to *Shigella flexneri* and to Shiga toxin will be protective. Lastly, we have begun to develop models to isolate lymphocytes and other functional cells such as Paneth cells from the gastrointestinal tract. By studying the location of B lymphoblasts committed to synthesizing IgA against enteropathogens, we will be able to develop more logical approaches for vaccine investigation.
Prior to development of model systems for mucosal immunity, it was difficult to determine whether a particular vaccine preparation would elicit a significant mucosal secretory IgA immune response. Further, it was totally unknown whether a mucosal memory response could be elicited and how such a response would be achieved. In the past seven years, our laboratory has established the chronically isolated ileal loop model in rabbits as a reliable probe for following the mucosal immune response to enteropathogens and their toxic products. We have studied several modes of immunization and several potential vaccine preparations with this model system. The emphasis in our studies has been to use routes of antigen administration which would be feasible in human immunization attempts. Occasionally, in order to demonstrate the maximal capabilities of the secretory immune system, we have used nonphysiologic routes of immunization. We have demonstrated that this model allows one to follow the kinetics of the secretory immune response to virtually any mucosal vaccine preparation. We have shown conclusively that oral immunization with a live strain of Shigella flexneri will be able to elicit a secretory IgA memory response. Parenteral immunization alone is ineffective in achieving this result. Further, in extensive studies we have proven that the strain of Shigella need not be invasive in order to elicit a secretory IgA memory response. Specifically, 2 noninvasive strains were found capable of eliciting excellent secretory IgA memory responses. Strain S. flexneri 2457-0 was not invasive by the Sereny test and has a negative isolated ileal loop test but nonetheless was capable of priming animals for a vigorous secretory IgA memory response. Interestingly, this strain does contain the 140 megadalton virulence plasmid for invasion. Therefore, we also performed studies on the S. flexneri M4243A strain which has been cured of the virulence plasmid. This microorganism has no invasive capabilities. It gave the strongest secretory IgA memory response seen to date in our laboratory. These findings indicate that vaccination against enteropathogens can be achieved using nonpathogenic strains of Shigella. Other investigations have shown that we can enhance the initial primary IgA response against enteropathogens if we carefully orchestrate the dosage schedule. Specifically, by giving the heat-killed preparation of Shigella one day prior to oral immunization we found significant enhancement of the primary IgA response. However, if the parenteral dose is given one week or earlier before the oral dose of live Shigella, no enhancement of the primary IgA response is seen. Adjuvants such as complete Freund's adjuvant and DEAE-dextran were ineffective in priming the mucosal immune response. Our most recent investigations have centered on understanding why some vaccine strains are more effective than others at eliciting mucosal immune responses. We are looking at the cellular events surrounding the humoral responses which result. Understanding these events will provide the key to a logical approach in the future for vaccine investigations. It will help to remove vaccination research from the field of trial and error. Other studies performed in this period demonstrated that bacteria such as Shigella are initially processed in M cells which overlie both Peyer's patches and isolated lymphoid follicles in the intestine. Indeed, our laboratory discovered the existence of these cells in isolated follicle epithelium. Lastly, we have begun studies on the functional significance of the secretory IgA
response to enteropathogens and their toxins. We have found that antigen-specific secretory IgA can prevent the toxic effects of Shiga toxin and may be able to prevent uptake of pathogenic strains of Shigella by M cells. Future studies will need to determine whether secretory IgA elicited by nonpathogenic vaccine strains will be able to achieve complete protection. These findings are relevant for not only the Shigella which were studied but all potential enteropathogenic agents.
Foreword

During the course of this work, the principal investigator has been greatly assisted by many capable colleagues. We wish to spend a minute paying appropriate recognition for their efforts. The specific individuals who helped with the studies were Roderick McDonald, Patricia Scott, Dianna Bauer, Pamela Porter, Scott Kern, Mitchell Wiatrak, Joseph Wassef, and John Carey. In addition, we have been fortunate to be situated at the University of Michigan whose excellent laboratory animal medicine department is largely responsible for the continued ability of our laboratory to perform difficult experimental surgery with a large (> 90%) survival rate of animals. Drs. Ringler and Peters have spent considerable time with us in making certain that conditions for the animals were proper and of the highest quality. Lastly, we have been fortunate to have the excellent secretarial services of Ms. Mary Ann Byrnes and Ms. Terri Throne.

In conducting the research described in this report, the investigators 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 Commission on Life Sciences, National Research Council (NIH No. 78-23, 1978 and NIH publication 85-23 revised 1985).

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Introduction

IgA was first described in 1959, yet not until 1965 was it demonstrated to occur mainly on mucosal surfaces (1,2). While much experimental evidence has been gathered in the ensuing two decades, we still lack many important details of how to best stimulate the mucosal immune response to many relevant enteropathogens and their toxic products. The formation of secretory IgA involves the unique collaboration between an epithelial cell which produces the 60,000 dalton glycoprotein secretory component and the plasma cell which produces IgA. Plasma cells produce IgA in the lamina propria of the gastrointestinal tract and other mucosal glands. It is transported to the surface epithelium where it attaches to secretory component, which protects the IgA from the proteolytic environment of the gut lumen (2). This allows IgA to function in the gut lumen while the IgG serum antibodies (which do not attach to secretory component) are rapidly degraded in this same environment.

Secretory IgA is stimulated best when antigens are applied to a relevant mucosal surface. The initial step in this process involves phagocytosis of antigens by specialized surface epithelial cells (M cells) which overlie Peyer's patches, the appendix, and isolated lymphoid follicles in the gut (3,4). This material is brought into the underlying lymphoid tissues. Early work from Cebra's laboratory indicated that the B lymphoblasts in the gut-associated lymphoid tissues (GALT) are especially enriched in precursors for developing the IgA response (5). Following their stimulation in the GALT, these B lymphoblasts migrate in turn to the mesenteric lymph nodes, the thoracic duct, and eventually lodge in the spleen where they undergo some degree of maturation (5-8). During their maturation, these B lymphoblasts come under the influence of various T cell populations which can affect the final outcome of this process. Kawanshi et al. described the presence of switch T cells in Peyer's patches which would encourage B lymphocytes to alter their surface isotype expression from mu to alpha heavy chain (9). Other workers have described helper T cells which could augment the IgA response of B lymphocytes which are already committed to IgA synthesis (10). The final site of lodging of these B lymphocytes may be influenced by the site of antigen stimulation (11). This is why oral immunization is the best route for stimulating mucosal immunity along the gastrointestinal tract and why inhalation antigen administration may prove to be the best route to stimulate mucosal immunity along the respiratory tract. Further, it has been learned that plasma IgA is transported into the bile and then to the upper small intestine. Biliary tract obstruction, for instance, results in decreased intestinal luminal IgA with an increase of secretory IgA in plasma (12).

The focus of our laboratory has been to develop a reliable animal model system to study specific aspects of the mucosal immune response. Several other laboratories have also developed model systems for following mucosal immune events. Considering the diversity of the model systems, markedly consistent information has been obtained from these studies.
Our laboratory was interested in following the kinetics of the development of secretory IgA responses against enteropathogenic bacteria and their toxic products. To allow us to collect sequential secretions, we developed a chronically isolated ileal (Thiry-Vella) loop model in rabbits (13). For this procedure, we operate on animals under conditions similar to that of human surgery. A 20 cm segment of ileum is isolated with its vascular supply intact. This isolated loop provides us with approximately 2 ml of secretions which we collect daily to analyze for specific antibody content using an enzyme-linked immunosorbent assay. Using this model system, we have followed secretory IgA responses to Shigella, Shiga toxin, and to cholera toxin (14-17). Our most extensive studies took advantage of the variety of strains of Shigella flexneri produced in the laboratory of Dr. Samuel B. Formal at the Walter Reed Army Institute of Research. Originally, we predicted that only strains that were invasive would be effective immunogens to give orally for stimulating the IgA response in intestinal secretions. In fact, our first studies on mucosal immunity to Shigella used the hybrid strain of S. flexneri and Escherichia coli, Shigella X16. This strain is capable of invading the surface epithelium but does not reproduce once within the host tissues. Therefore, no ulceration is produced. We demonstrated that by giving this antigen directly into the isolated intestinal loops, we could produce a strong local secretory IgA response. Similar responses were found with invasive strain M4243 and with a noninvasive strain 2457-0 when applied directly into the isolated ileal loops. Further, the presence of a Peyer's patch locally, and the dosage schedule are both found to be important factors influencing the development of the local immune response (19,20).

These early studies found that intestinal secretions from loops stimulated directly with various Shigella preparations would contain considerable antigen specific secretory IgA but little or no IgG directed against Shigella (19,20). We demonstrated that this was not due to rapid degradation of IgG (which is normally destroyed quickly in intact intestine), as the isolated loops are separated from the proteolytic effects of gastric acid, bile, and the proteolytic enzymes trypsin, pepsin, and chymotrypsin (21). Direct stimulation of the isolated loops by Shigella antigen resulted in little or no systemic (serum) IgG against Shigella unless the systemic immune response was previously primed by a parenteral (subcutaneous or intravenous) dose of Shigella (22).

It was extremely difficult, however, to study the mucosal memory response by directly stimulating the chronic isolated loops. There were two major difficulties in performing memory studies by direct intraloop immunization. First, it is technically difficult to maintain chronically isolated loops for longer than 45 days. Secondly, the effects that gastric acid, bile, and pancreatic enzymes would have on either the natural infection or on a potential oral vaccine are artificially bypassed by directly stimulating the chronically isolated intestinal loops. Although the findings by direct immunization of the loops were useful in studying the kinetics of the local immune response, the artificial method of stimulation may have limited the relevance to the study of the natural local immune response.
Therefore, in the present studies, rather than directly stimulating the isolated loops, we have used the loops as a probe for following the local immune response to orally or parenterally administered Shigella antigens. This approach takes advantage of the fact that when antigens stimulate the mucosal immune system in the gut, the B lymphocytes traffic around the systemic circulation as described above, and the B lymphoblasts eventually arrive back at the gastrointestinal site.

Using these isolated loops as a probe, we were able to demonstrate that a secretory IgA memory response could be elicited in isolated loop secretions after oral priming (23). This demonstration was key to development of logical oral vaccination studies. As other workers had suggested that multiple oral doses were more effective than a single dose in priming animals for mucosal immune responses, we primed animals by giving 3 weekly oral doses of Shigella X16. The animals were allowed to rest for 2 months after the 3rd oral dose and at that time, a chronically isolated ileal loop was created. The animals were then given the final oral challenge dose with Shigella X16. These animals demonstrated, for the first time in intestinal secretions, a striking secretory IgA memory response. Even 2 months after the last oral dose of Shigella antigen, there was a significant amount of IgA anti-Shigella LPS activity in the primed animals (23). The kinetics of the response after the oral challenge in the primed animals was remarkably strong by the 4th day (23).

In the past 2 years, we have shown that noninvasive antigens are also capable of eliciting this mucosal memory response (24,25). The most recent studies in our laboratory have focused on the functional significance of the secretory IgA response to prevent the pathogenic effects of the microorganisms. As described in this report, experiments using whole animal preparations and HeLa preparations were difficult to establish. We have had considerably more success by looking at the initial uptake of the Shigella, an event which is requisite to developing the ulceration. We have also been able to study with considerable success the functional significance of secretory IgA elaborated against Shiga toxin. Lastly, we have focussed on the initial processing events as a means for determining logically which vaccine preparations were the most successful. Using a combination of techniques from histology, immunohistology and electron microscopy, we have demonstrated that the mucosal immune response does not correlate with the invasiveness of the bacteria. However, secretory IgA may mediate protection by preventing the Shigella from binding to the surface epithelium. Lastly, we have looked at the Paneth cells, a poorly understood population of cells in the gastrointestinal tract which have relevance to microbial flora in the gut lumen. The studies described in this report demonstrate that these cells are clearly responding to the microbial flora of the gut lumen and that they can be isolated in vitro. Future studies will look at whether these cells will function in antigen processing of Shigella or whether they are part of the cytotoxic mechanisms for defeating potential infection following invasion.
Methods

Preparation of Chronically Isolated Ileal Loops. The surgical creation of chronically isolated ileal loops in rabbits has been described in detail previously (13). Briefly, 3 kg New Zealand white rabbits (specific pathogen free) are anesthetized with xylazine and ketamine. A midline abdominal incision is made and the terminal ileum is identified. Twenty centimeters of ileum containing a Peyer’s patch is isolated with its vascular supply intact. Silastic tubing (Dow-Corning) is sewn into each end of the isolated segment. The free ends of the tubing are brought out through the midline incision and are tunnelled subcutaneously to the nape of the neck where they are exteriorized and secured. Intestinal continuity is restored by an end-to-end anastomosis. 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 of air. With proper daily care, > 90% of our rabbits have completed experiments lasting 2 months.

Enzyme-linked Immunosorbent Assay (ELISA). Microtiter wells are coated with a solution containing S. flexneri lipopolysaccharide (LPS) (Westphal preparation). Immediately prior to testing serum samples or loop secretions, the LPS antigen solution is removed and the wells are washed with a phosphate-buffered saline solution (PBS) containing 0.05% Tween 20 (PT). The fluid to be assayed is diluted in the PT buffer and incubated in the coated and uncoated wells (the latter to control for nonspecific adsorption) for 4 hours. The plates are washed with PT and incubated with either alkaline phosphatase-conjugated sheep anti-rabbit IgA or sheep anti-rabbit IgG (both are isotype specific affinity column purified in our laboratory using methods previously described (21)). After an incubation of 4 hours, the wells are again washed with PT and the substrate reaction is carried out with p-nitrophenyl phosphate in carbonate buffer pH 9.8. The kinetics of the enzyme-substrate reaction are extrapolated to 100 minutes. The OD 405 nm (read on a Titertek Microelisa Reader) of the uncoated wells is subtracted from that of the coated wells. Specific IgG and IgA standards are processed on each plate with the unknown fluids as previously described (26).

The data are analyzed using the RSI software system. Data are presented as geometric means, as others have noted that this better reflects the logarithmic kinetics of the local immune response after immunization (27). For each day’s result, the variance is expressed together with the mean.

Antigen Preparations Used. Four antigen preparations were employed in the present studies: 1) Shigella flexneri M4243 (which can invade intestinal mucosa and persists in the epithelium), 2) Shigella X16 (a hybrid of S. flexneri and E. coli—which invades the intestinal mucosa but does...
not persist within the epithelium, 3) *S. flexneri* M2457-0 (which does not invade, but which possess the 140 megadalton virulence plasmid), 4) *S. flexneri* M4243A, (which lacks the virulence plasmid and shows no invasiveness). All strains are tested for invasion using the Sereny test. The Sereny test is performed weekly on strains to assure the invasive, or noninvasive activities for *Shigella* uptake studies.

In Vivo Assay for Uptake of *Shigella flexneri* by Follicle-Associated Epithelium and Villi. To determine the relationship between the virulence of the microorganism and its uptake by the isolated follicle epithelium, an in vivo assay procedure was employed. 10 cm isolated ileal loops were created in conventional New Zealand bred rabbits. A single dose containing 10⁸ *Shigella flexneri* was injected into this acute loop. At 30, 90, and 180 minutes, these loops were removed and frozen sections were prepared. These sections were fixed in methanol and stained with Giemsa. For each time, at least 10 sections of Peyer's patch and adjacent villi were examined for attachment and uptake of the *Shigella flexneri*. Histologically, these sections were divided into three areas: 1. the follicle associated epithelium overlying the dome areas in Peyer's patches (known to be enriched in M cells). 2. the villi between the dome areas within the Peyer's patch. 3. villi which were outside of the Peyer's patch area. Evaluation was performed using oil immersion light microscopy. Since the normal flora of the rabbit ileum contains less than 10⁴ microorganisms, for statistical purposes, less than 1% of the flora visualized were from other microorganisms. Further, the *Shigella flexneri* have a characteristic size and shape which, under the circumstances of this study, were readily recognizable using this technique. Electron microscopy was performed on some sections demonstrating the characteristic rod-shaped structure and the typical "M" cell location. Results were expressed as microorganisms per dome area or microorganisms per villus.

Mucosal Immune Response to Shiga Toxin. Two groups of rabbits were used in this study: two normal rabbits, three specific pathogen-free rabbits. Following creation of a chronically isolated ileal loop in each rabbit, three weekly intraloop doses of Shiga toxin were administered (the first dose being given on the day of surgery-antigen day 0). The Shiga toxin preparation used for this study was a post-DEAE fraction, provided by Dr. J. Edward Brown of the Walter Reed Army Institute of Research. Dr. Brown's laboratory found that this preparation contained 10⁴ units of toxin per ml of fluid. Each loop dose consisted of 0.5 ml of Shiga toxin fraction plus 3.5 ml of saline. As in our other studies, the ileal loop was flushed daily of mucus and secretion. The 4 ml of toxin preparation were then injected into the loop, followed by 2 ml of air. One of the tubes from the loop was immediately taped to keep the toxin from spilling out of the loop. Ileal loop secretions from each animal were collected daily for 1 month and assayed for specific IgA against Shiga toxin.

Samples of the flushes from the first two rabbits for several antigen days were sent to Dr. Brown who tested them for their anti-Shiga toxin activity using his in vitro assay.

Mononuclear Cell Isolation. At time of sacrifice, rabbits from various immunization groups had peripheral blood, Peyer's patches, mesenteric lymph nodes, spleen and axillary lymph nodes removed under aseptic con-
ditions. For the peripheral blood, the buffy coat was placed on lymphocyte separation medium and centrifuged at 400 x g at room temperature for 30 minutes. The cells at the interface were removed, characterized and used as mononuclear cell preparations. Tissues were cut into 1 cm square fragments with a sterile blade and placed on sterile wire mesh. The cells were carefully teased apart and passed through the mesh. This material was centrifuged at 400 x g at room temperature for 7 minutes. The pellet was gently resuspended and washed twice in RPMI 1640. The total cells and viability were determined. A Wright stain preparation was examined to determine the differential of the isolated cells.

Mononuclear Cell Phenotypes. Cell surface immunoglobulin positive and phagocytic cells were assayed together. 10⁶ viable mononuclear cells were suspended in 1 ml of a 1.1 micron latex bead/complete media suspension. This was incubated at 37°C in a humidified 95% air and 5% CO₂ incubator from 1 to 2 hours. The cells were then washed in a cold 4°C phosphate buffer of pH 7.4 in 0.1% sodium azide. Thereafter, it was incubated with 100 microliters of a 1:20 dilution of fluorescein-labeled goat anti-rabbit IgG or anti-rabbit IgM (Cappel) in an ice bath for 30 minutes. The cells were then washed three times in 2 ml of cold buffer and resuspended in 1 drop of glycerol:PBS 9:1. The number of surface immunoglobulin positive (slg) and phagocytic cells (at least 3 latex beads per cell) were enumerated using a Leitz microscope having both epifluorescence and phase contrast capabilities. At least 100 cells were counted for each calculation. Cytoplasmic immunoglobulin-containing cells were assessed using ethanol-fixed cytospin preparations; these were assayed with fluorescein-labeled anti-polyvalent rabbit immunoglobulins (Cappel). The cytospin was then washed 3 times with standard buffer, mounted with glycerol:PBS and at least 100 cells were counted.

In Vitro Mononuclear Cell Cultures. 10⁵ mononuclear cells were added to each row of a 96 well, polystyrene microtest III tissue culture plate (Becton Dickenson). The wells are flat-bottomed. Cultures were placed in a humidified, 95%/5% CO₂ 37°C incubator. At the times indicated in the result section, 3 wells for each tissue were aspirated. Cellular debris was removed by centrifugation at 440 x g for 15 minutes and the supernatants were stored at -20°C until assayed. Assays were performed using the above described ELISA technique.

Paneth Cell Studies. In each of 15 New Zealand white rabbits isolated ileal loops were created. One group of rabbits had the loop flushed daily per routine. Loop fluids and normal ileal contents were cultured to determine degree of bacterial colonization. In the second group of rabbits, an antibody solution containing 10 mg/ml neomycin sulfate and 2.5 mg/ml bacitracin in sterile saline together with 50 µg/ml of gentamicin were added to the isolated ileal loops. The nonabsorbable nature of these 3 antibiotics was a major requirement for their selection over other more commonly used agents. Animals were sacrificed at 2 weeks after surgery to determine the effect of the antibiotics on the flora and on the Paneth cell structure.

Coded Histologic Study Utilized Routine Hematoxylin and Eosin Stained Section. One section was examined for each tissue specimen. Paneth cell hyperplasia was estimated using an ocular micrometer with the measurement
of the height of the Paneth cell column (extension from crypt base into higher levels of the crypt) in 20 consecutive well-oriented crypts of each section. Crypt depth and villus height were similarly estimated by ocular micrometer, with 12 consecutive well-oriented crypt-villus pairs measured in each section. Average values were used to represent each parameter for each animal. Lamina propria heterophiles and epithelium mitotic figures were counted in 10 high power fields (400x) of the crypt region. Goblet cells and intraepithelial lymphocytes (IEL) were counted per 500 epithelial nuclei on the sides of well-oriented villi.

Electron Microscopy. All tissues for electron microscopy were minced with a fresh scalpel blade and fixed in 3% glutaraldehyde and PBS for 2 hours. Postfixation was carried with 2% S-collidine-buffered osmium tetroxide. 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. Thick sections (1 micron) were stained with toluidine blue. Electron microscopy was carried out on a Zeiss 109 fiberoptic transmission electron microscope.

In Vitro Assays for Adherence of S. flexneri. Two assay methods were explored. The first involved the use of whole ileum and colon tissue sections from rabbits. The second used a HeLa tissue culture line.

1. Sections of ileum and colon were opened and the contents gently expelled with saline moistened gauze. The bowel was cut into segments 1-2 cm. Following a brief rinse in two separate beakers of saline, the segments were placed into a tared cup containing 10 ml of modified Krebs Ringer Solutions (KRS) and weighed. The segments were placed in solutions containing Shigella for 20 minutes at 37°C with gentle shaking in a water bath. Thereafter, each segment was dipped 4 times (15 seconds each) in successive beakers of saline and placed into a cup containing 10 ml modified KRS. In each experiment, 1 segment was frozen to examine the location of the adhering shigella. Remaining segments were individually homogenized for 15 seconds using the Virtis blender. The homogenate was poured into another cup and allowed to settle for 10 minutes. Both the homogenate and the supernatant fluid (from the initial incubation) were used for plating on MacConkey agar. Viable counts of the homogenates were determined by plating 0.1 ml of serial dilutions onto MacConkey agar. The percentage of viable shigella adhering to the tissue sections was calculated by dividing the number of shigella per ml of KRS present after incubation by 100. Giemsa stains of the frozen sections were made to determine the location of the shigella on the tissue (serosal vs. epithelial surface).

2. HeLa cells were grown in Minimal Essential Medium (MEM) with 10% fetal calf serum containing 1% antibiotic-antimycotic (Gibco Laboratories). Sixty thousand HeLa cells were grown overnight to form a contiguous monolayer on Lab-Tek tissue culture chamber slides (Miles Scientific). Following a gentle rinse with MEM, S. flexneri M4243 were added to the chamber in the indicated concentrations and allowed to incubate for 30 minutes at 37°C (multiplication period). Slides were fixed in ethanol and stained with Giemsa. For each
preparation, 100-200 HeLa cells were counted and rated for bacterial involvement.

\[
\begin{align*}
0 &= \text{no rods attached} \\
+/- &= 1 \text{ rod/HeLa cell} \\
1+ &= 2-4 \text{ rods/HeLa cell} \\
2+ &= 5-10 \text{ rods/HeLa cell} \\
3+ &= > 10 \text{ rods/HeLa cell}
\end{align*}
\]

In Vivo Assays for Adherence of S. flexneri. Two assay systems were used. The first involved the use of intact rabbits with an oral challenge route. The second involved the surgical creation of a RITARD system and direct inoculation of bacteria into the jejunum.

1. Rabbits were fasted for 48 hours prior to challenge with bacteria. Oral doses of S. flexneri M4243 were given (via orogastric tube) to rabbits anesthetized by xylazine and ketamine. For two days prior to challenge and 2-4 days after challenge, animals had rectal temperatures recorded at 9 a.m. and 3 p.m. and total white blood cell count and differentials were obtained. Daily cultures of stool were made on MacConkey's agar. At time of sacrifice, histologic sections of jejunum, ileum (including a Peyer's patch) and colon were routinely obtained. These were graded for inflammation and bacterial invasion. Also, cultures on MacConkey agar were made of the jejunal, ileal and colonic contents.

2. RITARD model. Rabbits were fasted for 24 hours and anesthetized with xylazine and ketamine. The abdomen was opened under aseptic conditions and sterile umbilical tape was used to create a slip knot around the terminal ileum immediately anterior to the mesoappendix. This served to partially obstruct the flow of chyme. A 10 ml sample of S. flexneri M4243 was injected directly into the lumen of the jejunum. The incision was closed in 2 layers and one end of the slip knotted tape was brought out through the incision. Two hours after the inoculation of bacteria, the slip knot was released. Temperatures, cultures and white blood counts were performed as in the oral challenge rabbits. Histologic sections and cultures were obtained at time of sacrifice.
Results

Establishment of the Chronically Isolated Ileal Loop Model as a Probe for the Mucosal Immune Response.

The Immunization schedule used in the first 6 groups of rabbits for these studies is outlined in Table 1.

Table 1. Immunization Schedule

<table>
<thead>
<tr>
<th>Group</th>
<th>Antigen</th>
<th>Dose</th>
<th>Route</th>
<th>Day(s) Given</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Live Shigella X16</td>
<td>$10^{10}$</td>
<td>oral$^{(2)}$</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>Killed Shigella X16</td>
<td>$10^{10}$</td>
<td>oral</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>Live Shigella X16</td>
<td>$10^{10}$</td>
<td>oral</td>
<td>0,7,14</td>
</tr>
<tr>
<td>IV</td>
<td>Live Shigella X16</td>
<td>$10^{10}$</td>
<td>oral</td>
<td>-75,-68,-61,0</td>
</tr>
<tr>
<td>V</td>
<td>Killed Shigella X16</td>
<td>$10^{10}$</td>
<td>oral</td>
<td>0,7,14</td>
</tr>
<tr>
<td>VI</td>
<td>Killed Shigella X16</td>
<td>$10^{10}$</td>
<td>oral</td>
<td>-75,-68,-61,0</td>
</tr>
</tbody>
</table>

$^{(1)}$ Day of surgical creation of isolated loops = day -1 for all groups.
$^{(2)}$ Shigella placed in stomach via orogastric feeding tube. Isolated loop not directly exposed to shigella.

Figure 1 demonstrates the mean IgA anti-Shigella response found in secretions from animals given a single oral dose of $10^{10}$ live Shigella X16 (Group I). The kinetics of the IgA anti-Shigella response in these secretions paralleled those previously described by our laboratory when the isolated ileal loops were directly stimulated with a single dose of Shigella (14) even though the isolated loop in Group I was never directly exposed to the Shigella. These findings confirmed our preliminary hypothesis that the chronically isolated ileal loop could be used as a probe to follow the kinetics of the local secretory IgA response to orally administered antigens. In addition, these results established the normal response of a large group (11 rabbits) of unprimed animals to a single oral dose of Shigella. This information was used in subsequent comparisons with the secretory IgA response using different dosage schedules and antigen preparations.
Figure 1. Mean IgA anti-Shigella response in ileal loop secretions from rabbits immunized orally with a single dose of $10^{10}$ live Shigella X16 on day 0. Standard air of the means (SEM) indicated.

To determine whether live antigen is necessary to stimulate the mucosal immune system, a single oral dose of $10^{10}$ heat-killed Shigella X16 was given to each of 12 rabbits (Group II). The anti-Shigella response obtained from these secretions of these isolated loops (again not directly exposed to antigen) in these rabbits is depicted in Figure 2. Overall, the response paralleled but was weaker than that of the Group I animals.
Many investigators have found that multiple immunizations are necessary to achieve optimal mucosal immune responses. These are similar to our own findings when we directly immunized the isolated loops with antigen. In the Group III animals, we administered $10^{10}$ live Shigella X16 orally to 8 rabbits and followed the mucosal immune responses in their isolated loops. As shown in figure III, the kinetics of the response after the first dose of Shigella was similar to that seen after the single dose of live or killed Shigella X16. However, after the 2nd and 3rd dose an increase in the IgA anti-Shigella was found. Five of the 8 rabbits gave significant increases in the IgA anti-Shigella activity in these secretions. The heterogeneity of the response is evident from the SEM indicated in Figure 3.
Figure 3. Mean IgA anti-Shigella response in ileal loop secretions from rabbits immunized orally on days 0, 7, 14 and with $10^9$ live Shigella X16. SEM indicated.

In contrast to the response to live Shigella, the IgA anti-Shigella response of 7 rabbits given 3 weekly oral doses of $10^9$ heat-killed Shigella showed no increase in the activity as compared to those given a single oral dose (Figure 2). This gave us our first hint that heat-killed Shigella preparations though immunogenic may not be able to prime animals for a mucosal memory response. This would put significant limitations on the ability to use such preparations for vaccination.

Demonstration of secretory IgA memory response in intestinal secretions.

The most dramatic information we obtained from the early studies on the isolated loop as a probe involved the demonstration of the secretory IgA memory response. Until this time, there was only scant information in the literature which suggested that a secretory IgA response existed. Pierce and his colleagues were able to demonstrate increases in the number of antibody containing cells (ACC) following priming with cholera toxin preparations (28). However, no one had shown a boost in the secretory IgA response in intestinal secretions or an alteration in the kine-
tics of that response prior to this time. We immunized a group of 7 rabbits that did not have chronically isolated ileal loops with 3 weekly oral doses of $10^9$ live *Shigella* X16 (Group IV-Table 1). After the third dose, the animals were allowed to rest for 60 days, then a chronically isolated ileal loop was created in each animal and the animals were given a single oral challenge with $10^{10}$ live *Shigella* X16. The IgA anti- Shigella response in the secretions of these animals following their single oral rechallenge with $10^{10}$ live *Shigella* are compared in Table 2 to the responses of the nonprimed animal that received a single oral challenge (Group I). It should be noted that after 60 days had passed since the animals received their oral challenge, a low level of IgA activity was still present in intestinal secretions from the primed animals. Following the single oral challenge, secretions from the primed animals displayed a rapid increase of IgA anti-Shigella activity. In the history of this model system, such a dramatic increase in IgA had not been seen. The response peaked at the fourth day after challenge. In the meantime, the nonprimed animals displayed the typical weak primary IgA response described earlier in this report.

Table 2. IgA Memory Response in Rabbit Ileal Loop Secretions After Oral Priming with Live *Shigella* X16

<table>
<thead>
<tr>
<th>Day After Challenge</th>
<th>Not Primed (2)</th>
<th>Primed with Live X16 (3)</th>
<th>Significance (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>.05 ± .008</td>
<td>.19 ± .08</td>
<td>N.S. (6)</td>
</tr>
<tr>
<td>2</td>
<td>.05 ± .01</td>
<td>.34 ± .14</td>
<td>&lt;.03</td>
</tr>
<tr>
<td>3</td>
<td>.05 ± .02</td>
<td>.38 ± .14</td>
<td>&lt;.04</td>
</tr>
<tr>
<td>4</td>
<td>1.10 ± .03</td>
<td>1.29 ± .41</td>
<td>&lt;.03</td>
</tr>
<tr>
<td>5</td>
<td>1.20 ± .14</td>
<td>1.04 ± .33</td>
<td>&lt;.03</td>
</tr>
<tr>
<td>6</td>
<td>1.51 ± .24</td>
<td>1.15 ± .28</td>
<td>N.S.</td>
</tr>
<tr>
<td>8</td>
<td>.59 ± .31</td>
<td>.77 ± .21</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

(1) Day 0 = day of final antigen challenge.
(2) Animals given $10^9$ live *Shigella* X16 orally on day 0 (n=11).
(3) Animals given $10^9$ live *Shigella* X16 orally on days -75,-68,-61 prior oral challenge on day 0 (n=7).
(4) As determined by Student's t-test.
(5) Results expressed as mean O.D. 400nm/100min. ± standard error of mean (S.E.M.) of IgA antibodies specific for shigella antigen as determined by ELISA.
(6) Not significant.

In contrast to the striking mucosal memory response seen with oral priming, the group of 11 rabbits that were primed with 3 oral doses of $10^9$ heat-killed *Shigella* X16 (Group VI) gave no evidence of a mucosal IgA anamnestic response when challenged with the same antigen. The IgA anti-*Shigella* responses in the secretions of these animals following their single oral rechallenge are compared in Table 3 to the responses of non-primed animals that had received a single oral challenge with the same antigen. Further, no residual response was noted in the orally primed group.
Table 3. Lack of IgA Memory Response After Oral Priming with Killed Shigella X16

<table>
<thead>
<tr>
<th>Day After Challenge (1)</th>
<th>Not Primed (2)</th>
<th>Orally Primed with Killed X16 (3)</th>
<th>Significance (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>.040(5) ± .008</td>
<td>.057 ± .02</td>
<td>N.S. (6)</td>
</tr>
<tr>
<td>2</td>
<td>.046 ± .011</td>
<td>.040 ± .14</td>
<td>N.S.</td>
</tr>
<tr>
<td>4</td>
<td>.089 ± .016</td>
<td>.108 ± .05</td>
<td>N.S.</td>
</tr>
<tr>
<td>6</td>
<td>.140 ± .031</td>
<td>.173 ± .063</td>
<td>N.S.</td>
</tr>
<tr>
<td>8</td>
<td>.161 ± .049</td>
<td>.120 ± .041</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

(1) Day 0 = day of final antigen challenge.
(2) Unprimed animals given $10^7$ killed Shigella X16 orally on day 0 (n=12).
(3) Animals primed with $10^{10}$ killed Shigella X16 on days -75,-68,-61 prior to oral challenge on day 0 (n=11).
(4) Significance assessed by Student's t-test.
(5) Results expressed as mean O.D. 400nm/100 min. ± S.E.M. for shigella antigen as determined by ELISA.
(6) N.S. = Not significant.

The heightened response of the triple oral dose of live Shigella X16 and the memory response of the Group IV animals as compared to the lack of a memory response in animals given a triple oral dose of killed Shigella X16 may relate to the fact that these bacteria can multiply in the gastrointestinal tract and, therefore, the actual dose of shigella antigen would be greater with the live than with the killed shigella. Alternatively, or in addition, the Shigella X16 strain is known to invade the epithelium locally (although it does not persist following this invasion). This invasion may allow for a more efficient presentation of antigen and the heightened response results. Therefore, in the next series of studies we review the ability of noninvasive bacteria to elicit a mucosal memory response.

The dosage schedule in Table 4 shows the various groups we used to study the effects of noninvasive bacteria and adjuvant preparations on the mucosal immune response.
Table 4. Immunization Schedule

<table>
<thead>
<tr>
<th>Group</th>
<th>Antigen</th>
<th>Dose</th>
<th>Route</th>
<th>Day(s)(1) Given</th>
</tr>
</thead>
<tbody>
<tr>
<td>VII</td>
<td>Live \textit{S. flexneri} 2457-0</td>
<td>$10^{10}$</td>
<td>oral(2)</td>
<td>0</td>
</tr>
<tr>
<td>VIII</td>
<td>Live \textit{S. flexneri} 2457-0</td>
<td>$10^{10}$</td>
<td>oral</td>
<td>0,7,14</td>
</tr>
<tr>
<td>IX</td>
<td>Live \textit{S. flexneri} 2457-0</td>
<td>$10^{10}$</td>
<td>oral</td>
<td>-75,-68,-61,0</td>
</tr>
<tr>
<td>X</td>
<td>Heat-killed \textit{Shigella} X16</td>
<td>$10^8$</td>
<td>subcutaneous</td>
<td>0,1</td>
</tr>
<tr>
<td></td>
<td>Heat-killed \textit{Shigella} X16</td>
<td>$10^8$</td>
<td>intravenous</td>
<td>4-8,14</td>
</tr>
<tr>
<td>XI</td>
<td>Heat-killed \textit{Shigella} X16</td>
<td>$10^8$</td>
<td>subcutaneous</td>
<td>-21,-20</td>
</tr>
<tr>
<td></td>
<td>Heat-killed \textit{Shigella} X16</td>
<td>$10^8$</td>
<td>intravenous</td>
<td>-17,-13,-7</td>
</tr>
<tr>
<td></td>
<td>Live \textit{Shigella} X16</td>
<td>$10^{10}$</td>
<td>oral</td>
<td>0</td>
</tr>
<tr>
<td>XII</td>
<td>Live \textit{Shigella} X16</td>
<td>$10^{10}$</td>
<td>oral</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>with DEAE-dextran</td>
<td>5 gm/dl</td>
<td>oral</td>
<td></td>
</tr>
<tr>
<td>XIII</td>
<td>Live \textit{Shigella} X16</td>
<td>$10^{10}$</td>
<td>oral</td>
<td>-75,-68,-61,0</td>
</tr>
<tr>
<td></td>
<td>Antibiotic</td>
<td></td>
<td></td>
<td>-2,-1,0,1,2,3</td>
</tr>
</tbody>
</table>

(1) Day of surgical creation of isolated loops = day-1 for all groups.
(2) \textit{Shigella} placed in stomach via orogastric tube. Isolated loop not directly exposed to \textit{Shigella}.

The local IgA response in isolated loop secretions following a single oral dose of $10^{10}$ live \textit{S. flexneri} strain 2457-0 (noninvasive) is shown in Figure 4. The kinetics of the IgA anti-shigella response is similar to that described with X16 except that by day 24, the response has not deteriorated.
Figure 4. Mean IgA responses to Shigella in secretions from rabbits given a single oral dose of \(10^8\) noninvasive, live, \(S\). flexneri strain 2457-0 on day 0 (Group VII). IgA activity ± S.E.M. indicated.

Multiple immunizations were given with the noninvasive 2457-0 strain on day 0, 7 and 14 (Group VIII). As shown in Figure 5, there was no significant increase in the response observed with this regimen. This differed from the situation with Shigella X16 wherein a significant increase was seen in the latter days.
To determine whether a local IgA memory response could be elicited by immunization and challenge with noninvasive S. flexneri the Group IX animals were primed with 3 weekly oral doses of $10^{10}$ live S. flexneri 2457-0. 60 days after the third oral dose, a chronically isolated ileal loop was created and the rabbits were challenged with a single dose of the same bacteria. As shown in Table 5, a significant IgA anti-shigella response was seen very quickly in animals with previous oral priming. A significant increase over background levels was found as early as day 3 with a marked increase in the IgA anti-shigella activity by day 4. Responses from day 3, 4, 5, and 6 were highly significantly greater in the primed than in the unprimed rabbits. These findings confirm that a non-invasive strain of shigella could elicit a mucosal memory response.
Table 5. IgA Anti-Shigella in Loop Secretions

<table>
<thead>
<tr>
<th>Day After Challenge</th>
<th>Not Primed (2)</th>
<th>Primed (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>.061 ± .017(4)</td>
<td>.387 ± .267</td>
</tr>
<tr>
<td>1</td>
<td>.146 ± .088</td>
<td>.216 ± .109</td>
</tr>
<tr>
<td>2</td>
<td>.109 ± .039</td>
<td>.276 ± .126</td>
</tr>
<tr>
<td>3</td>
<td>.176 ± .070</td>
<td>.571 ± .232</td>
</tr>
<tr>
<td>4</td>
<td>.432 ± .153</td>
<td>2.030 ± .400</td>
</tr>
<tr>
<td>5</td>
<td>.894 ± .242</td>
<td>2.434 ± .493</td>
</tr>
<tr>
<td>6</td>
<td>1.580 ± 1.389</td>
<td>2.671 ± .498</td>
</tr>
</tbody>
</table>

(1) Day 0 = Day of final challenge.
(2) Unprimed Animals given $10^6 S. flexneri 2457-0 (N=16).
(3) Rabbits primed orally with $10^7 S. flexneri 2457-0 on Days -75, -68, -61 prior to oral challenge on Day 0 (N=10).
(4) Results expressed as mean O.D. nm/100 min. ± S.E.M.

Immunogenicity of heat-killed Shigella given subcutaneously.

Since traditional methods of immunization have utilized parenteral administration of heat-killed antigen preparations, the Group IV animals were given heat-killed Shigella X16 subcutaneously on day 0, 1 and intravenously on days 4-8 and 14. The mean IgG and IgA activity to Shigella in secretions from these animals is shown in Figure 6. Clearly a weak primary IgA response was all that was elicited by this dosage regimen. This dosage was chosen as it elicits a strong serum IgG and IgA response (22).

Use of adjuvants to enhance the IgA response following oral immunization.

Other groups have found that DEAE-dextran was able to serve as a mucosal adjuvant (29). Therefore, to establish the baseline for future memory response experiments, we looked at the effect of DEAE-dextran on the primary local IgA response to Shigella. Group XII rabbits were given DEAE-dextran along with the single oral dose of live Shigella X16. As shown in Figure 8, no enhancement of the response was seen. Indeed, there was some suggestion that a decrease in the response had occurred. However, owing to the large S.E.M. this could not be determined with certainty.
Figure 6. Mean IgG (open circle) and IgA (closed circle) activity to Shigella in secretions from Group X rabbits. When a single oral dose was added on day 0 (Group XI) no enhancement of the IgA response was found in secretions (Figure 7).

Figure 7. Mean IgG (open circle) and IgA (closed circle) activity to Shigella in secretions of Group XI rabbits.
Figure 8. Mean IgA anti-Shigella activities in secretions from rabbits fed a single live, oral dose of $10^8$ Shigella X16 on day 0 (open circle) compared to responses from rabbits given DEAE-dextran along with the single oral dose of $10^8$ Shigella X16 on day 0 (closed circle).

**Effect of erythromycin on the mucosal immune response.** During the course of these experiments, a group of animals became infected with Pasteurella multocida and were treated with erythromycin. Anecdotally, we noted a decrease response from this group of rabbits. While we originally assumed that this was due to their illness, we decided to perform a control experiment to be certain that the erythromycin did not have a deleterious effect on the mucosal immune response. Therefore, the Group XIII rabbits received the typical priming doses of live Shigella X16 and challenge dose also received erythromycin on the two days preceding and three days following the challenge dose of live Shigella X16. As shown in Figure 9, a significant decrease in the IgA memory response was consistently observed in these animals. We believe that this effect is specific to erythromycin.
Figure 9. Mean IgA anti-Shigella activity in the secretions from orally primed animals challenged with a single dose of live Shigella X16. The group of animals given erythromycin (Group XIII) at time of challenge (open circles) had a significantly weaker response than did those animals which did not receive antibiotics (closed circles).

Live Shigella are needed as challenge dose for the mucosal memory response. We have hypothesized that the erythromycin affect may have resulted from a decreased viability of Shigella in the animals so-treated. Therefore, it was important to determine whether live or killed Shigella are needed to elicit an appropriate mucosal memory response upon rechallenge. To perform these studies, the experiments outlined in Table 6 were performed. In Figure 10 is shown the results from animals in Groups XIV and XV. The animals in Group XIV were given the typical priming regimen which consistently elicits a mucosal memory response. However, following challenge by heat-killed Shigella given subcutaneously no memory response was seen. Indeed, the response did not differ significantly from those of the Group XV animals which were given only the subcutaneous dose of Shigella.
Table 6. Immunization Schedule

<table>
<thead>
<tr>
<th>Group</th>
<th>Antigen</th>
<th>Dose</th>
<th>Route</th>
<th>Day(s) (1) Given</th>
</tr>
</thead>
<tbody>
<tr>
<td>XIV</td>
<td>Live Shigella X16</td>
<td>$10^{10}$</td>
<td>oral (2)</td>
<td>-75, -68, -61</td>
</tr>
<tr>
<td></td>
<td>Heat-killed Shigella X16</td>
<td>$10^{10}$</td>
<td>subcutaneous</td>
<td>0</td>
</tr>
<tr>
<td>XV</td>
<td>Heat-killed Shigella X16</td>
<td>$10^{10}$</td>
<td>subcutaneous</td>
<td>0</td>
</tr>
<tr>
<td>XVI</td>
<td>Heat-killed Shigella X16</td>
<td>$10^{10}$</td>
<td>oral</td>
<td>-75, -68, -61</td>
</tr>
<tr>
<td></td>
<td>Live Shigella X16</td>
<td>$10^{10}$</td>
<td>oral</td>
<td>0</td>
</tr>
<tr>
<td>XVII</td>
<td>Live Shigella X16</td>
<td>$10^{10}$</td>
<td>oral</td>
<td>-75, -68, -61</td>
</tr>
<tr>
<td></td>
<td>Heat-killed Shigella X16</td>
<td>$10^{10}$</td>
<td>oral</td>
<td>0</td>
</tr>
<tr>
<td>XVIII</td>
<td>Heat-killed Shigella X16</td>
<td>$10^{12}$</td>
<td>oral</td>
<td>0, 7, 14</td>
</tr>
<tr>
<td>XIX</td>
<td>Heat-killed Shigella X16</td>
<td>$10^{12}$</td>
<td>oral</td>
<td>-75, -68, -61</td>
</tr>
<tr>
<td></td>
<td>Live Shigella X16</td>
<td>$10^{10}$</td>
<td>oral</td>
<td>0</td>
</tr>
</tbody>
</table>

(1) Day of surgical creation of isolated loops = day -1 for all groups.
(2) Shigella placed in stomach via orogastric tube. Isolated loop not directly exposed to Shigella.
Figure 10. Mean IgA anti-Shigella activity in secretions of Group XIV (open circles) and Group XV (closed circles) rabbits. These findings indicate that a challenge with parenteral heat-killed Shigella is not able to boost even a significantly primed mucosal immune system.

It was important to determine whether the lack of mucosal memory response produced by our earlier studies with heat-killed Shigella was due to a failure of priming versus a failure of the challenge dose by the heat-killed Shigella X16. Therefore, the experiments in Groups XVI-XIX were performed. When animals were primed with heat-killed Shigella and challenged with live Shigella X16 (known to be an effective challenge microorganism) no mucosal memory response was seen (Figure 11). Similarly, when animals were challenged with heat-killed Shigella following priming with an appropriate microorganism (live Shigella X16), no mucosal memory response was seen (Figure 11). These studies conclusively show that in order to prime for a mucosal memory protective response against Shigella that live bacteria must be used. Heat-killed Shigella given orally provide neither an appropriate priming nor challenge dose for the mucosal memory response.
Figure 11. Mean IgA anti-Shigella activity in secretions of Group XVI (closed circles) and Group XVII (open circles) rabbits.

It was possible that the bacteria given were too few in number to achieve a strong mucosal immune response. Therefore, the Groups XVIII and XIX rabbits were given enormous doses of heat-killed Shigella X16 orally (Table 6) and then the latter group was challenged with live Shigella. Once again, the heat-killed Shigella X16 proved totally ineffective for eliciting a mucosal immune response (data not shown).
Role of parenteral adjuvant in enhancing the primary secretory IgA response of the intestine.

Since our earlier studies indicated that a small primary local IgA response is detectable in secretions within a week of stimulation by live, oral Shigella X16, we explored the role of parenterally administered heat-killed Shigella X16 with and without complete Freund's adjuvant in stimulating the mucosal immune response. However, we first needed to establish that the heat-killed Shigella were immunogenic for mucosal immune responses.

Immunogenicity of heat-killed Shigella for a mucosal immune response. Since our earlier studies demonstrated that heat-killed Shigella were ineffective for stimulating a mucosal memory response, it was important to determine whether they had lost key antigenic determinants for eliciting this response. Therefore, a single oral dose of $10^{10}$ heat-killed Shigella X16 was injected directly into each of five Peyer's patches (0.2 ml/Peyer's patch) at the time of surgical creation of the isolated ileal loop in these animals. The seven animals in this Group developed significant increases in the secretory IgA anti-Shigella activity in their loop secretions over day 0 values by the fourth day after surgery (Figure 12). This was considerably sooner than responses which were achieved when antigen preparations were given orally. In a few secretions, weak IgG activity against Shigella was detected (data not shown). In the serum, however, the opposite specific antibody activity was seen. As shown in Table 7, the IgG anti-Shigella LPS activity rose to a geometric mean of 1.038 and did not significantly decline by the end of this study at one month. At contrast, the serum IgA activity to Shigella was weak throughout the study. These findings indicate that the heat-killed Shigella preparation used in our experiments was immunogenic for both the local IgA and systemic IgG response following immunization directly into Peyer's patches. Therefore, antigenic determinants requisite for stimulating mucosal immunity are present in these preparations.

<table>
<thead>
<tr>
<th>Days Post-Immunization</th>
<th>IgA</th>
<th>anti-LPS(2)</th>
<th>IgG</th>
<th>anti-LPS(2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-7</td>
<td>.263</td>
<td>(.228-.305)</td>
<td>.479</td>
<td>(.363-.630)</td>
</tr>
<tr>
<td>8-14</td>
<td>.268</td>
<td>(.239-.299)</td>
<td>1.038</td>
<td>(.922-1.165)</td>
</tr>
<tr>
<td>15-21</td>
<td>.206</td>
<td>(.199-.213)</td>
<td>.927</td>
<td>(.814-1.058)</td>
</tr>
<tr>
<td>22-28</td>
<td>.192</td>
<td>(.184-.200)</td>
<td>.824</td>
<td>(.570-1.193)</td>
</tr>
</tbody>
</table>

(1) Data not available for preimmunization. For comparison, Tables 3 unimmunized rabbits have shown values of .018 and .017 for IgA and .015 and .016 as Geometric means of unimmunized rabbits.

(2) Data expressed as Geometric means with variance as described in the Methods section.
Figure 12. IgA anti-Shigella LPS activity (circles) and IgG anti-Shigella activity (squares) in secretions from rabbits given intraplantar injections on day 0. S.E.M. are indicated.

The overall kinetics of the immune response achieved following combined parenteral immunization with heat-killed Shigella X16 and oral immunization with live Shigella X16 follow those of the primary mucosal immune response described in Group 1. However, by giving the heat-killed Shigella one day prior to oral antigen administration, we were able to achieve a significant enhancement of the primary mucosal immune response. Further, in these animals, there was a significant amount of IgG anti-Shigella LPS activity present in the intestinal secretions. The response for IgG was more variable than that of secretory IgA, however, the kinetics paralleled those of the primary secretory IgA response. As shown in Figures 13 and 14, IgA and IgG activity respectively was present in intestinal secretions within the first week from animals primed with both parenteral and oral Shigella. This effect was not further enhanced by using complete Freund's adjuvant to stimulate the parenteral response.
Figure 13. IgA anti-Shigella response in secretions given a single oral dose of live Shigella on day 0 (closed circles) and from rabbits given a combined parenteral dose of antigen on day -1 with a single oral dose on day 0 (open circles). S.E.M. are indicated.

Figure 14. Mean IgG anti-Shigella activity in isolate loops secretions from the two groups of rabbits in Figure 13.
Role of antigen form in the mucosal memory response—virulence plasmid. Although previous studies in our laboratory found that the noninvasive strain S. flexneri 2457-0 is able to elicit both a vigorous primary and mucosal memory response (see above), this bacteria possesses the 140 megadalton virulence plasmid. Further, in some clinical trials, it has reverted to a pathogenic form making it unsuitable as a mucosal vaccine. These findings raise the question as to whether in the experimental situation expression of the virulence plasmid by a few of the bacteria would account for the mucosal memory results obtained. Therefore, in the present studies, a single dose of Shigella M4243A, was given orally on day 0. The resulting intestinal IgA response is shown in Figure 15. This strain proved to be a highly immunogenic bacteria despite the lack of 140 megadalton virulence plasmid. The secretory IgA levels produced were significantly greater than those seen with a single dose of the locally invasive Shigella X16 strain. As with the other studies, the local IgG response was trivial and systemic IgG and IgA against Shigella were lacking.

Figure 15. Comparison of IgA anti-Shigella activity in secretions from animals given a single oral dose of live Shigella X16 (triangles) than those given M4243A (circles).
For the memory response studies with this nonpathogenic strain, the same dosage schedule was used as in previous memory studies performed with the invasive and noninvasive stains. As shown in Figure 16, an impressive secretory IgA memory response was found in the animals given this regimen. A residual secretory IgA response remained even 60 days after the last oral dose with this strain. Further, following the oral challenge dose on day 0, a striking secretory IgA memory response occurred. These responses were significantly greater than those elicited by a single dose of invasive Shigella X16 on all days tested and greater than the primary response to the M4243A on all days except 2 and 6-10 (here the large standard errors prevented significance. These findings indicate conclusively that noninvasive Shigella can be effective mucosal immunogens. Furthermore, the 140 megadalton virulence plasmid is not needed for establishing a primary and secondary secretory IgA response to Shigella. It must be cautioned, however, that this plasmid may be necessary to achieve mucosal immunity which will prevent uptake of the bacteria. However, the latter remains to be determined.

Figure 16. IgA memory response from M4243A.
The last and most recent mucosal immune humoral response studies performed in these systems have used Shiga toxin as immunogen. In Figure 17 is shown the data from intestinal secretions of two rabbits given direct intraloop immunization with Shiga toxin. We found that by day 10, a significant increase in anti-Shiga toxin activity over background was seen in both rabbits. Booster doses of Shiga toxin were given on days 7 and 14. The antibody activity continued to rise through the end of the experiment at one month. Secretions from these animals were sent to Dr. Edward Brown for analysis of the ability of the IgA anti-Shiga toxin to prevent the in vitro pathogenic effects of this molecule. As shown on the Y2 axis, there was an excellent correlation of the IgA anti-Shiga toxin activity with the inhibition titer determined by Dr. Brown's assay. These findings indicate that there is a great potential for secretory IgA to prevent the cytopathogenic effects of Shiga toxin. Future studies will be directed to confirming these results in a larger group of animals and to establishing whether a secretory IgA memory response against Shiga toxin can be elicited.

Comparison of Inhibition Titer with IgA anti-Shiga Toxin

Figure 17. IgA anti-Shiga toxin by ELISA (Y1) and anti-Shiga toxin titer (Y2) activity in loop secretions.
Uptake of Shigella by Intestinal Epithelium.

During the course of these studies, we observed that when invasive strains of Shigella were administered to acute intestinal loops, ulcerations would most frequently occur over Peyer's patches after 18-48 hours. These ulcerations were much more frequent than those overlying villi. We also observed focal ulcerations in the small intestine. Histologic examination revealed that these often had isolated lymphoid follicles beneath them. Therefore, we chose to study isolated lymphoid follicles to determine if they have a specialized surface epithelium (M cells) which may function to take up Shigella and other enteropathogens. Isolated lymphoid follicles were difficult structures to study by electronmicroscopy since they were not visible grossly or with the aid of a dissecting microscope. For these studies, guinea pigs were allowed to ingest colloidal carbon (India ink) with their drinking water for a period of several months. One hour prior to sacrifice, a bolus of India ink was placed in the small intestine. The ink collected in Peyer's patches and in isolated lymphoid follicles. These allowed the follicles to be visualized grossly (Figure 18). Therefore, we could dissect the follicular areas out in order to study the surface epithelial cells. Specialized M cells were readily identified in the epithelium overlying the structures (Figure 19). This indicates that Shigella may use these M cell areas for initial invasion.
Figure 18. Dissecting 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.
Because of these observations, we have recently set out to determine the relationship between the initial uptake of different strains of Shigella and the immune response to Shigella. As shown in Figure 20, after 90 minutes within the intestinal lumen, there is a marked difference between the uptake of virulent M4243 strain, the less virulent Shigella X16 and avirulent strains 2457-0 and m4243A. The uptake of these strains of Shigella by the dome area epithelium correlates well with the inherent invasive capabilities of the individual strains. There is no correlation, however, with the secretory IgA response that results when these strains are administered as oral immunogens. This indicates that antigen-specific secretory IgA can be elicited equally well by pathogenic and nonpathogenic strains.
Figure 20. Uptake of Shigella by epithelium overlying dome areas of Peyer's patches correlate with the inherent pathogenicity of the strain. Each bar represents the average number of bacteria per dome area in a rabbit. Invasive M4243 had the most uptake. However, the strongest IgA anti-Shigella activity was elicited in intestinal secretions by the least pathogenic strains 2457-0 and M4243A₁.
Paneth Cells and Mucosal Defense.

Another poorly understood cell type in the intestine is the Paneth cell. In our isolated ileal loop model, we have repeatedly observed a striking hypertrophy and hyperplasia of Paneth cells which occurs as the loops age (13). This is accompanied by an atrophy of villi with an increase in crypt depth. Whereas the known trophic effects of chyme are lost in the isolated loops, we have found that isolation is not the only variable characterizing the model. We have shown that within days of their creation, there is extensive bacterial colonization of these isolated ileal loops (16). The present studies found that by controlling the bacterial overgrowth with antibiotics, we can prevent Paneth cell changes. For these studies, antibiotics are placed directly into one of two ileal loops created in the same rabbit. Nonabsorbable antibiotics were used. Therefore, results for these animals include an antibiotic loop (AL), a saline loop (SL), and the control ileum (CI). As shown in Table 8, there was a significant reduction in the Paneth cell hyperplasia and in the crypt depth in the antibiotic loops as opposed to the saline loops. No differences were found in the numbers of mitoses in either loop for the control ileum. In both the antibiotic and saline loop there was an increase in number of goblet cells compared to the control ileum. These findings indicate that Paneth cell hyperplasia is largely in response to the microbial flora of the gut. This suggests a role for the Paneth cell in responding to microorganisms in the intestine. In preliminary studies, we have been able to separate Paneth cells from our hyperplastic loops (Figure 21). Evaluating their ability to both phagocytose and to destroy microorganisms which have been variously coated with IgA or IgG anti-Shigella.
Figure 21. This electron micrograph shows a Paneth cell in tissue culture at 48 hours after isolation. These epithelial cells along with M cells represent the major phagocytic epithelial cells along the gut lumen. In addition, Paneth have unique microbicidal capabilities that will be the subject of our future studies.
Functional significance of secretory IgA to interfere with enteropathogens

Several experiments were performed to determine the functional significance of antigen specific secretory IgA. This proved to be the most difficult portion of our experimental model systems. We attempted to develop both in vivo and in vitro models to follow Shigella adhesion and invasion.

In vitro studies to follow Shigella adhesion. Two major problems were found with the intact tissue system for studying adhesion to colonic and ileal mucosa. First, histologic studies of the sections revealed that the Shigella flexneri M4243 infrequently adhered to the epithelium and was not found attached to the serosa. In the latter studies we noticed that the Shigella were taken up by the specialized surface epithelium. However this represented only a small number of the bacteria. For instance, a dose response study revealed that when $10^5$ or more bacteria were included in the culture containing no serum or intestinal secretions, less than 1% will adhere to the tissue sections. Thus, in any attempted protection studies with antigen-specific IgA, we would be forced to measure extremely minor differences. Because of this, we chose to establish tissue culture lines and other means for assessing the percentage of bacteria adhering to the involved epithelium.

Attachment and invasion of Shigella into HeLa cells. The HeLa cell system proved to be relatively reliable in terms of generating consistent numbers of infected cells. Using the conditions outlined in the methods section, an average of 54.1% of HeLa cells were involved by more than 10 bacteria per cell while an average of 4.5% had no attached bacteria. When normal rabbit serum was incubated together with the S. flexneri M4243, there was a significant inhibition of the involvement of HeLa cells by bacteria. In this group, only 26.5% of HeLa cells were involved by more than 10 bacteria while an average of 24.4% had no involvement. Similarly, when immune serum against M4243 was used, only 25.8% of the HeLa cells were involved by more than 10 bacteria and an average of 49.1% of HeLa cells were totally uninvolved. This indicated that there was an inhibitory influence on this attachment. However, there was significantly more uninfected cells in the group treated with immune serum versus normal serum (p<.01). Unfortunately, use of this system proved too inefficient at determining whether intestinal secretions could inhibit uptake of Shigella by the HeLa cells.

In vivo studies to follow the pathologic effects of S. flexneri. Oral challenge was performed in intact rabbits to determine the effects of pathogenic Shigella on temperature, peripheral blood leukocyte count, and differential count. However, we noted that there was too little difference in temperature or leukocyte count of the different animals following challenge with Shigella. Unless an extraordinarily large number of animals could be used, we felt that this was an inefficient way to define the functional capabilities of secretory IgA.

A RITARD (removable intestinal tie-adult rabbit diarrhea) model for Shigella invasion was also attempted. The RITARD model has been used in our laboratory to control variables which we presumed were affecting the oral challenge system. Although the animals for the oral challenge study were fasted, they always had considerable gastric and intestinal contents.
This may have diluted the dose of antigen. Further, even with bicarbonate, the gastric acid and pepsin may have had enough anti-bacterial activity to prevent invasion. With the RITARD model, the Shigella are directly injected into the jejunum, thereby avoiding the gastric acid and the considerable dilution affect of lagomorph gastric contents. However, even with the RITARD model system, we were not satisfied with consistency in results.

Therefore, our most recent studies have centered around using acutely isolated loops and studying the initial invasion event. Protection against invasion is, after all, what we need to show. In these studies, we have found (as mentioned above) that uptake of Shigella by follicular epithelium overlying Peyer's patches and isolated follicles, correlates with pathogenicity rather than with immunogenicity of the microorganism. In preliminary studies, we have found that antigen specific IgA isolated from our intestinal loop model system is able to prevent uptake of the Shigella. In the next two years, we will pursue these studies to determine if we can quantify the amount in activity of IgA required to prevent disease in this model system.

**Cellular immune studies.** Most vaccination programs of mucosal or systemic immunity have relied on trial and error to a large extent. In the present studies, we have begun to evaluate the kinetics of the cellular immune response elicited by regimens which we have proven to be effective for stimulating mucosal immunity. The goal of this line of investigation is to determine the location and number of cells which are committed to IgA synthesis against the enteropathogen of interest following immunization. By understanding the kinetics of the cellular immune response, we should be able to logically tailor future vaccination efforts and have a quantifiable parameter to determine the success of those regimens without waiting for the long-term results of the previous experiments. This will make vaccine testing much faster, more logical, and more reliable.

For these studies we isolated mononuclear cells from Peyer's patches, mesenteric lymph nodes, spleen, and peripheral lymph nodes. We have grown these cells in tissue culture and have found that antigen specific IgA and IgG can be detected in the various cell preparations. When rabbits were given the combined parenteral and oral schedule described above, we found that we could reliably detect IgG and IgA activity against the immunizing antigen. For instance, spleen mononuclear cells from rabbits eight days following immunization had the largest amount of anti-Shigella activity. As can be seen in figures 22 and 23, the optimum cell concentration range was from 2-8 x 10⁶ viable cells per ml for both IgA and IgG anti-Shigella activity. Increasing time of incubation did not raise the amount of anti-Shigella activity at suboptimal cell concentration to that seen with optimal cell concentrations. Maximal anti-Shigella activities were usually reached between 4 and 14 days of incubation. These findings parallel our humoral immune studies, and indicate that parenteral priming together with oral immunization is able to stimulate both systemic IgG and IgA anti-Shigella B lymphocytes in several lymphoid tissues. Further studies will determine the location of IgA-committed cells when animals have been primed for the mucosal memory response.
Figure 22. IgG anti-Shigella activity. Spleen mononuclear cell concentrations (x 10^6/ml) are: 0.5(circle with a dot); 1.0(filled circle); 2.0(open circle); 4.0(filled triangle); 8.0(open triangle); 16.0(open square); and 32.0(filled square). The IgG anti-Shigella LPS activity was approximately 10 times greater than that for IgA as shown in Figure 21.
Figure 23. IgG anti-Shigella LPS production. Spleen mononuclear cell concentrations are as in Figure 20.
In the past seven years, our laboratory has developed the chronically isolated ileal loop model as a probe for following the mucosal immune response in intestinal secretions. Using this system we have documented the existence of a secretory IgA memory response in intestinal secretions, evaluated several potential vaccine strains with the observation that secretory immunity is best elicited by live oral bacteria (though they do not need to be invasive) and learned that careful orchestration of parenteral with mucosal stimulation will enhance the strength of the primary secretory IgA response. During these studies, we have made several other observations on the biology of the interaction of the intestinal epithelium with enteropathogens. We demonstrated that M cells (the major intestinal antigen sampling device) exist over isolated follicles throughout the intestine and that they may serve as the portal of entry for Shigella flexneri. Paneth cells have been found to correlate with bacterial flora, and, while growing these cells in tissue culture, we found them to have phagocytic capabilities. Lastly, we have begun to isolate the mucosal lymphoid populations to define more precisely the relationship between immunization with enteropathogens and the development of the cellular response. This should provide a more logical approach to development of vaccine regimens for the mucosal immune response. Overall, these studies have advanced our understanding of the mechanisms for developing mucosal immunity to enteropathogens and the functional significance of these responses. They are relevant for any agent which gains access via a mucosal route or which produces a toxin that functions directly on the mucosal surface.
References


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