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AN INVESTIGATION OF THE MEMORY RESPONSE OF THE

LOCAL IMMUNE SYSTEM TO SHIGELLA ANTIGENS

ANNUAL REPORT

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→When the mucosal immune system is appropriately stimulated, the secretory IgA response which results can protect the intestine from damage by microorganisms or their toxic products. Our laboratory has found that oral immunization with virulent or avirulent Shigella flexneri can elicit a significant memory mucosal IgA response. In the present studies, we determine the mechanism of initiation of that immune response. We have found that both invasive and noninvasive S. flexneri are taken up by these specialized surface epithelial cells, M cells, and are packaged into vesicles. This explains why both virulent and avirulent S. flexneri are able to stimulate the mucosal immune response. However, we have also found that the virulent M4243 strain is able to replicate within the follicle-associated epithelium and produces ulceration initially in these sites. Therefore, the M cells serve as a double-edged sword. They ingest antigen including live microorganisms from the gut lumen to process this antigen for a secretory IgA response. However, when pathogenic strains which

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Summary

The mucosal immune response to enteropathogens is known to be important in host defense. Our laboratory has been conducting investigations to determine the optimal means to stimulate a memory mucosal immune response to enteropathogens and their toxic products. During the past year, our investigations have concerned the role of antigen form with uptake of virulent Shigella flexneri by the specialized surface epithelial cells, M cells. We have found that uptake of Shigella by this early antigen sampling epithelium takes place regardless of the virulence potential of the microorganism. Noninvasive and invasive Shigella were both taken up by these M cells. While the kinetics was somewhat greater with the virulent M4243 strain used in these studies than with the avirulent strains, the former likely reflected replication of the bacteria following ingestion. This was suggested by the ulcerations present over the dome areas (M cell areas) of Peyer's patches at 18 hours while adjacent villi were damaged but structurally intact. No ulcerations were seen with any of the avirulent strains despite the fact that they were all taken up by the M cells within 90 minutes. This indicates that the M cells serve both as the initial antigen sampling site for Shigella and as the portal of entry for virulent Shigella. These findings also apply to other enteropathogens. We have studied the cellular basis for the memory mucosal immune response which we have previously demonstrated in intestinal secretions. These studies have identified the Peyer's patches as the main reservoir for memory B cells within the gut-associated lymphoid tissues. Following oral antigen challenge in primed animals, a rapid proliferation of these memory B cells occurs within the Peyer's patches. They then are transported to the mesenteric lymph nodes and to the spleen where they reside briefly for one or two days. Thereafter, some of the cells repopulate the Peyer's patches while others migrate to the lamina propria as mature IgA secreting plasma cells. By using in vitro culture techniques, we can more quickly identify the presence of a memory mucosal response in animal model systems. Lastly, we have conducted collaborative studies on the potential to induce significant mucosal immune responses to Shiga toxin. Shiga toxin produces a strong secretory IgA response following intraintestinal immunization, while almost no local or serum IgG is elicited. The secretory antibodies to shiga toxin were able to interfere with the action of shiga toxin in vitro. This substantiates the potential for an appropriately primed secretory immune response to prevent the pathologic effects of enterotoxins.

Foreword

In conducting the research described in this report, the investigators followed the "Guide for Care and Use of Laboratory Animals" prepared by the The Committee on Care and Use of Laboratory Animals of The Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council (NIH Publication 85-23 Revised 1985).

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Introduction

The discovery that IgA is the main antibody on mucosal surfaces provided the key for beginning definitive work to understand the biology of the mucosal immune system (1). While many tissues (bronchial mucosa, mammary glands, conjunctiva, genitourinary tract, etc.) are involved with the mucosal immune response, the gastrointestinal tract is the major site of antigenic stimulation and immune response for secretory IgA (2).

It is well known that parenteral administration of antigen will result in the formation of a systemic immune response directed to that antigen. Depending on the characteristics of the antigen, its dose and the genetic capabilities of the animal, a humoral and/or cellular immune response will result. The same is true of antigens which are administered to a mucosal surface, including the gastrointestinal tract. During the past decade, several animal model systems have been developed to study the details of the production of secretory IgA against orally adminis- tered antigens. Since secretory IgA is overwhelmingly the predominant immunoglobulin along mucosal surfaces and plays a major role at that site for host defense, much work has been directed at understanding initiation of the secretory IgA response to enteropathogens.

Our laboratory has been studying several aspects of the secretory IgA response to enteropathogens. We have developed a chronically isolated ileal loop model in rabbits as a probe to study secretory IgA response (3). This Thiry-Vella loop model system has become a standard method to study the intestinal IgA response to cholera toxin, Shigella flexneri, Salmonella typhi, and Shiga toxin (3-7). This model system has established that a secretory IgA memory response can be elicited following multiple oral immunizations with live vaccine preparations.

The mechanism of antigen processing in the gastrointestinal tract is beginning to be established. Initial processing is thought to occur in one of several major structures of the gut-associated lymphoid tissue. These consist of Peyer's patches, isolated lymphoid follicles, the appendix, and mesenteric lymph nodes. These structures have a common characteristic feature of having lymphocytes (which are precursors for IgA secreting plasma cells) in an aggregate or follicle covered by spe- cialized antigen sampling cells known as M cells. Following oral adminis- tration of an antigen, M cells take up luminal macromolecules and micro- organisms for antigen processing. These M cells can take up macromole- cules such as horseradish peroxidase within a few minutes of their appli- cation to the intestinal lumen (8-10). Some of our present studies have concerned the relevance of the uptake of Shigella flexneri by these cells and the role that this process plays both in antigen processing as well as in the pathogenicity of this microorganism. It has not been clear what the route of cellular migration is following the uptake of entero- pathogens by the gastrointestinal tract. In studies using erythrocytes and protein antigens, it is known that the antigenic material is brought to the underlying lymph node tissue in the GALT. At this stage of develop- ment, most B lymphocytes in the GALT express surface IgM and/or IgD. Immunoregulatory T lymphocytes are present in GALT which influence the development of IgA precursor B lymphocytes (11-13). Little is known about

the relationship of these regulatory T cells in stimulating the secretory IgA response to <u>Shigella flexneri</u>. Work from Kawanishi <u>et al</u>. indicates that switch T cells exist which can alter the surface immunoglobulin expression of the Peyer's patch B lymphocytes from IgM to IgA. In the present studies, we concentrate on determining the location of cells which are capable of producing secretory IgA specifically to <u>Shigella flexneri</u>. Other laboratories have described the role of helper T cells in the IgA immune response. These helper T cells encourage differentiation of B lymphocytes which already express surface IgA toward becoming mature plasma cells (12). In addition to regulatory T cells, it is clear that the B lymphocytes within GALT have an inherent genetic proclivity for becoming IgA plasma cells (13). By studying the <u>in vitro</u> capability of lymphocytes from GALT to produce specific IgA anti-Shigella LPS following oral priming, we are determining the location of cells precommited to make IgA against enteropathogens following mucosal priming with antigen. When completed, these investigations will provide a faster and a more logical approach for developing vaccines against several enteropathogenic agents than what is presently available.

In other studies this year, we have concentrated on the role of antigen form and its uptake by M cells for establishing the mucosal immune response. This work demonstrates that nonpathogenic strains of Shigella are taken up and processed by M cells effectively to stimulate a mucosal memory response. However, the M cells themselves may serve as the portal of entry for enteropathogenic strains of Shigella. In the present studies on acute rabbit ileal loops, we demonstrate that the follicular dome regions are the site of mucosal ulceration in dysentery. This likely relates to the selective invasion of M cells by the pathogenic bacteria. Future work will determine whether such ulcerations can be prevented in those animals having a strong secretory IgA response to Shigella antigens. These finding are relevant to both traditional enteropathogens and newer agents such as the human immunodeficiency virus which causes AIDS. Sneller and Strober have hypothesized that the M cells may also serve as the site of uptake of this agent (14).

Methods

<u>Preparation of Chronically Isolated Ileal Loops</u>. The surgical creation
of chronically isolated ileal loops in rabbits has been described in
detail previously (1). 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 conti-
nuity 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 silas-
tic tubing. Mucus is separated by centrifugation. The slightly opaque,
colorless supernatant is studied for specific immunoglobulin content and
activity. 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.

<u>Enzyme-linked Immunosorbent Assay (ELISA)</u>. Microtiter wells are coated
with a solution containing <u>S. flexneri</u> lipopolysaccharide (LPS) (Westphal
preparation). Immediately prior to testing serum samples or loop secre-
tions, 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 adsorp-
tion) for 4 hours. The plates are washed with PT and incubated for 4
hours 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 (15)). 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 tested fluids as previously described
(16).

The data are analyzed using the RS1 software system. Data are pre-
sented as geometric means, as others have noted that this better reflects
the logarithmic kinetics of the local immune response after immunization
(17). For each day's result, the variance is expressed together with the
mean.

<u>Antigen Preparations Used</u>. Four antigen preparations were employed in
the present studies: 1) <u>Shigella flexneri</u> M4243 (which can invade intes-
tinal mucosa and persists in the epithelium), 2) <u>Shigella</u> X16 (a hybrid
of <u>S. flexneri</u> and <u>E. coli</u>-which invades the intestinal mucosa but does

not persist within the epithelium, 3) S. flexneri 2457-0 (which does not invade, although possesses the 140 megadalton virulence plasmid), 4) S. flexneri M4243A$_1$ (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 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 2 x 10^9 Shigella flexneri was injected into this acute loop. At 30, 90, and 180 minutes, these loops were removed and samples were fixed for EM studies and frozen sections were prepared. These sections were fixed in ethanol and stained with Giemsa. For each time period, 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 2 areas: 1. the follicle associated epithelium overlying the dome areas in Peyers patches (known to be enriched in "M" cells). 2. villi which were outside of the Peyers patch area. Evaluation was performed using oil emersion light microscopy. Since the normal flora of rabbit ileum contains <10^4 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. The Bioquant Biometrics Image Analyzer (Nashville, Tennessee) with an IBM computer was used to measure the actual length in millimeters of the lining epithelium over the villi and over the dome regions of the Peyer's patches. The average of 100 areas for dome and villus areas from representative rabbits was calculated. This allowed us to directly express data as bacteria/mm of surface epithelium. This permits a direct relationship of villus surface area to follicle-associated epithelium surface area. 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 mm of dome epithelium or microorganisms per mm of villus epithelium.

Mucosal Immune Response to Shiga Toxin. Two groups of rabbits were used in this study: two conventional rabbits and 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 given on the day of surgery-defined as antigen day 0). The Shiga toxin preparation used for this study was a post-DEAE fraction, provided by Dr. Ed. Brown of the Walter Reed Army Institute of Research. Dr. Brown's laboratory found that this preparation contained 10^7 LD50 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 followed by 2 ml of air. The end of one tube 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 their anti-Shiga toxin protective ability using his in vitro assay.

<u>Mononuclear Cell Isolation.</u> 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 asceptic conditions. 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^3 fragments with a sterile blade and placed on sterile wire mesh. The cells were carefully teased apart and passed through steal 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.

<u>In Vitro Mononuclear Cell Cultures.</u> 10^5 mononuclear cells were added to each row of a 96 well, polystyrene microtest III tissue culture plate with flat bottom wells (Becton Dickenson). Cultures were placed in a humidified, 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.

<u>Electron Microscopy.</u> Tissues were minced to approximately 1 mm^3 and fixed in 3% gluteraldahyde-formaldehyde in 0.1 molar cacodylate buffer, pH 7.3. The samples were postfixed in 2% osmium tetroxide. After staining en block with 2% uranyl acetate, tissues were dehydrated in alcohol and embedded in epon. One micron thick sections were cut and stained with toluidine blue and examined for uptake of Shigella. Areas of follicle-associated epithelium which contained Shigella were chosen for thin sectioning. Thin sections (approximately 800 angstrom's thick) were then cut from the selected areas on a Porter-Blum MT-2 ultramicrotome. These sections were stained with lead citrate and examined with a Zeiss 109 transmission electron microscope. Photomicrographs were taken of the characteristic rod-shaped bacteria in the follicle-associated epithelium.

I. Determination of M Cell Uptake of Shigella in the Acute Loop Model
    System.

         In previous studies performed using our chronically isolated
    ileal loop model in rabbits, we demonstrated that either oral or
    intraloop immunization consistently initiated a secretory IgA re-
    sponse to Shigella. Our initial assumption with this model was
    that strains of Shigella which were invasive would provide the best
    mucosal immunogens by virtue of their ability to penetrate the
    surface epithelium. Yet, when we used strains of Shigella with
    various invasive capabilities, we observed that both invasive and
    noninvasive strains of Shigella (see Table I) were able to elicit
    equivalent primary and mucosal memory responses (18). Even strains
    such as S. flexneri M4243A$_1$ (which is noninvasive and lacks the
    virulence plasmid) has been shown to give a vigorous memory mucosal
    response (7). Since either invasive or noninvasive strains produced
    equivalent mucosal immune responses to Shigella LPS, we hypothesized
    that both strains must be processed in the same manner by the gut-
    associated lymphoid tissues.

Table I. Characteristics of Shigella
Used in the Present Studies

Strain	Virulence Plasmid	Sereny Test	Intestinal Invasion
S. flexneri M4243	+	+	+
Shigella X16	+	o	+
S. flexneri 2457-0	+	o	o
S. flexneri M4243A$_1$	o	o	o

We know from the work of Owen (8) that there are specialized surface
epithelial cells overlying lymphoid follicles in Peyer's patches
which can take up macromolecules and microorganisms from the gut
lumen. He has termed these cells "M" cells due to their irregular
microvillus folds. They are intimately associated with wandering
lymphocytes in the epithelial layer. M cells have also been demon-
strated in the epithelium overlying lymphoid follicles in the ap-
pendix, isolated follicles throughout the gut, and in the bronchus.
In the present studies, we have systematically reviewed strains of
Shigella with varying capabilities of invasiveness with regard to
their uptake by the surface epithelium. For the present studies, we
allowed the four strains of Shigella listed in Table 1 to incubate
for 30 minutes, 90 minutes, or 18 hours in the acute loops. At the
30 minute time period, there was almost no demonstrable attachment
or uptake of S. flexneri to either the follicular epithelium in
Peyer's patches or to the villus epithelium. No damage or evidence
of inflammation was seen at this early time. A rare Shigella was

seen in the apical portions of cytoplasm of M cells in the M4243
strain group. However, even in that group, only rare uptake was seen
in all of the sections examined. Too few Shigella were seen to give
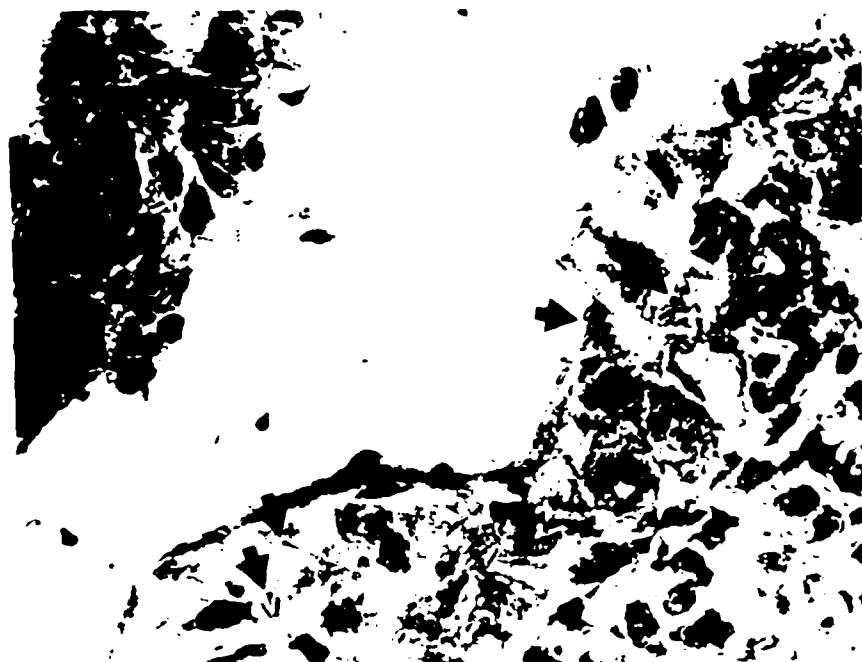reasonable quantification figures.

Figure 1.   Shigella present within surface epithelium.

        By 90 minutes, all four strains of Shigella showed readily
demonstrable uptake over the dome regions of the Peyer's patches. To
be included in a count, we required that the entire Shigella be lo-
cated within the cytoplasm (Figure 1). Bacteria which were adherent
to the surface epithelium but not clearly present within the cyto-
plasm were not counted. The bacteria were taken up by the M cells and
packaged in vesicles. Most of the bacteria seen at the 90 minute time
period by ultrastructural studies were contained within membrane-
lined vesicles (Figure 2), although some vesicles in the loops given
the pathogenic strain showed early breakdown. The pathogenic strain,
S. flexneri M4243 had significantly greater uptake of bacteria in
the dome regions than did the three nonpathogenic strains (Figure 3).
In several areas, the M4243 strain showed numerous bacteria within
the same M cell (Figure 1). The 3 nonpathogenic strains studied
were taken up with equal efficiency regardless of their invasive
capabilities or the presence of the 140 megadalton virulence plasmid
(Figure 3). All strains examined had relatively few Shigella within
the villus epithelium, however the uptake was significantly greater
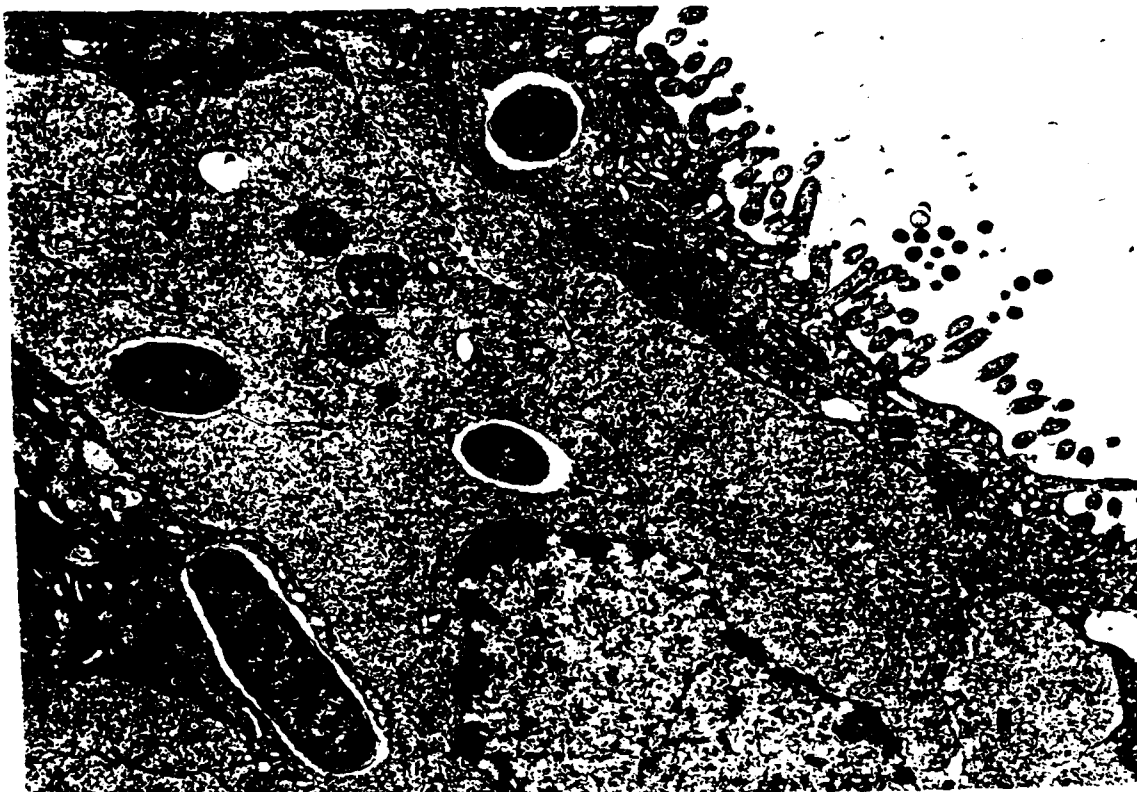with the pathogenic M4243 strain as compared to the nonpathogenic
strains.

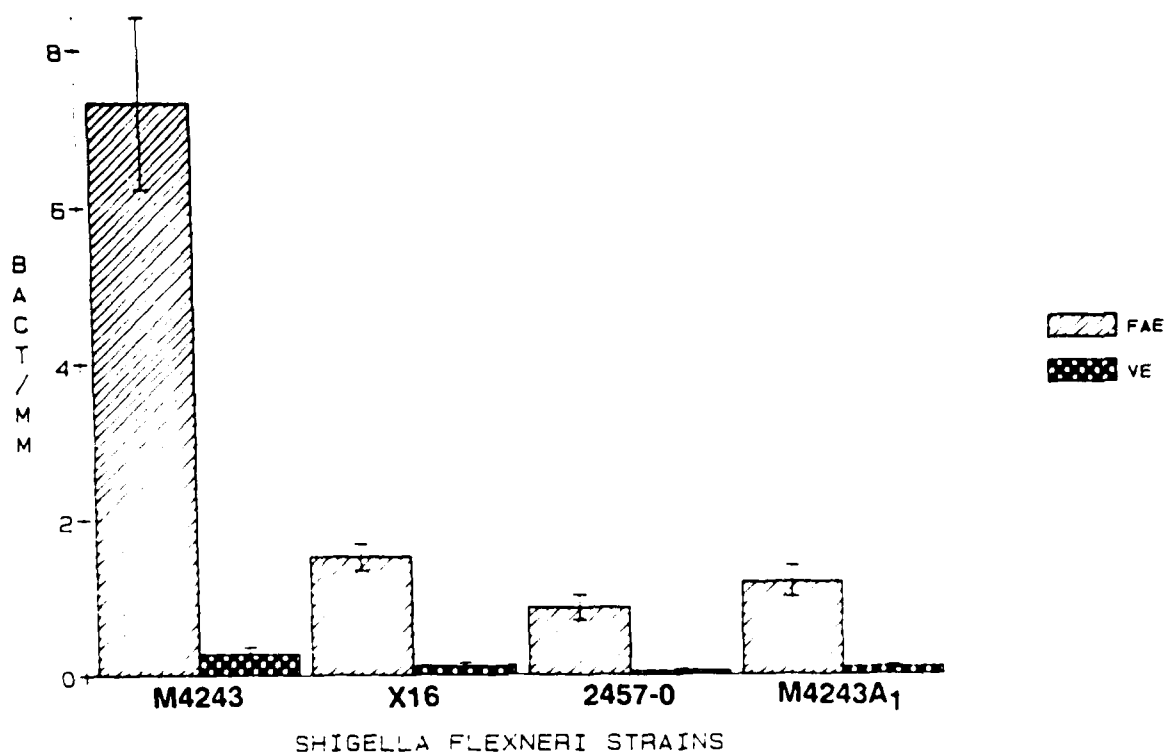Figure 2.   Electron micrograph showing shigella present within membrane-lined vesicles.

Figure 3.   Comparison of uptake of the four strains by surface epithelium.

Since both pathogenic and nonpathogenic strains of Shigella were preferentially taken up by the specialized M cells in the follicle-associated epithelium which overlies Peyer's patches and isolated follicles, it is likely that M cells do not distinguish between Shigella on the basis of antigens encoded by the virulence plasmid. Since all four strains have been found to elicit significant mucosal immune response in our previous studies where direct intestinal stimulation was given in chronically isolated ileal loops, and since the three noninvasive strains could prime for a mucosal memory response regardless of their ability to invade the surface epithelium or of the presence of the 140 megadalton virulence plasmic, we believed that the strains would be sampled with equal efficiency by the surface M cells. The findings in our acute loop studies are consistent with this hypothesis. There was no significant difference in the uptake of Shigella X16 (invasive), S. flexneri 2457-0 (non-invasive but containing the virulence plasmid), and of S. flexneri M4243A$_1$ (noninvasive, lacking the virulence plasmid). There was, however, a significant difference in the uptake of the pathogenic S. flexneri M4243 strain versus the avirulent strain. Since we believed that this reflected successful replication of this bacteria within the tissue following uptake, we followed this process for 18 hours. This would allow replication to continue and pathologic effects to occur in those sites.

Figure 4.   Ulceration is present over lymphoid follicles.

After 18 hours of incubation, profound mucosal ulceration was seen exclusively with the S. flexneri M4243 strain. The acute loops incubated for 18 hours with this bacteria showed a hemorrhagic surface with marked acute inflammation throughout the lamina propria (Figure 4). Ulceration was present predominantly in the dome regions over the Peyer's patches. Although there was mucosal damage in the adjacent villi, the surface epithelium was, in general, intact. With the pathogenic M4243 strain, myriads of microorganisms were seen in the exudate over the ulcer and within the tissues attesting to their successful replication (Figure 5). In marked contrast, Shigella were not found within the surface epithelium of the non-pathogenic strains at this time. The three nonpathogenic strains showed no ulceration after the 18 hour incubation. With the Shigella X16 strain, there was some hemorrhage in the lumen, however, the epithelium overlying villi and overlying the dome regions of Peyer's patches was intact.
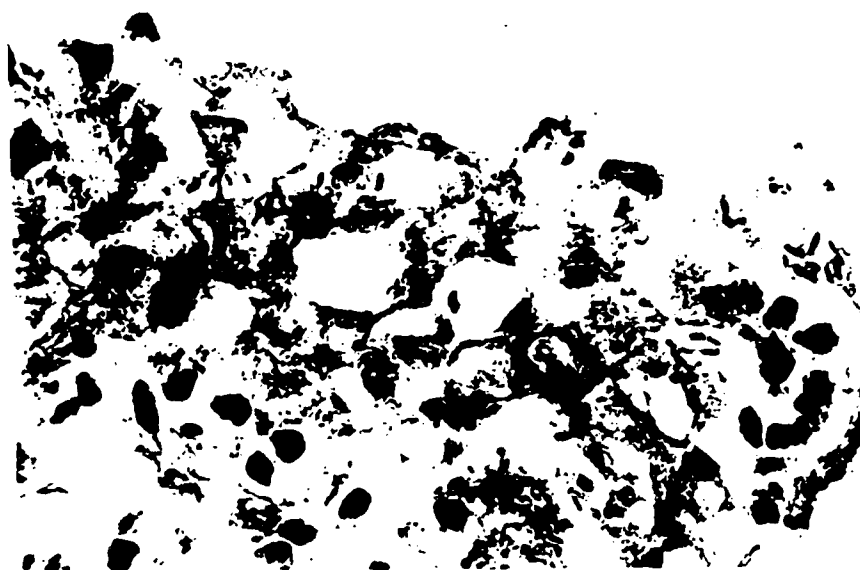

Figure 5.   Shigella proliferate in the ulcer exudate.

These findings indicate that in addition to being the site for antigen sampling and stimulation of the mucosal immune response, M cells serve as a preferential portal of entry for pathogenic micro-organisms. Indeed, M cells have been proposed by others as portal of entry for intestinal pathogens including the human immunodefi-ciency virus (14).

It will be important to determine whether the uptake of viru-lent Shigella by the epithelium overlying lymphoid follicles can be prevented by the production of antigen-specific secretory IgA. This would provide a logical method of preventing mucosal invasion and dysentery. Studies on the ability of secretory IgA to prevent uptake or to opsonize bacteria for phagocytosis are underway in our labora-tory.

II. Location of IgA Precursor B Lymphocytes Specific for Shigella Lipopolysaccharide Following Mucosal Memory Priming.

The purpose of these studies was to determine the distribution of IgA precursor B lymphocytes with specificity for Shigella flexneri LPS. From our previous work, it was clear that oral immunization with various strains of Shigella flexneri resulted in the production of specific secretory IgA against the immunizing strain. To estab-lish that a memory mucosal response has occurred, however, required performing complicated surgical creation of chronically isolated Thiry-Vella loops in these animals. This procedure is laborious and time consuming. It is also expensive to keep animals for as long as one or two months after the surgery to determine if memory has been achieved. While the system yielded useful information about the kinetics of the primary and secondary mucosal immune responses to enteropathogens, it would be valuable and informative if we could determine the location of antigen reactive cells following different priming regimens and what their migration pattern after oral chal-lenge with antigen. This information would allow us to use the cellular kinetics to predict stimulation of mucosal immunity by oral and/or parenteral priming regimens.

In the present studies, we used Shigella flexneri M4243A$_1$ (which does not contain the virulence plasmid) for immunization. Three intragastric immunizations were given one week apart at 60, 67 and 74 days prior to dissection. One to ten days before dissec-tion, the rabbits were given a single challenge dose of live Shigella flexneri M4243A$_1$. The rabbits were sacrificed and the lymphoid populations in the spleen, Peyer's patches, and mesenteric lymph node were sampled as described in the Methods section.

Mitogen Studies. Dose response curves were created to determine the optimal mitogen dose for cell culture from each of the lymphoid organs of interest. For this, 0.1 ml of cells (4 x 10^6/ml were dis-tributed into 96 well culture plates with or without mitogen. The doses of mitogen used were: pokeweed mitogen dilutions: 1:5, 1:10, 1:50, 1:100, 1:300, 1:500, 1:1,000, 1:5,000, and 1:10,000; Con A dosages (µg/ml): 0.01, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0,

20.0, 100.0. Each treatment was set up with four replicate wells.
The cocultures were allowed to grow at 37°C, 5% CO2 in humidified
air for 24, 48, or 72 hours. Six hours before harvest, 0.1 μCi
tritiated thymidine was added to each well. The cells were harvested
with an automatic harvester onto glass fiber filter papers (Belco,
#7735-10024). The dried papers were placed into scintillation vials
with 5ml of Aqueous counting fluid (ACS) and counted on a Beckman LS
7000 scintillation counter. Values for each treatment were taken as
mean disintegrations per minute (DPM) of four replicates minus the
total count. The results of the PWM stimulation of rabbits lympho-
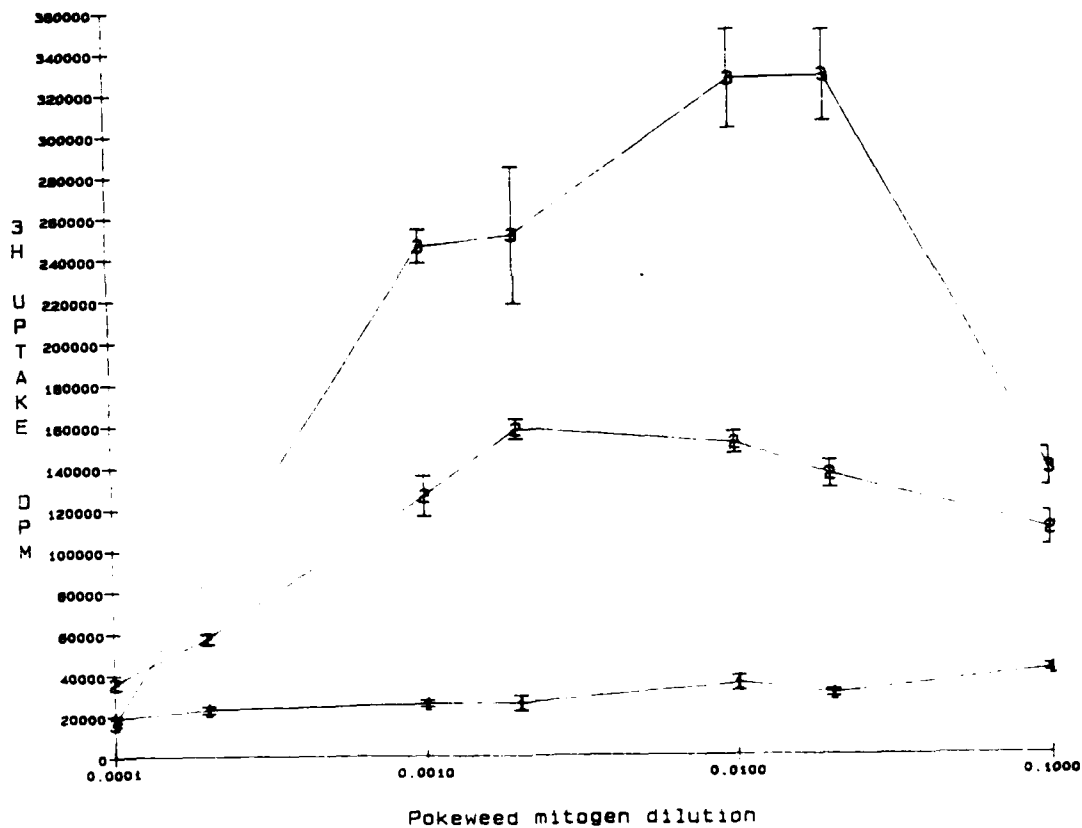cytes is shown in Figures 6, 7 and 8.

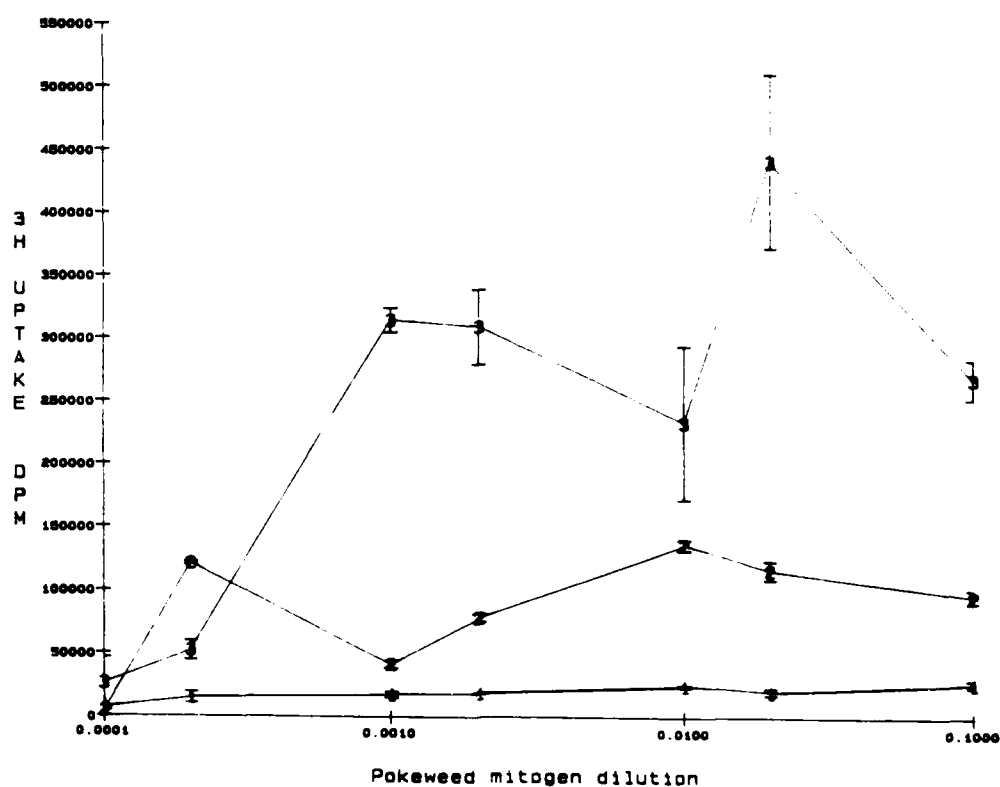Figure 6.   PWM stimulation of rabbit spleen cells is shown.

- 14 -

Figure 7.   PWM stimulation of rabbit mesenteric lymph node cells is shown.
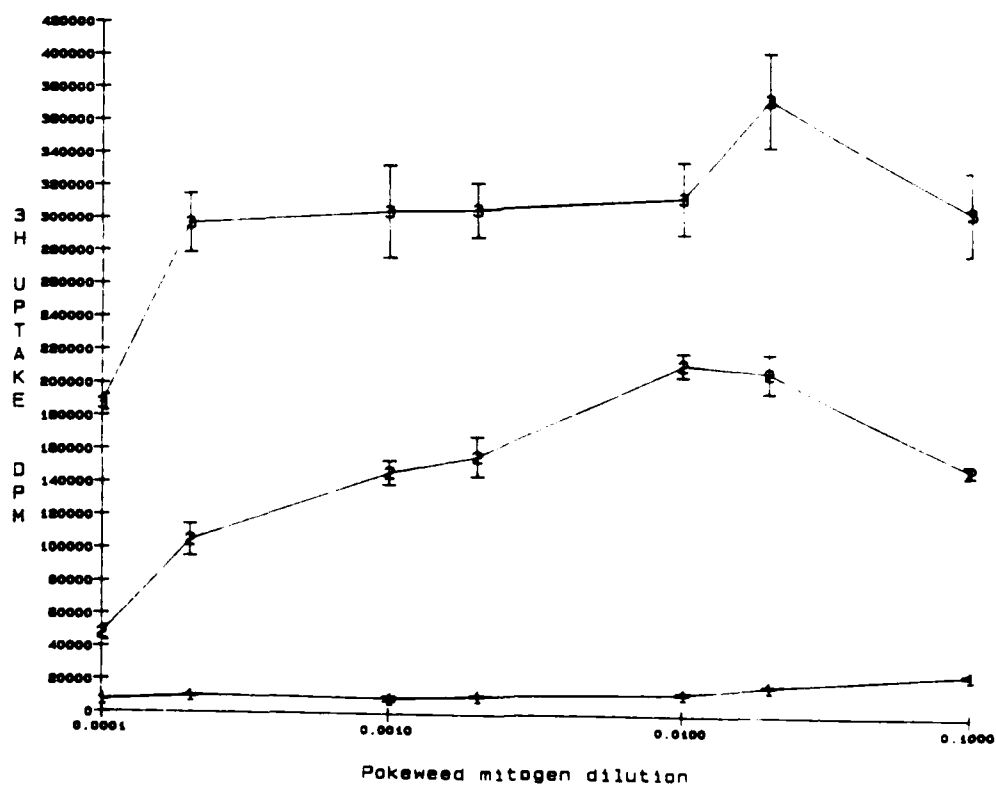

Figure 8.   PWM stimulation of rabbit Peyer's patch cells is shown.

These studies will be continued to look at the specific responses in culture supernatant during the next year. In addition, control animals that have not received prior oral priming with live Shigella will also be examined. By using this approach, we will establish an inexpensive method to test whether animals are primed for a mucosal memory response and will provide insight into the mechanisms by which secretory IgA response is formed to this and other enteropathogens. This model system permits the convenient correlation of kinetic humoral data in sequential intestinal secretions with cellular events within specific lymphoid compartments.

III. <u>Mucosal Immune Response to Shiga Toxin</u>.

We have begun collaborative investigations with Dr. Ed Brown's laboratory on the functional significance of mucosal immune responses to Shiga toxin. The preliminary studies indicate that by the 10th day following direct intraloop immunization with Shiga toxin a significant secretory IgA response to Shiga toxin is demonstrated. Future studies will concern using an oral immunization route to elicit IgA anti-Shiga toxin. These early findings are very similar to those previously reported by our group when examining the mucosal immune response to cholera toxin. They indicate that Shiga toxin may also serve as a mucosal immune adjuvant. No protein other than cholera toxin has given such a strong secretory IgA response in our model for mucosal immunity as has the present Shiga toxin preparation.

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