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

**Annual Report**

**Jan. 1983**

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

Annual Report

David F. Keren, M.D.

January 1983

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SUMMARY

In the present studies, we have used our chronically isolated ileal loop model in rabbits as a probe to study variables involved in eliciting primary and mucosal memory responses to shigella antigens. In our previous studies, we documented that a local IgA memory response to shigella antigens could be elicited by priming rabbits with three oral, weekly doses of $10^{10}$ live Shigella X16. No such local IgA memory response in intestinal secretions was found when heat-killed shigella were given in the same dosage orally. Last year, we found that the antigen preparation does not need to invade the surface epithelium in order to stimulate local immunity. This was demonstrated by the finding that the noninvasive strain Shigella flexneri 2457-0 was effective in priming for a mucosal memory response.

In the present studies, we demonstrate that heat-killed shigella are not effective as a challenge dose and therefore inappropriate to use to test whether a mucosal memory response is present or not. Therefore, the original studies using the $10^{10}$ dose of orally-administered heat-killed Shigella X16 were repeated this time with a subsequent live challenge dose. Nonetheless, it was found that this dosage was insufficient to prime for a mucosal memory response even with appropriate challenge. Since total dosage may be an important factor (the live bacteria have been shown to multiply within the gut) a second group of rabbits was immunized with $10^{12}$ heat-killed shigella in three weekly oral doses. This dosage was found to be ineffective at eliciting either a primary or a mucosal memory response. We believe that the antigenicity of these heat-killed preparations is intact as we can elicit systemic immune responses when the shigella are given subcutaneously. Future studies will determine whether these antigens in an adjuvant mixture can prime for a more vigorous mucosal response.

We have also completed significant morphologic studies this year on the capabilities of isolated lymphoid follicles to process enteropathogens for a mucosal immune response. Heretofore, there had been no functional studies of the capabilities of these numerous isolated follicles. In the present work, we demonstrate that the specialized follicle-associated epithelium overlying these structures contain specialized "M" cells which can pick up and process luminal macromolecules. It is likely that these structures are of key importance in the immune response to shigella and other enteropathogens.

Lastly, our collaborative efforts this year have included studies of the efficacy of outer membrane protein preparations, and their ability to, solely or in combination with lipopolysaccharides, provide an effective stimulation for mucosal immune responses. Our previous results had shown that only poor local immune responses can be elicited to lipopolysaccharide alone given orally. In the present studies, however, a vigorous local response to LPS preparations can be elicited when outer membrane protein is used in combination. These findings point up the crucial importance of further investigating the role of adjuvants in enhancing mucosal immune responses. Our presently ongoing functional studies will help determine
what degree of mucosal stimulation is needed to practically protect the individual against the effects of enteropathogens. These studies should tell us to what extent it is necessary to pursue the use of adjuvants to enhance mucosal immune responses.
FOREWARD

During the course of this work, the author was greatly assisted by Roderick McDonald, Liz Strubble, Patricia Scott, Mitchell Wiatrak, Arthur Rosner, and Scott Kern. In addition, the excellent Laboratory Animal Medicine Department at The University of Michigan continues to provide excellent care for our animals. The help of these individuals especially Drs. Ringler and Peters is deeply appreciated. The excellent assistance of MaryAnn Byrnes in preparing this and other manuscripts related to this project is appreciated.

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

This third annual report includes work completed from 1 February, 1982 to the present (31 January, 1983).

The primary focus of our work has been to use our well-characterized, chronically isolated ileal loop model in rabbits as a probe to follow the kinetics of the local immune response of the bowel to _Shigella flexneri_ antigens. The feasibility of using chronically isolated intestinal loops as probes to study local immunity has evolved from several laboratories that have demonstrated the role of antigen stimulation of mucosal immunity and lymphocyte trafficking in the bowel. In the bowel, antigen is taken up by specialized follicle associated epithelial cells that cover lymphoid tissue throughout the bowel (including appendix and Peyer's patches)(1,2). Our recent studies, reported herein, demonstrate that these specialized surface epithelial cells that are key to antigen processing are also located in the epithelium overlying isolated lymphoid follicles (3). The abundance of the latter structures throughout the gastrointestinal tract suggest that they may play the major role in stimulating mucosal immunity. After antigen is taken-up into the lymphoid tissue, IgA precursor B lymphocytes and regulatory T lymphocytes in the isolated follicles and Peyer's patches are stimulated (4-7). These stimulated lymphocytes migrate into the systemic circulation, mature in the spleen, and eventually travel back to the mucosal surface of the gastrointestinal tract as well as to other mucosal surfaces (bronchial mucosa, mammary glands, etc.)(8-11).

Our most useful recent contribution has been the demonstration, in intestinal secretions, that an IgA memory response to _Shigella flexneri_ antigens could be elicited in animals primed by oral immunization with 3 weekly doses of live _Shigella X16_ (a hybrid of _S. flexneri_ and _E. coli_) (12). Furthermore, we have found that non-invasive, live _S. flexneri_ strain 2457-0 is also able to elicit a mucosal memory response when given in 3 weekly doses orally.

However, when heat-killed _Shigella X16_ was given as a priming and challenge dose orally, using the same dosage schedule as with the live bacteria, no mucosal memory response resulted. Indeed, only a blunted primary IgA response was produced (12).

In addition, our initial studies on parenteral immunization, reported last year, demonstrated that such peripheral stimulation was not able, by itself, to achieve a significant local IgA stimulation against the immunizing antigen. In the present studies, we present the effects of strong systemic (serum IgG) immunity on development of a local immune response, and the booster effect of a single parenteral dose on an orally primed animal. In addition, it was important for us to determine why the 3 doses of heat-killed _Shigella X16_ given orally were ineffective in our previous studies (12).
The present studies explore whether this was due to inadequate total dosage or inadequate challenge dosage.

METHODS

Preparation of Chronically Isolated Ileal Loops

The surgical creation of ileal Thiry-Vella loops in rabbits has been described in detail previously (13). In brief, while 3 kg New Zealand White Rabbits are anesthetized with Rompum and Ketamine, a midline abdominal incision is made and the terminal ileum is identified. A 20 cm. segment of ileum containing a grossly identifiable Peyer's patch is isolated with its vascular supply intact. Silastic tubing (Dow-Corning) is sewn into each end of the isolated segment. This tubing is brought out through the midline incision and tunnelled subcutaneously to the nape of the neck where it is exteriorized and secured. Intestinal continuity is restored by an end-to-end anastomosis and the midline incision is closed in two layers.

Each day about 2-4 ml of secretions and mucus that collect in the ileal loops are expelled by injecting 20 ml of air into one of the silastic tubes. The slightly opaque, colorless fluid and mucus expelled from the tubing is studied for specific immunoglobulin content. A subsequent flush with 20 ml of sterile saline helps to remove adherent mucus. This saline is then removed by repeated gentle flushes with air. With proper daily care, 80-90% of rabbits can complete experiments lasting 1-2 months.

Enzyme-linked Immunosorbent Assay (ELISA)

Briefly, microtiter plates are coated with a solution containing Shigella flexneri lipopolysaccharide (Westphal preparation). Immediately prior to testing serum samples or loop secretions, the antigen solution is removed and the wells are washed with a phosphate-buffer containing Tween 20 (PT). The fluid to be assayed is diluted in the PT buffer and incubated in the coated wells and in uncoated wells (to control for non-specific adsorption) for four hours on a horizontal rotary shaker. The plates are washed with PT and incubated with either alkaline phosphatase-conjugated goat anti-rabbit IgG or IgA overnight on the shaker. Following another PT wash, substrate reaction is carried out with the nitrrophenyl phosphate in carbonate buffer. The OD 405 nm of the substrate reaction is determined using a TiterTek MicroELISA Reader. Kinetics of the enzyme-substrate reaction are extrapolated to 100 mins. The OD 405 nm of uncoated wells are subtracted from the OD 405 nm of coated wells. Specific IgG and IgA standards are processed daily with unknown fluids as previously described (14).
Table I. Immunization Schedule

<table>
<thead>
<tr>
<th>Group</th>
<th>Antigen</th>
<th>Dose</th>
<th>Route</th>
<th>Day(s)(^{(1)}) Given</th>
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<tr>
<td>I.</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>II.</td>
<td>Heat-killed Shigella X16</td>
<td>(10^{10})</td>
<td>subcutaneous</td>
<td>0</td>
</tr>
<tr>
<td>III.</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>IV.</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>V.</td>
<td>Heat-killed Shigella X16</td>
<td>(10^{12})</td>
<td>oral</td>
<td>0, 7, 14</td>
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<tr>
<td>VI.</td>
<td>Heat-killed Shigella X16</td>
<td>(10^{12})</td>
<td>oral</td>
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<td>Live Shigella X16</td>
<td>(10^{10})</td>
<td>oral</td>
<td>0</td>
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\(^{(1)}\) 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.
RESULTS

Although our previous work has shown that a parenteral immunization without adjuvant using the present antigen system is ineffective in priming the mucosal immune response for a subsequent mucosal challenge (15), data from other workers indicates that individuals previously primed by natural infections with a mucosal enteropathogen can have a booster effect locally by subsequent parenteral administration of that antigen (16). Therefore, we examined the intestinal secretions from animals given a regimen previously shown by us to prime for a mucosal memory response— that is, 3 oral doses of \(10^{10}\) live Shigella X16 (12). After waiting 60 days from the third dose a single parenteral challenge dose of \(10^{10}\) heat-killed shigella was given subcutaneously. As control, a second group of rabbits was given a single subcutaneous dose of \(10^{10}\) heat-killed shigella one day after creation of chronically isolated ileal loops. As shown in figure 1, neither group was able to produce a vigorous secretory IgA response in intestinal secretions against the Shigella following the single parenteral challenge with Shigella X16. This contrasts markedly with the IgA memory response seen when a single live oral dose is used to challenge the animals. Therefore, using the present system the parenteral dose in orally-primed animals was not able to boost the mucosal IgA or IgG (data, not shown) responses to shigella antigens.

Figure 1. Mean IgA anti-shigella in loop secretions of Group I (open circles) and Group II (closed circles) rabbits.
In our previous studies (12), we have demonstrated that when rabbits are immunized with 3 oral doses of killed Shigella X16 no local IgA memory response results when they are subsequently challenged with a single oral dose of this same heat-killed preparation. The obvious conclusion from these studies is that the rabbits were not effectively primed for a local IgA memory response by this heat-killed preparation. However, it is possible that these animals indeed were primed for the IgA memory response, but the challenge with heat-killed Shigella X16 was insufficient to elicit this response. This possibility could be functionally important as, in a natural situation following vaccination with the killed Shigella X16, the individual would, of course, be challenged with live Shigella flexneri. Therefore, to determine whether these animals were effectively primed by heat-killed shigella for challenge with a live agent, a group of 10 rabbits was given 3 oral doses of $10^6$ heat-killed Shigella X16. Sixty days after the last of these oral doses, a chronically isolated ileal loop was created in each rabbit. The next day the animals were given a single challenge dose orally with $10^{10}$ live Shigella X16. This is the same dose that elicited a vigorous local IgA memory response in our previous studies when animals were primed effectively with three live oral doses of Shigella X16 (12). In figure 2 is shown the local IgA response in intestinal secretions from these rabbits following their challenge dose. Only a typical IgA primary response was seen in these animals. This indicates that at a dose of $10^{10}$, the heat-killed Shigella X16 preparation is ineffective in priming the mucosal immune system.

![Graph](image-url)

**Figure 2.** Mean IgA anti-shigella in loop secretions of Group III (closed circles) and Group IV (open circles) rabbits.
However, although a mucosal memory response was not seen, animals given a live Shigella challenge did show a stronger early IgA response than did animals challenged orally with heat-killed shigella in our previous studies (12). Therefore, to determine whether heat-killed shigella provided an adequate stimulus, 10 rabbits were given 3 weekly oral doses of live $10^{10}$ Shigella X16. We have shown previously that this regimen consistently results in an effective priming for a mucosal IgA memory response (12). Sixty days after the last weekly oral dose of live Shigella X16, a chronically isolated ileal loop was created in each rabbit. The day following, the animals were given a single oral challenge with $10^{12}$ heat-killed Shigella X16. In figure 2, the responses in intestinal secretions are compared to the responses of those animals primed with heat-killed Shigella X16 and challenged with live Shigella X16. It is obvious that the heat-killed shigella provided an inadequate stimulus for the IgA memory response - even in animals given an appropriate priming dose. We believe the difference in the early response to the two antigen preparations relates to the ability of the live preparation to invade the surface epithelium initially.

We felt it was important to resolve the question of why the heat-killed vaccine was ineffective in priming the mucosal response. Two of the most likely factors responsible for the different effects of heat-killed vs. live antigen vaccine preparations would be that 1) local invasion was required of the bacteria or 2) that the live bacteria were able to multiply in a gastrointestinal tract and the dose of the live bacteria is, in effect, much greater than that of the heat-killed bacteria. The first possibility that invasion is necessary was ruled out in the studies described in our previous annual report. In those studies, we found that the noninvasive Shigella flexneri strain 2457-0 was able to elicit and challenge for as great a local IgA memory response when given live as when the locally invasive live Shigella X16 was used. Therefore, since we also demonstrated in our original studies that Shigella X16 is capable of multiplying within the lumen of the gastrointestinal tract (12), it is probable that the dose of the live bacteria was considerably greater than that of the heat-killed shigella.

Therefore, in the present studies, an oral priming dose of $10^{12}$ (two logs greater than the previous priming dose) heat-killed Shigella X16 was given. The schedule was the same as that used previously - 3 oral doses given weekly of heat-killed Shigella X16 in chronically isolated ileal loops that were prepared one day prior to the first oral dose of $10^{12}$ heat-killed shigella. As shown in figure 3, only a weak local immune response resulted from this immunization schedule.

To determine whether the dose of $10^{12}$ heat-killed Shigella X16 enhances the mucosal memory response, a second group of 10 rabbits was immunized with 3 weekly oral doses of $10^{12}$ heat-killed shigella. Sixty days after the third oral dose of $10^{12}$ heat-killed shigella, a chronically isolated ileal loop was created in each animal. The next day animals were
Figure 3. Mean IgA anti-shigella in loop secretions from Group V (too few animals at present for standard errors).

challenged with a single dose of live (since this has been shown to give maximal response) Shigella X16. In figure 4 the results of this challenge show only a weak primary IgA response resulting. Therefore, we believe that the antigenicity of the heat-killed preparation should be studied further using an adjuvant in future studies. This must be studied with the knowledge from our recent studies that local immune response can be elicited even to relatively subtle antigens in bacterial cells (17).
In addition, we conducted morphologic studies this year to determine the functional capabilities of the specialized surface epithelial cells over isolated lymphoid follicles to process macromolecules for a local immune response. These structures have been difficult to study by electron microscopy because they are not visible grossly or with the aid of a dissecting microscope. For this work, guinea pigs were allowed to ingest India Ink with their drinking water. One hour prior to sacrifice a bolus of India Ink was placed in the small intestine. The India Ink collected in Peyer's patches and in isolated lymphoid follicles. These visualized isolated follicles were processed for electron microscopy. Specialized "M" cells were readily identified in the epithelium overlying these structures (figure 5). This indicates that our earlier studies which suggested such a function of these cells (18) were correct.

These findings are of particular significance in studying shigella as our previous observations have shown that these bacteria preferentially adhere to the dome regions over Peyer's patches (15). However, it is clear that the major site of pathology in humans is in the large bowel where no Peyer's patches normally exist. With our demonstration that the isolated follicles have the same "M" cells as Peyer's patches, a reasonable site of initial invasion can be hypothesized.
Lastly, we have spent some time this year characterizing the variables associated with microELISA technology. A preprint of this work is appended. In brief, we showed that U-shaped and V-shaped wells can be used because the shape of the well was found to give a trivial change in measured optical density as compared with that caused by accumulation of the product of the enzyme-substrate reaction. Further, we documented the nature of plate-to-plate variance and the necessity for intraplate controls on each microtiter plate processed. These practical studies are extremely useful for our future work with microELISA systems.
LITERATURE CITED


