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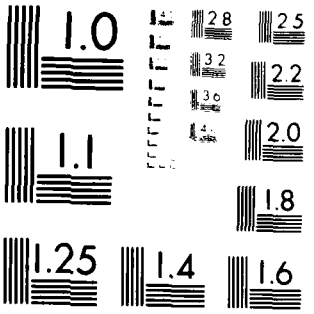

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An Investigation of the Memory Response of the Local Immune System to Shigella Antigens

Annual Report

David F. Keren, M.D.

January 1981

For the period September 1, 1980 - January 31, 1981

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD 17-80-C-0113

The University of Michigan
Ann Arbor, Michigan 48109

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SUMMARY

The present studies used our chronically isolated ileal loop model in rabbits to study the mucosal IgA memory responses of the gastrointestinal tract to orally administered shigella antigens. The secretory IgA response was followed by performing an enzyme-linked immunosorbent assay (ELISA) (sensitive to 1.5 ng/ml of isotype specific antibody) on daily secretions collected from the isolated ileal loops. Both live and heat killed shigella X16 (a locally invasive, nonpathogenic hybrid of Shigella flexneri and Escherichia coli) were used to induce mucosal immune responses. We first established that the kinetics of the IgA mucosal immune response in chronically isolated loops of a group of eleven rabbits stimulated orally with live shigella parallels our previously published results on the IgA response of rabbits immunized directly in the isolated loop with live shigella. A single dose of killed shigella in a group of 12 rabbits produced a similar, though somewhat weaker IgA anti-shigella response in the isolated loop secretions.

Three weekly oral doses of live shigella resulted in a slightly stronger IgA anti-shigella response after the third oral dose of shigella. No such increase was noted when three oral doses of killed shigella were administered.

The most dramatic finding we present is that of a strong mucosal secretory IgA memory response in a group of rabbits stimulated with three oral doses of live shigella and allowed to rest for two months prior to creation of their isolated ileal loops. A single oral dose of live shigella produced a remarkable IgA anti-shigella response within 48 hours of challenge in all the animals studied. This is the first demonstration of a mucosal memory response in intestinal secretions to shigella antigens and is exciting information in relation to possible oral vaccination against shigella and other enteric pathogens. Lastly, when a group of rabbits was primed with three weekly oral doses of killed shigella, no memory response was found in any animal upon rechallenge. This indicates that the form of the antigen is of considerable importance in eliciting a mucosal memory response. The role of antigen form, parenteral priming and a possible mucosal immune adjuvant (DEAE dextran) in stimulating the mucosal memory response will be studied during the next year.

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) AN INVESTIGATION OF THE MEMORY RESPONSE OF THE LOCAL IMMUNE SYSTEM TO SHIGELLA ANTIGENS.		5. TYPE OF REPORT & PERIOD COVERED Annual Report September 1, 1980 - January 31, 1981 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) David F. Keren, M.D.		8. CONTRACT OR GRANT NUMBER(s) DAMD-17-80-C-0113
9. PERFORMING ORGANIZATION NAME AND ADDRESS Department of Pathology The University of Michigan Medical Center Ann Arbor, MI 48109		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62770A.3M162770A871.AD.095
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research & Development Command, Fort Detrick, Frederick, MD 21701		12. REPORT DATE January 1981 13. NUMBER OF PAGES 17
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Shigella flexneri, secretory IgA, mucosal immunity, oral immunization, parenteral immunization, mucosal anamnestic response enzyme-linked immunosorbent assay (ELISA), Thiry-Vella ileal loops in rabbits.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Rabbits were immunized orally with 3 doses of live noninvasive, Shigella flexneri strain 2457-0. Sixty days after the third oral dose a chronically isolated ileal loop was created surgically in each rabbit. The local IgA and IgG anti-shigella responses in secretions and sera were followed with a sensitive enzyme-linked immunosorbent assay. A significant local IgA memory response was found in the secretions of the orally-primed rabbits. Parenteral

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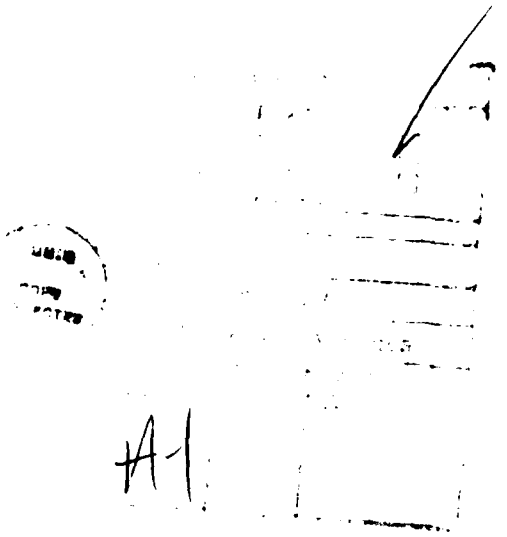
immunization alone results in erratic and weak local IgA production. Further, with a dosage schedule was that achieved serum IgG activity to shigella antigens, parenteral immunization was not able to prime the rabbits for local, intestinal IgA memory response. In other studies, it was found that erythromycin interferred with development of IgA memory responses and adjuvant (DEAE-dextran) had no significant effect on the primary local IgA response to orally-administered Shigella flexneri. The effect of this proposed adjuvant on the IgA memory response remains to be investigated the present studies demonstrate that a local IgA memory response to Shigella flexneri can be elicited by oral priming with a live, noninvasive strain. Further, parenteral vaccination was ineffective in priming for a mucosal IgA memory response.

FOREWARD

During the course of this work, the author was greatly assisted by Patricia Scott, Diana Bauer, Pamela Porter, Scott Kern, and the excellent Laboratory Animal Medicine Department personnel. Their help is deeply 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 No. (NIH) 78-23, 1978).



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INTRODUCTION

This first annual report covers work completed from 1 September 1980 (project starting date) to the present (31 January 1981).

In our original proposal, we suggested that the chronically isolated ileal loop model in rabbits could be used to study the mucosal secretory IgA memory response of the bowel to shigella. Previously, we have demonstrated that a local secretory IgA response to shigella could be detected in secretions from the loops after direct intraloop challenge with live or acetone killed, invasive (strains X16, M4243) or noninvasive (strain 2457-0) shigella (1,2). Further, the presence of a Peyer's patch locally, and the dosage schedule are important factors influencing the development of the local immune response (3).

These previous studies have shown that the intestinal secretions from loops stimulated directly with various shigella preparations will contain considerable secretory IgA but no or only little IgG directed against the shigella (1,2,3). We have demonstrated recently that this is 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 chymotypsin (4). Our recent studies have also shown that a single parenteral or a single oral priming with shigella was ineffective at enhancing the subsequent local immune response when the same antigen was rechallenged directly in chronically isolated loops (2). Direct stimulation of the isolated loops by shigella antigens has resulted in little or no systemic (serum) IgG against shigella unless the systemic immune response was previously primed by a subcutaneous or intravenous dose of shigella (3). Lastly, our most recent report presented evidence which suggested that a secretory IgA memory response could be elicited after priming the animal intraloop with three weekly doses of shigella antigens (2).

The difficulties in studying the mucosal memory response by directly stimulating the chronically isolated loops are twofold. First, it is technically difficult to maintain chronically isolated loops for longer than 45 days. Those animals that do survive for long periods (we have kept some for 10 months) usually have several superinfections and often require resurgery to keep their isolated loops intact. Obviously, these are undesirable events in an experimental model. Secondly, the effects that gastric acid, bile, and the pancreatic digestive enzymes would have on either the natural infection or on a potential oral vaccine are artificially bypassed by directly stimulating the chronically isolated intestinal loop. Although the findings by direct immunization of these loops have been useful in studying the kinetics of the local immune response, the artificial method of stimulation may limit the relevance to the study of the natural local immune response.

Therefore, rather than directly stimulating the isolated loops, the present studies used the chronically isolated loops as a probe for following the local immune response to orally or parenterally administered shigella antigens. The feasibility of this approach evolves from studies of antigen stimulation of mucosal immunity and lymphocyte trafficking in the bowel. In the bowel, antigen is taken up by specialized epithelial cells that cover the dome regions of Peyer's patches (5,6). This antigen then stimulates

IgA precursor B lymphocytes and regulatory T lymphocytes in the Peyer's patches and other gut associated lymphoid tissues (GALT - Peyer's patches, isolated lymphoid follicles, appendix, mesenteric lymph nodes) (7,8,9,10). After antigen stimulation in GALT, these stimulated lymphocytes migrate to the systemic circulation and lastly migrate back to the mucosal surface of the gut and other mucosal surfaces such as the mammary gland, bronchial mucosa and unstimulated portions of the gastrointestinal tract (11,12,13). The dosage schedules for the studies included in this annual report are shown in Table 1.

Table 1. IMMUNIZATION SCHEDULE

Group	Antigen	Dose	Route	Day(s)[1] Given
I	Live Shigella X16	10^{10}	oral[2]	0
II	Killed Shigella X16	10^{10}	oral	0
III	Live Shigella X16	10^{10}	oral	0,7,14
IV	Live Shigella X16	10^{10}	oral	-75,-68,-61,0
V	Killed Shigella X16	10^{10}	oral	0,7,14
VI	Killed Shigella X16	10^{10}	oral	-75,-68,-61,0

[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.

Methods: Preparation of Chronically Isolated Ileal Loops.

The surgical creation of ileal Thiry-Vella loops in rabbits has been described in detail previously (1). In brief, while 3-4 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 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 cc 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 cc of sterile saline helps to remove adherent mucus. This saline is then removed by repeated gentle flushes of air. With proper daily care, 80-90% of rabbits can complete experiments lasting 1-2 months.

Enzyme-Linked Immunosorbent Assay (ELISA).

In the past year we have modified our previously described enzyme-linked immunosorbent assay (ELISA) for detecting IgA and IgG antibodies to bacterial products (2,8). This assay has been converted to a microelisa technique that can use a smaller volume of sample (1/5 of the macrosystem) and allows one to process more samples simultaneously (about five times as many samples can be processed due to the use of multiwell pipet equipment). The same reagents are used with the substitution of Griener polystyrene microtiter plates for the polystyrene tubes of the macroelisa system.

Briefly microtiter wells are coated with a solution containing shigella lipopolysaccharide (Westphal preparation). Immediately prior to testing serum samples or loop secretions, the antigen solution is removed and wells are washed with a phosphate buffer containing Tween 20 (PT). The fluid to be assayed is diluted in the PT buffer and incubated in the coated wells and in uncoated wells (to control for nonspecific adsorption) for four hours on a horizontal rotary shaker. The plates are washed with PT and incubated 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 nitrophenyl phosphate in carbonate buffer. Kinetics of the enzyme-substrate reaction are extrapolated to 100 minutes. 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 the unknown fluids as previously described (8).

The use of this microelisa system has allowed us to process specimens more efficiently. Consequently, the large numbers of loop fluids and sera generated by the present experiments to study the mucosal memory response can be processed almost as quickly as they are produced. Microelisa

results were originally read on a single well manual microelisa reader designed by Thomas Clem and Robert Yolken at NIH. However, the inefficiency of reading one well at a time decreased the usefulness of this technique. Fortunately, the Ligand Assay Laboratory at The University of Michigan has allowed us to use their automated Titer Tek microelisa reader for the past four months. This machine is both faster and more sensitive than the manual Yolken-Clem design.

RESULTS

Figure 1 demonstrates the mean IgA anti-shigella response found in secretions of animals given a single oral dose of 10^{10} live shigella X16 (Group I). The kinetics of the IgA response is remarkably similar to those previously described by us when the isolated ileal loops were directly stimulated with a single dose of shigella (3) 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 loops could be used as a probe to follow the kinetics of the local secretory IgA response to orally administered antigens. In addition, these results establish the normal response of a large group (11 rabbits) of unprimed animals to a single oral dose of shigella. This information will be used in comparison with the secretory IgA responses in animals primed by various regimens.

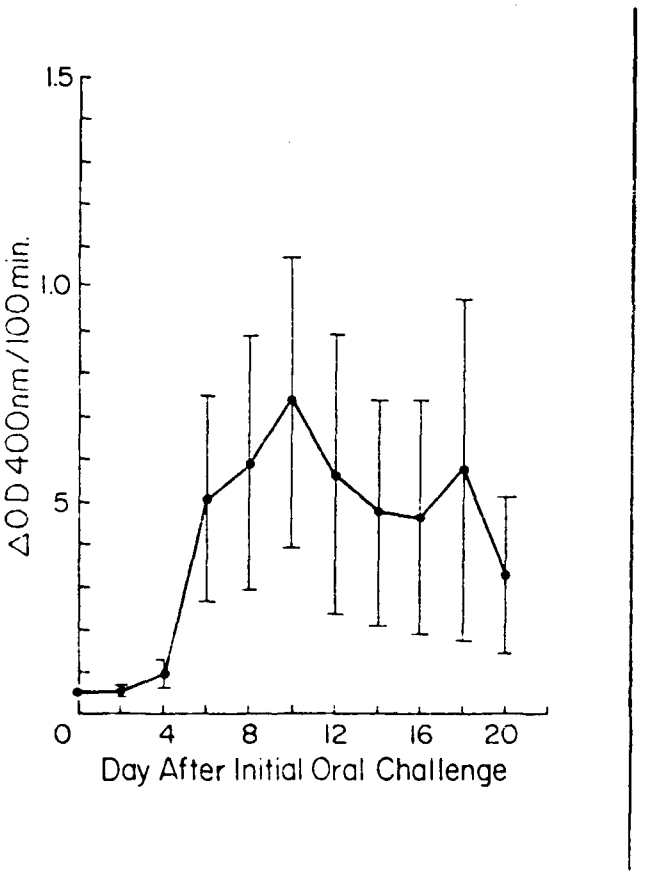

Fig. 1. Mean IgA anti-shigella response in ileal loop secretions from rabbits immunized orally with a single dose of 10^{10} live shigella X16 on day 0. Standard error of the means (SEM) indicated.

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 IgA anti-shigella response obtained from secretions of isolated loops (not directly exposed to antigen) in these rabbits is depicted in figure 2. The overall response was similar to that following oral immunization with live shigella X16 (figure 1). This confirms our most recent studies which indicate that killed shigella X16 is an effective immunogen at least for the primary mucosal immune response whether delivered directly into the isolated loop or given orally to a previously unprimed animal (2).

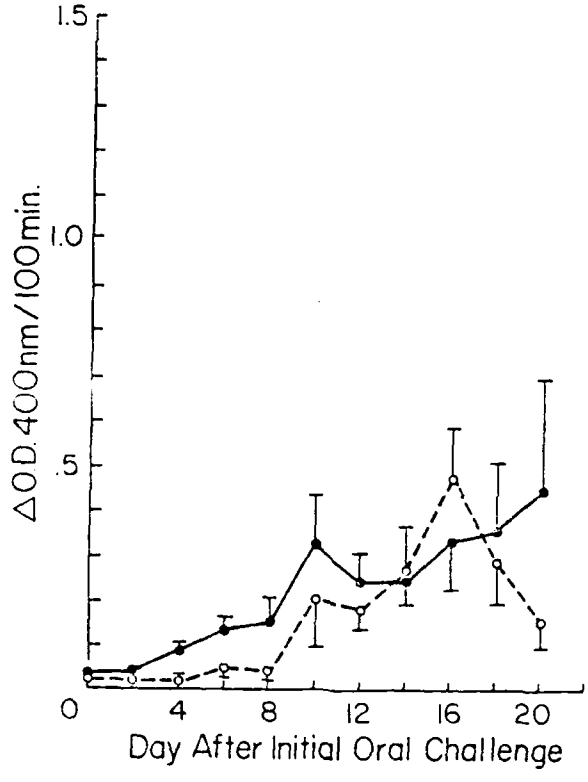

Fig. 2. Mean IgA anti-shigella response in ileal loop secretions from rabbits immunized orally with 10^{10} heat killed shigella X16 on day 0 only (closed circles) or on days 0, 7 and 14 (open circles). SEM indicated.

Since we have shown previously that multiple intraloop immunizations result in a stronger local immune response to shigella than a single stimulation, we administered 10^{10} live shigella X16 orally to eight rabbits and followed the mucosal immune response in their isolated loop (not directly stimulated with shigella) secretions. As shown in figure 3, the kinetics of the response after the first dose of shigella X16 was similar to those seen after a single oral dose of live or killed shigella X16 (figures 1 and 2). After the second dose (given on day 7 in figure 3) a slight decrease in mean IgA anti-shigella was seen (not statistically significant). This may have represented binding of secretory IgA to the live shigella, as by day 12 and 14 the mean IgA anti-shigella had increased. Following the third oral dose of 10^{10} live X16 a considerable increase in the secretory IgA directed against shigella was found in five of the eight rabbits. The heterogeneity of the response is evident from the standard error of the mean indicated in figure 3. Nonetheless, all eight rabbits in this group were able to produce at least a five-fold increase over their baseline level of IgA anti-shigella.

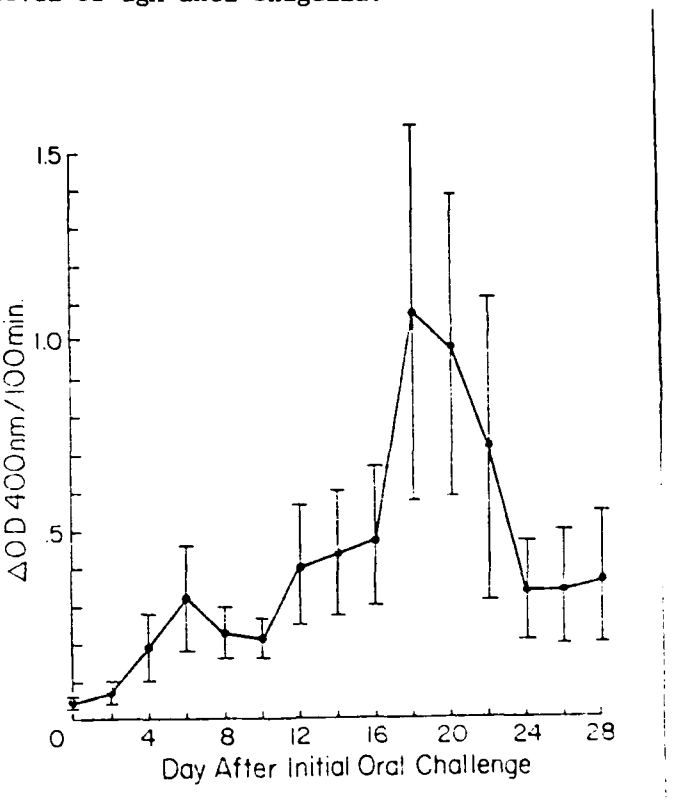

Fig. 3. Mean IgA anti-shigella response in ileal loop secretions from rabbits immunized orally on days 0, 7 and 14 with 10^{10} live shigella X16. SEM indicated.

In contrast to the response to live shigella, the IgA anti-shigella response of 7 rabbits given three weekly doses of 10^{10} heat killed shigella showed no increase in reactivity as compared to those given a single dose. Whereas an increased response was found after the third oral dose of 10^{10} live shigella X16 to the Group II rabbits, no such increase was found after a third oral dose of killed shigella X16 (figure 2).

The most dramatic information we have obtained comes from studies that have just been completed. A group of seven rabbits that did not have chronically isolated ileal loops were primed by giving three weekly oral doses of 10^{10} live shigella X16. 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 responses in the secretions of these animals following their single oral re-challenge with 10^{10} live shigella X16 are compared in Table 2 to the responses of the nonprimed animals that received a single oral challenge. It should be noted that although sixty days have passed since the animals received their oral challenge, a low level of IgA activity was present in 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 their secretions. This response peaked at the fourth day after challenge. In the meantime, the unprimed animals display the typical primary local IgA response that we described earlier to this antigen. By the eighth day after the single oral challenge the secretory IgA responses of both groups to shigella are similar.

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

Day After Challenge[1]	Not Primed[2]	Primed with Live X16[3]	Significance[4]
0	.05[5] ± .008	.19 ± .08	N.S.[6]
2	.05 ± .01	.34 ± .14	$<.03$
3	.05 ± .02	.38 ± .14	$<.04$
4	.10 ± .03	1.29 ± .41	$<.03$
5	.20 ± .14	1.04 ± .33	$<.03$
6	.51 ± .24	1.15 ± .28	N.S.
8	.59 ± .31	.77 ± .21	N.S.

(1) Day 0 = day of final antigen challenge.

(2) Animals given 10^{10} live shigella X16 orally on day 0 (n=11).

(3) Animals given 10^{10} live shigella X16 orally on days -75,-68,-61 prior to oral challenge on day 0 (n=7).

(4) As determined by Student's t-test.

(5) Results expressed as mean O.D. 400nm/100 min. ± standard error of mean (S.E.M.) of IgA antibodies specific for shigella antigen as determined by ELISA (14).

(6) Not significant.

In contrast, the group of eleven rabbits that were primed by three oral doses of 10^{10} killed shigella X16 (Table 1 group VI) 60 days prior to rechallenge with a single oral dose of 10^{10} killed shigella showed no evidence of a mucosal IgA anamnestic response. 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 nonprimed animals that received a single oral challenge with the same antigen. No residual response was noted in the orally primed group and the same, slow primary type mucosal IgA response was found in both groups.

Table 3. LACK OF IgA MEMORY RESPONSE AFTER ORAL PRIMING WITH KILLED SHIGELLA X16

Day After Challenge[1]	Not Primed[2]	Orally Primed With Killed X16[3]	Significance[4]
0	.040[5] ± .008	.057 ± .02	N.S.[6]
2	.046 ± .011	.040 ± .14	N.S.
4	.089 ± .016	.108 ± .05	N.S.
6	.140 ± .031	.173 ± .063	N.S.
8	.161 ± .049	.120 ± .041	N.S.

(1) Day 0 = day of final antigen challenge.

(2) Unprimed animals given 10^{10} 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 (14).

(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 can invade the bowel locally (although it does not persist following this invasion). This may allow for a more efficient presentation of antigen and the heightened response seen. It will be important to compare these results to those obtained when live invasive Shigella flexneri strain 2457-0 or live invasive pathogenic strain M4243 are used in oral challenge.

As in our previous studies which directly immunized isolated ileal loops with shigella X16 (1-3), no or trivial IgG against shigella was found in either loop fluids or sera of any animal receiving the live or killed shigella X16 orally. Our recent studies on the stability of IgG or IgA in the isolated loops indicate that this is more likely due to the lack of synthesis and/or transport of IgG into secretions than to rapid degradation in the isolated ileal loops (4).

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