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ANTIBODY FORMATION AND RELEASE IN ILEAL LOOPS EXPOSED TO ENTEROTOXIN (U)

Final Report

John H. Yardley, M.D.
David F. Keren, M.D.
Stanley R. Hamilton, M.D.

November 1977

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Cholera; cholera toxin; cholera toxoids; choleragenoid; enterotoxin; enzyme-linked immunosorbent assay (ELISA); IgA secretion; intestinal immunity; keyhole limpet hemocyanin (KLH); local immunity; suppression of local immunity; Thiry-Vella ileal loops in rabbits.

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

Formation of local antibody to cholera toxin (CT) was studied in fluid from Thiry-Vella intestinal loops in rabbits. IgA anti-CT production was maximal in the fluid when only intraloop (i.l.) immunization was used, and was minimal after subcutaneous (s.c.) immunization alone. Combined i.l. and s.c. routes led to intermediate production of IgA anti-CT in the fluid, suggesting suppression of local immune response to CT by the s.c. inoculations. The IgA anti-CT levels corelated with neutralizing capacity, and challenge of locally immunized loops with CT showed protection against the biologic effects (excess fluid production).

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18. 19-21, 1977) (obtainable from Geographic Medicine Branch, US-Japan Cooperative Medical Science Program, NIAID - NIH). An article has been accepted by Infection and Immunity (Feb. 1978).

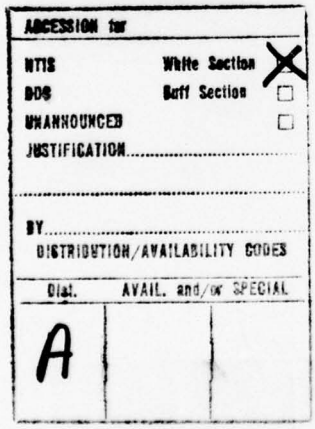
20. ABSTRACT (continued)

The protection was independent of serum IgG-anti-CT levels.

In other experiments it was shown that local immunization to CT was also suppressed by s.c. inoculation with cholera toxoids (choleragenoid and glutaraldehyde toxoid). This finding was consistent with an immunogenic (presumably suppressor T-cell mediated) suppression of local immunity by s.c. inoculation. Preliminary studies with another antigen system (keyhole limpet hemocyanin) also suggested that suppression of local immunity by systemic immunization may be a more general phenomenon.

These observations support strongly the concept that use of local immunization for protecting human beings against enteric infections could be markedly superior to systemic immunization. Furthermore, the studies also suggest that systemic immunization could be disadvantageous under some circumstances.

In another series of experiments using freshly prepared intestinal loops in unimmunized rabbits, it was shown that stimulation of small intestinal mucosa by CT was associated with significantly greater reduction in IgA content of crypt epithelial cells in the loops than in unstimulated control loops. IgA content of the choleraic intestinal fluid was measured in two types of experiments -- one that was accompanied by biopsies of loops and another in which biopsies were omitted. In the first experiment there was no change in total fluid IgA after CT stimulation, but the second study, which was considered much more reliable, showed increased total IgA release after CT. The effect of CT on the IgA system was found to be more complex than the apparent relationship between goblet cell mucin depletion and stimulation of increased fluid production by CT. However, release of IgA caused by CT could enhance protection in the host that has been previously exposed to the enterotoxin or infectious agent.



SUMMARY

Our overall goal was to study formation and processes of release into the intestinal secretion of antibody to cholera toxin (CT). Three main lines of investigation were pursued during the period of this project (July 1, 1974 - August 31, 1977): a) Effects of local and systemic immunization with cholera toxin in rabbits having Thiry-Vella (T-V) intestinal loops. b) Experiments with cholera toxoids and another antigen system (KLH) to further examine immune suppression phenomena noted in the experiments of section a). c) Investigations to determine the degree, if any, to which CT can stimulate the small intestine to release stored antibody (sIgA) into the lumen.

A) Study of Cholera Toxin (CT) Immunization in T-V Loop Rabbits:

Chronically isolated Thiry-Vella (T-V) ileal loops in rabbits were used to study the local and systemic immune response to purified cholera toxin (CT). Immunization consisted of intraloop (i.l.), subcutaneous (s.c.) or combined i.l. and s.c. inoculation of purified CT. Fluid from the loops and sera were tested for neutralization of CT by the blueing test and for relative content of isotype-specific (IgA and IgG) anti-CT. To demonstrate protection against CT, fluid production by the chronic T-V loops was measured after challenge with CT; an "acute" loop prepared from adjacent intestine at the time of challenge was also tested in some animals.

The highest neutralizing titers in loop fluids were found in animals receiving i.l. or i.l. and s.c. inoculations whereas titers in sera were highest in rabbits receiving s.c. or i.l. and s.c. inoculations. IgA anti-CT in fluids became greatest after i.l. inoculation alone and was lowest in s.c. animals. Combined s.c. and i.l. immunization was accompanied by reduced content of IgA anti-CT in fluids as compared with that obtained with i.l. inoculation alone. This finding strongly suggested a suppressive effect on local immunization by s.c. inoculation. While this suppression may have been due to a direct (toxigenic) effect of CT on lymphocytes, an immunogenic mechanism, probably mediated through suppressor T cells, is favored. Little IgG anti-CT was detected in any loop fluids, but high levels were found in sera after two s.c. inoculations or four i.l. inoculations. Neutralization titers for the fluid specimens showed much better correlation with IgA anti-CT values than with IgG anti-CT.

The chronic and acute T-V loops showed protection against fluid production after CT in systemically and locally immunized animals. However, IgG anti-CT usually appeared in both loops, and leakage of serum antibodies because of surgical manipulation was felt, therefore, to invalidate these protection results as a demonstration of local immunity. In challenge studies in undisturbed chronic loops, only local immunization alone was found to result in definite protection.

B) Further Study of Suppression of Local Immune Response to Cholera Toxin (CT) Using Toxoids; Comparative Study of Suppression Using Keyhole Limpet Hemocyanin (KLH) and CT:

Experiments were performed to determine: a) Whether production of IgA anti-CT in loop fluids after i.l. CT was also suppressed when cholera toxoids were used for priming and booster systemic (s.c.) immunization. b) Whether local immune response to an unrelated antigen, keyhole limpet hemocyanin (KLH), could be similarly modified either by s.c. immunization with KLH or CT.

The experimental techniques were comparable to those used in the initial studies on CT. It was found that systemic administration of either the natural toxoid of CT (choleragenoid) or glutaraldehyde toxoid caused suppression of local immune response comparable to that found previously with CT given s.c.

There was greater variation in results when KLH was used for the antigen. However, the findings suggest some suppression of local production of IgA anti-KLH when KLH was also given s.c. On the other hand, s.c. administration of CT probably caused enhanced production of IgA anti KLH by intestinal loops. This was an unexpected finding, but could be accounted for by non-specific biologic effects of the CT, perhaps mediated through the adenyl cyclase mechanism.

C) Study of IgA Content of Secreted Fluid and Intestinal Epithelium and Comparison With Fluid Production and Goblet Cell Mucin:

The fluid produced by the intestine following exposure to CT is mucin-rich, and the histological counterpart of this finding is depletion of mucin from goblet cells. The secreted fluid also contains IgA. In the present study, the IgA content of the crypt epithelium and lamina propria plasma cells was compared with net fluid production and with mucin content of goblet cells after exposure to CT. In addition, these morphological results were compared with measurements of IgA content of the secreted loop fluid. The methods used were: a) In all experiments, freshly prepared intestinal loops in unimmunized rabbits were challenged with either CT or a control solution. b) Biopsies were performed at varying intervals after challenge and content of IgA in epithelium and plasma cells was assessed using immunofluorescent technique. c) Goblet cell mucin was assessed using Alcian blue staining of the same biopsies. d) A radioimmunoassay technique was used to measure IgA content of the secreted fluid.

The results showed that release of mucin from goblet cells was related to cholera toxin stimulation and that decline in mucin content tended to parallel increased net fluid production. IgA content of crypt epithelium by immunofluorescence also declined with cholera toxin stimulation, but the relationship was more complex than with goblet cell mucin since there was also

some loss of epithelial IgA in control loops. No change in plasma cell IgA was seen. Fluid IgA concentration declined with rising fluid production in the cholera loops such that increased total IgA production corresponding to the loss of IgA from the epithelium could not be demonstrated in the initial series of experiments where the loops were also biopsied. However, in a redesigned experiment using a washout procedure IgA concentration did not fall as net fluid production rose in the cholera loops and total fluid IgA release, therefore, was seen to increase. No early stimulation of IgA release was demonstrated in either experimental system.

FOREWARD

During the course of this work the authors were greatly assisted by Mrs. Gertrude Brown, Mr. Gerald Horne, Mrs. Cariel Edwards, Dr. Hannibal Edwards, Mrs. Nancy Folker, Miss Deborah Grady, Mr. Udo Wahn, Mr. Grady Barnes, Mr. Douglas Milligan and Miss Martha Jones. Their help is deeply appreciated. Dr. John Boitnott participated in the experiments on IgA release by crypt cells. The cooperation of Dr. John Cebra and Ms. Stella Robertson in supplying essential reagents and their helpful advice and criticism during this study is gratefully acknowledged.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal, Resources, National Academy of Sciences - National Research Council.

TABLE OF CONTENTS

I. LOCAL (IgA) IMMUNE RESPONSE BY THE INTESTINE TO CHOLERA TOXIN AND ITS PARTIAL SUPPRESSION WITH COMBINED SYSTEMIC AND INTRA-INTESTINAL IMMUNIZATION.

A. Introduction

Although local immunity can protect the intestine against diarrheagenic organisms and their toxins, little is known about how best to stimulate such immunity. Cholera toxin (CT), an important cause of diarrheal illness, is a convenient antigen for studying local immunity because CT induces a vigorous humoral immune response and its bioactivity permits direct testing of protective effects by local immunity. After its release into the lumen during natural infection with V. cholerae, the toxin must first attach to the luminal side of the epithelium to cause fluid production, and local immunity to CT may be able to protect the host by modifying this process (1).

Most studies of local immunity to CT have been conducted by administering the toxin parenterally, orally, or by intraluminal inoculation into the intact small intestine (1-10). However, CT can also be presented directly to the small intestine by using chronically isolated Thiry-Vella (T-V) loops (11-13). Whereas chronic T-V loops are clearly not a completely "natural" in vivo system, they offer several important advantages for investigating local immune phenomena: a) Repeated intraluminal inoculations with antigen can be performed easily so that various parameters of antigen preparation, dose, and administration are readily examined. b) The isolated loops permit detailed serial study of intestinal secretions from undisturbed, intact mucosa without the possible variable of protein degradation by pancreatic proteases. c) The T-V loops permit study of other fundamental aspects of local immunity such as the role played by Peyer's patches. d) In regard to CT, the chronic T-V loops provide a convenient means to test for protection by local immunity.

In the present studies, rabbits with chronic T-V ileal loops were immunized with CT using intraloop, subcutaneous, and combined intraloop-subcutaneous inoculation. Sera and secreted fluid from the loops were tested for neutralization of toxin by blueing test and for IgA and IgG antibody to CT. These findings were compared with each other and with the fluid production after challenge of the T-V loops by CT.

B. Materials and Methods

1) Preparation and Care of Loops. Using a previously described technique (14), an isolated loop of distal ileum 20 cm. in length and containing a Peyer's patch was prepared in outbred New Zealand white rabbits of mixed sex weighing 3-4 Kg. Stated briefly, Silastic tubing (Dow Chemical Corp., Midland, Mich.) was sewn into each end of the isolated segment of bowel. Intestinal continuity was restored by end-to-end anastamosis. The two tubes from the T-V loop were passed through the muscle wall via the abdominal incision, and tunneled subcutaneously to the nape of the neck. Fluid normally accumulated in the ileal loop, and daily flushing with saline prevented excessive mucus accumulation. Previous studies have shown that isolated ileal loops in rabbits undergo atrophy of villi with an increase in size and number of Paneth cells, but there are no histologic alterations in lymphoid tissue, including Peyer's patches (14).

2) Immunization of Animals and Specimen Preparation: Rabbits were inoculated subcutaneously (s.c.) and/or intraloop (i.l.) with purified CT (obtained from Geographic Medicine Branch, NIAID, Bethesda, Md. or from Schwarz-Mann Division of Becton, Dickinson, Inc., Orangeburg, N.Y.). For i.l. inoculations, 100 ug CT in 4 ml 0.01 M neutral phosphate buffered saline (PBS) was placed in the loop and the distal (efferent) tube was clamped for 24 hours. For s.c. inoculations, 30 ug CT in 1 ml. PBS was used.

Two series of experiments were conducted (series A & B) and the group designations and inoculation schedules are summarized in Table I.

TABLE I

Immunization Schedules

	Experimental Groups[1]	Cholera Toxin Inoculations[2] Intraloop (IL)	Subcutaneous (SC)
SERIES A	IL (5)	Days 0,7,14,21	NONE
	SC (6)	NONE	Days -7 & 14
	IL + SC (6)	Days 0,7,14,21	Days -7 & 14
	Controls (5)	NONE	NONE
SERIES B	IL (7)	Days 0,7,14	NONE
	SC (8)	NONE	Days -21 & 0
	IL + SC (6)	Days 0,7,14	Days -21 & 0
	Controls (6)	NONE	NONE

1- Numbers of animals in parentheses.
2- Day 0 was day of first IL inoculation.

In both series weekly i.l. inoculations were begun three days after loop preparation and s.c. inoculations were spaced three weeks apart. In Series A four i.l. inoculations were used and s.c. inoculations were begun one week before the time for initial i.l. inoculations. In Series A, vehicle (PBS) alone was given at appropriate times for omitted inoculations, and a control group receiving only PBS was included. Series B differed from Series A by utilizing only three i.l. inoculations and by beginning s.c. inoculations three weeks before initial i.l. inoculation. Series B thus provided study of fewer i.l. inoculations and of earlier s.c. inoculations and permitted examination of loop fluid for antibodies for a longer period after the last i.l. dose of CT.

Loop fluids were collected on the day of first i.l. inoculation and every 3-4 days thereafter. Specimens were frozen at -20°C and after thawing mucus and any cell debris were removed by low speed centrifugation, yielding clear supernatants which were used for all studies. Blood for serum was collected prior to first inoculation, and at weekly intervals thereafter.

3) Skin Test for Neutralizing Capacity Against CT: The assay method used was similar to the one described by Benenson, et al. (15). Sera diluted 1/10, 1/100, 1/400, 1/1600, and fluid specimens diluted 1/4, 1/16, 1/64, 1/256, 1/1024 were mixed with equal volumes of PBS with .02% gelatin containing 2-4 ng of purified CT per 0.05 ml. The amount of CT used was adjusted to maintain a uniform response with a standard immune rabbit serum. After incubation at 37° for 30 minutes, the specimen-CT mixtures were injected intradermally into rabbits in triplicate, using 0.1 ml. at each site. Eighteen hours later 5% Pontamine Sky Blue 6B (as Chicago Blue 6B, Matheson, Coleman & Bell, Norwood, Ohio) in saline, 0.12 ml/100 g body weight, was given i.v. Neutralization endpoint was the highest dilution that showed no blueing and/or greater than 50% reduction in average wheal diameter. Geometric mean titers and their standard errors (SEM) were calculated for comparable collection times.

4) Materials and Reagents for Enzyme Linked Immunosorbent Assay (ELISA) for Antibodies to CT: The procedures were modified from those developed by Engvall and Perlman (16) and applied to CT by Holmgren and Svennerholm (17). Polystyrene tubes (Type 2052, Falcon Plastics, Rutherford, N.J.) were exposed at 37° C for 3¼ hours to 1 ug purified CT contained in 0.5 ml PBS + 0.02% sodium azide. These CT-coated tubes were stored at 4° C without removing fluid until needed. The rinse solution and diluent for specimens was PBS containing 0.02% sodium azide and 0.05% Tween-20 (Fisher Scientific Co., Pittsburgh, Pa.) (PBS-Tween). Goat anti-rabbit IgG and goat anti-rabbit IgA for conjugation to alkaline phosphatase were provided by Dr. John Cebra and Ms. Stella Robertson. These had been prepared by immunizing goats with the Fc portion of rabbit serum IgG or with colostrum-derived sIgA that had been "washed" with guanidine. Goat sera were purified to their IgG fraction using ammonium sulphate precipitation followed by DEAE cellulose column chromatography. Antibodies to light chains and to heavy chains of unwanted isotypes were removed by affinity chromatography with cyanogen bromide-activated Sepharose coupled to whole IgG, IgA or IgM molecules.

In order to conjugate the goat anti-rabbit IgG and anti-rabbit IgA to alkaline phosphatase, 2 mg. of the goat IgG in 0.1 - 0.2 ml. PBS was combined with 0.55 mg of centrifuged precipitate of calf intestine alkaline phosphatase (Type VII, 990 u/mg, Sigma Chemical Co., St. Louis, Mo.). After dialysis against PBS, 0.01 ml. of PBS containing glutaraldehyde (Biological grade, 50% W/W, Fisher Scientific Co., Pittsburgh, Pa.) was added to the goat anti-Ig-phosphatase mixture at a concentration that yielded a final concentration of 0.7% glutaraldehyde. The coupling reaction was allowed to take place for 2 hrs. at room temperature and caused the solution to become slightly cloudy. Dialysis against PBS was followed by dilution to 5 ml. in Tris buffer, 0.2 M, pH 8.0, containing 5% human albumin, 0.001 M magnesium chloride, and 0.02% sodium azide. The resulting stock rabbit anti-Ig-phosphatase conjugate solution was sterilized by filtration (0.45 um); it could be stored at 4° C for several months without loss of activity.

The anti-Ig-phosphatase solutions were tested for isotype specificity and for optimal working dilution by means of ELISA reaction. This was done by using polystyrene tubes that had been coated with 1 ug purified rabbit IgA, IgG or IgM (kindly supplied by Dr. John Cebra and Ms. Stella Robertson) in the same manner as with CT. Tests were conducted as described below for anti-CT activity except that various dilutions of the anti-Ig-phosphatase conjugate solution were placed immediately into the Ig-coated tubes for 16 hours. Both the anti-IgA and anti-IgG phosphatase conjugates were found to be highly specific, showing only minimal cross-reaction with other isotypes.

5) ELISA Test Procedure: All steps were conducted at room temperature on a horizontal rotary shaker set at low speed (30-60 RPM), and tubes were rinsed in three changes (1 ml x 2 and 5 ml x 1) of PBS-Tween for 5 min. between each step. After initial rinsing, the CT-coated tubes were exposed for 6 hours to 0.5 ml. samples of fluid supernatant of serum appropriately diluted in PBS-Tween. Tubes were next exposed for 16 hours to 0.5 ml. of the anti-Ig-phosphatase solution diluted 1:50 to 1:150. The dilution used had usually given maximal or near maximal response against samples of the appropriate isotype. The final step was addition of 1 mg. of nitrophenyl phosphate substrate (Type 104, Sigma Chemical Co., St. Louis, Mo.), 1 mg/ml in 0.05 M pH 9.8 in carbonate buffer containing magnesium chloride, 0.001 M. The reaction was stopped at 1 hour with 1 ml. of 0.2 N sodium hydroxide; spectrophotometric absorbance was determined at 400 nm. Specimens from animals in each of the immunization and control groups were tested in each run. Control samples of immune fluid and serum were included in each run, and their absorbance values were adjusted to an arbitrary absorbance of 2.00 by a correction factor. This factor was then applied to the specimen values to compensate for day-to-day variations in the test system.

Preliminary studies were conducted on fluid and serum samples from several appropriately immunized animals to determine optimal dilutions for routinely performing the assays. Serial specimen dilutions were tested by ELISA, and semilog plots of the inverse of dilution (titer) against absorbance for each specimen were made. It was thereby found that the relative absorbances of the specimens at any given dilution generally showed the same relationship as the titers of the specimens, i.e., the specimens with the highest titers usually had the highest absorbances at any given dilution. On this basis the lowest dilution in which the ELISA system was not saturated was chosen as the test dilution. IgA anti-CT in fluids and sera and IgG anti-CT in fluids were assayed at 1:40 while IgG anti-CT in serum was assayed at 1:1600. Thus, the absorbance values of the IgA test system could not be compared directly with those for IgG. However, the time course of the IgA- or IgG anti-CT response could be followed in both serum and fluid, and the relative amounts of IgA- and IgG anti-CT could be compared with the neutralization activity of the specimens. The coefficients of variation for the test systems as calculated from standard deviations of the differences using the control specimens were about 8%.

6) Challenge with CT at Termination of Experiments: These studies were conducted on the final day of each experiment (days 24-28 in Series A and day 35 in Series B).

In Series A animals, and in the SC group from Series B, the peritoneal cavity was re-entered. The spleen, mesenteric lymph nodes, and portions of the chronic T-V loop and undisturbed small intestine were removed for later studies. The chronic T-V loop was then reattached to tubing and an additional "acute" loop measuring 15-20 cm. was prepared from intact intestine near the original anastomotic site in the ileum. The purpose of the "acute" loop was to determine if immunization of the chronic T-V loops had brought about detectable immunity to CT elsewhere in the intestine. Tubes attached to the "chronic" and "acute" loops were brought out through the abdominal wall. After body temperature had been restored to 37° C, each loop was challenged with 25 mg of crude cholera toxin (Lot 001, Wyeth Laboratories, Philadelphia, Pa.) dissolved in 3 ml. PBS. The toxin-containing solutions were removed ½ hour later and fluid production by each loop was monitored for an additional 4½ hours by expelling the fluid with air at ½ hour intervals. Fluid specimens collected from the 3rd to 5th hours were pooled and assayed by ELISA for IgA and IgG anti-CT. At the end of the experiment the animal was reopened, loops were measured, and fluid output during the final two hours of the experiment was calculated as uL./cm./hr.

A second series of challenge experiments utilized four groups of animals: IL animals from the Series B experiments; IL + SC animals from Series B; 5 rabbits (designated SC-Ch) given 30 ug CT on days -7 and +14 and followed for 28 days in the same manner as SC animals from Series A; 6 control rabbits that were kept for 35 days without prior exposure to CT by any route. For all animals the challenge studies were performed as follows: after the usual collection of fluid and saline rinse on the last day of each experiment (Day 28 or 35), the otherwise undisturbed loops were exposed to 50 mg crude cholera toxin (Lot 001, Wyeth Laboratories, Philadelphia, Pa.) in 3 ml. PBS for ½ hour. Fluid was thereafter collected from the loops at ½ hour intervals for another 5½ hours. Fluid obtained between the 2nd and 4th hours was pooled and assayed by ELISA for IgA and IgG anti-CT. Fluid outputs as uL./cm./hr. for the 4th through 6th hours were calculated after measuring loop lengths.

C. Results

1) Toxin Neutralization Tests: Neutralizing activity against CT was found in loop fluids from all three immunized groups in the Series A experiments. Higher mean titers were reached, however, in animals receiving i.l. CT (IL and IL + SC Groups) than in rabbits inoculated only s.c. (SC Group) (Fig. 1). Indeed, mean titers for loop fluids never rose above 1:4 in the SC Group. Mean titers for fluids in the IL + SC Group tended to be lower than in the IL Group and most of the high titers (> 1:64) were in animals from the IL Group.

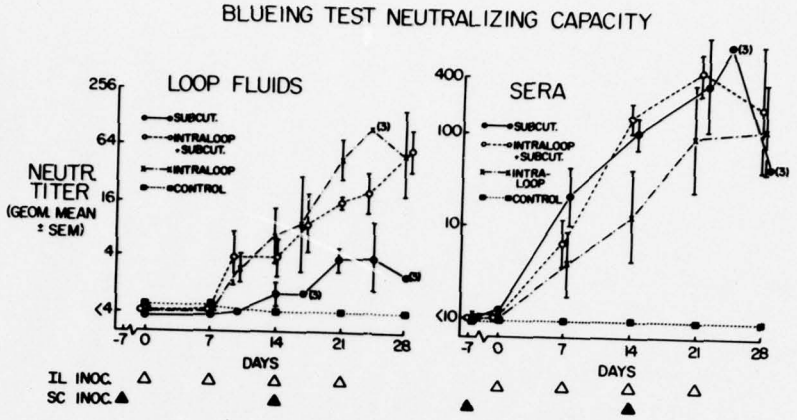


Fig. 1 Series A experiments. Capacity of fluids and sera to neutralize cholera toxin by blueing test.

Serum antitoxin titers (Fig. 1) in Series A showed patterns of elevation which were different from those seen in loop fluids. While neutralizing capacity was evident in sera from all three groups by day 7, values rose more rapidly and reached their highest mean levels in animals receiving s.c. inoculations (SC and IL + SC Groups). Mean serum titers for the IL Group showed a slower rise, but from Day 21 onward they were not significantly different in the three groups.

2) ELISA Assay for IgG- and IgA-Anti-CT: In Series A, IgG anti-CT was eventually detected in sera after all three immunization schedules, while PBS inoculated controls were uniformly negative (Fig. 2). On the other hand, loop fluids showed very little IgG anti-CT activity even though the tests were done at 40 times greater concentration than sera. Thus, systemic immunization took place with i.l. as well as s.c. inoculation of CT, but very little of the resulting IgG anti-CT activity could be detected in the loop fluids.

In contrast to IgG anti-CT, IgA anti-CT showed high mean ELISA values for fluids from IL animals in Series A. On the other hand, very low IgA anti-CT was found in fluids from SC animals (Fig. 3). Immunization by combined i.l. and s.c. inoculation (IL + SC Group) led to mean ELISA values for IgA anti-CT in fluid which were higher than those seen with s.c. immunization, but from day 10 onward they were consistently lower than the IL group (Fig. 3). These findings suggested that combined s.c. and i.l. immunization with CT had a suppressive effect on local immunization. In sera, low absorbance values were obtained by ELISA for IgA anti-CT by all three immunization schedules.

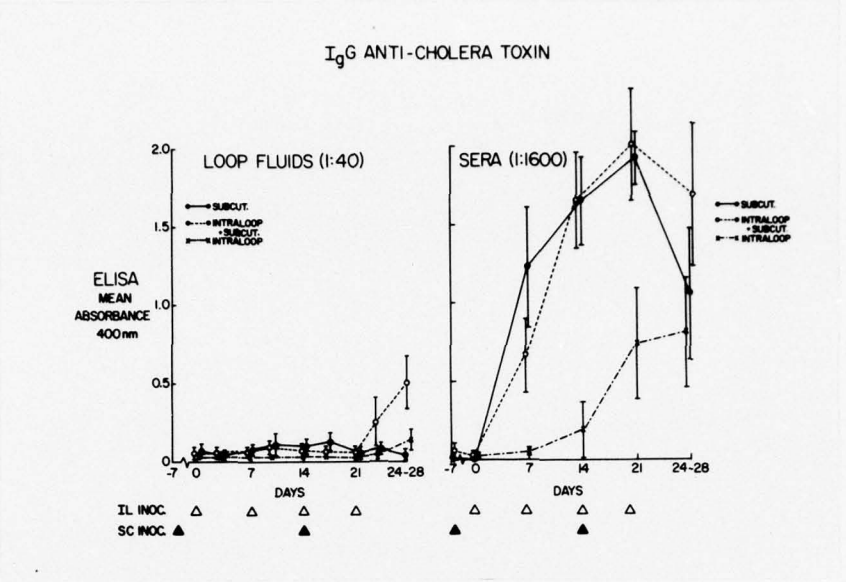


Fig. 2 Series A experiments. Relative content of IgG anti-CT in fluids and sera as indicated by ELISA.

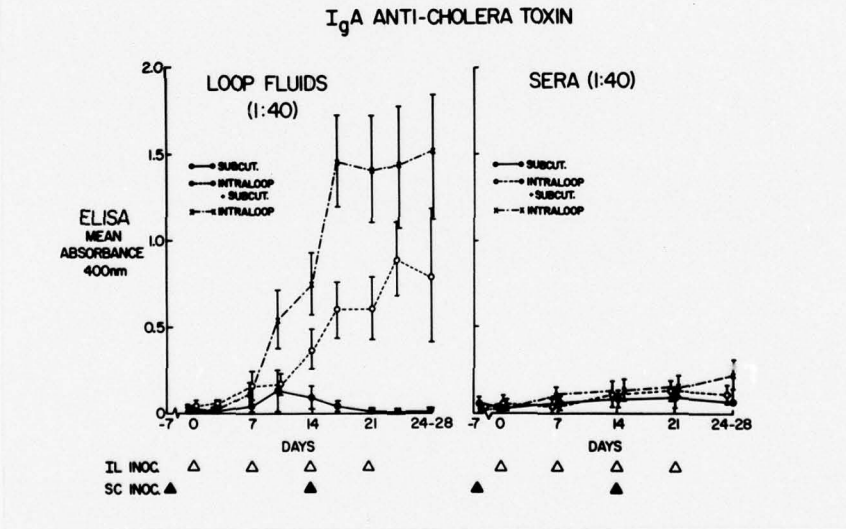


Fig. 3 Series A experiments. Relative content of IgA anti-CT in fluids and sera as indicated by ELISA.

In fluids from Series B, IgA anti-CT was detectable after two i.l. inoculations of CT, and was sustained for three weeks after the last (third) inoculation (Fig. 4). Furthermore, s.c. inoculation alone with CT beginning two weeks sooner than in Series A still led to only slight elevation in mean fluid IgA anti-CT levels. However, in the IL + SC group suppression of IgA anti-CT was more striking than in Series A since IgA anti-CT levels rose only slightly as compared to IL animals. As in Series A, mean serum IgA anti-CT did not rise significantly in Series B.

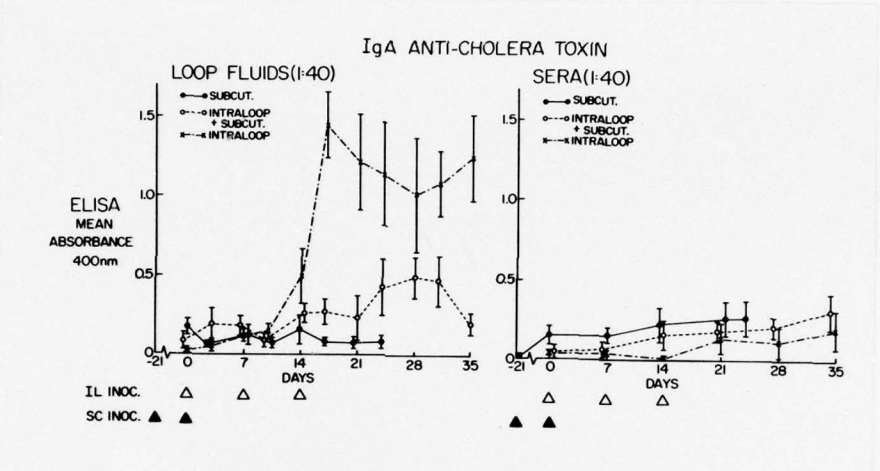


Fig. 4 Series B experiments. Relative content of IgA anti-CT in fluids and sera as indicated by ELISA.

IgG anti-CT levels in Series B animals were low in fluids from all experimental groups and elevated in sera from SC and IL + SC animals (Fig. 5). However, serum IgG anti-CT from IL + SC animals were significantly lower than in the counterpart Series A group. No reason for this finding could be determined.

3) Correlation of Skin Tests with IgA anti-CT: Individual neutralizing capacities by skin test correlated much better with IgA anti-CT activities ($r = 0.77$), than they did with IgG anti-CT activities ($r = 0.24$) when they were compared for all fluids in the Series A experiments (Fig. 6).

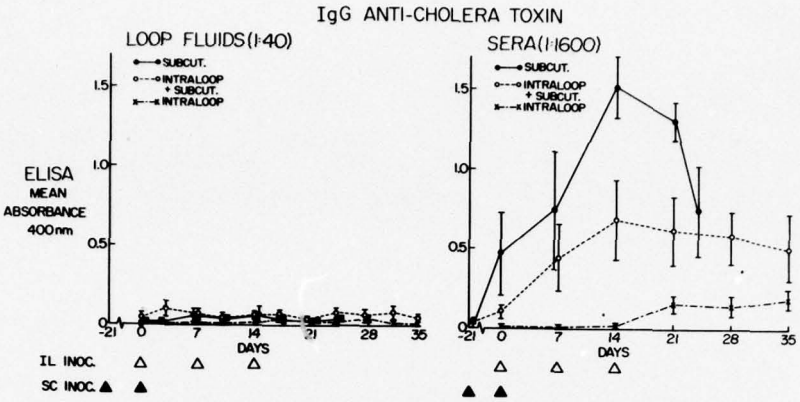


Fig. 5 Series B experiments. Relative content of IgG anti-CT in fluids and sera as indicated by ELISA.

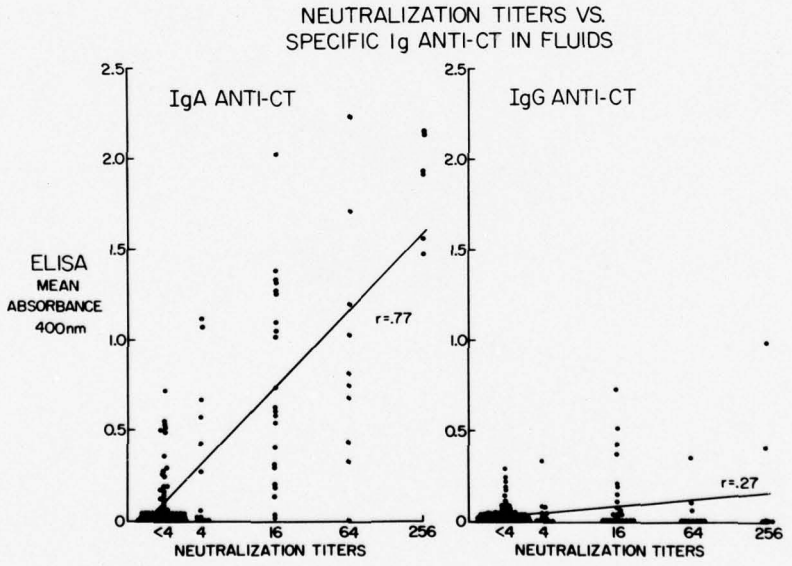


Fig. 6 Comparison of neutralization titers with corresponding IgA anti-CT and IgG anti-CT ELISA values for all fluids in Series A experiments. (The absorbance values cannot be directly compared due to the different assay systems used.) Only IgA anti-CT shows a high correlation coefficient.

4) Challenge of T-V Loops with CT: Mean fluid outputs on challenge of both the chronic and newly prepared acute T-V loops with 25 mg crude toxin are shown in Table II along with ELISA values for the fluids. Irrespective of route of immunization, fluid outputs from both chronic and acute loops were below those noted for controls. This consistent pattern of reduction in output resulted in all likelihood from immunity to CT, but the reductions were not necessarily relevant to local (IgA-mediated) immunity. Whereas only IL + SC animals showed an elevated mean ELISA level for IgG anti-CT in fluid collected from the chronic loops prior to challenge (final day specimens), fluid obtained from the chronic and acute T-V loops after challenge regularly showed high ELISA values for IgG anti-CT in all experimental groups. Thus, leakage of IgG anti-CT into fluid, presumably as a result of surgical manipulation, could readily account for the protection from challenge with CT noted in T-V loops where IgA anti-CT levels were found to be low.

TABLE II

Challenge Experiments - Chronic and Acute Loops

Exp. Group	Mean output ± SEM (μL./cm./hr) Chronic Loop	Acute Loop	Anti-CT Isotype	Fluid anti-CT Values[1] Chr. Loop (Final Day)	Chr. Loop (Challenge)	Acute Loop	Serum Anti-CT Values[2]
IL (Series A)	57±18	37±13	IgA	1.53±.36	.88±.51	0 (n=1)	.23±.09
			IgG	.15±.08	1.17 (n=2)	.09 (n=1)	.83±.35
IL + SC (Series A)	70±24	87±37	IgA	.96±.27	.70±.25	.06±.06	.09±.05
			IgG	.39±.14	.58±.25	.62±.26	1.69±.46
SC (Series A)	73±10	65±17	IgA	.01±.01	.01±.01	0	.14±.08
			IgG	.03±.02	.83±.28	.49±.29	1.06±.43
SC (Series B)	39±13	59±11	IgA	.08±.04	.05±.03	.05±.02	.26±.11
			IgG	.04±.01	.35±.01	.65±.21	.74±.28
CONTROLS (Series A)	138±31	157±19	IgA	0	0	0	0
			IgG	0	0	0	0

1 - Mean ELISA absorbance ± SEM at 1:40

2 - Mean ELISA absorbance ± SEM at 1:40 (IgA) and 1:1600 (IgG) on final day.

ELISA data on challenge fluids from the acute loops in IL and IL + SC animals indicated that little if any IgA anti-CT could be detected there in response to immunization via the chronic loops. (Fluid from the acute loops could be assayed in only one IL animal , however, since an insufficient volume was secreted from the others.)

Table III shows fluid outputs and ELISA values after challenge of undisturbed chronic T-V loops using a larger amount (50 mg) of crude toxin in order to enhance output. Mean output from IL animals was significantly below that noted in controls ($p < 0.05$). On the other hand, IL + SC and SC-Ch animals showed mean outputs which were lower than but which were not significantly different from the control value ($p > 0.1$). Furthermore, high mean ELISA values for IgG anti-CT were not observed in challenge fluids from loops of the IL + SC and SC-Ch groups even though mean serum levels in those animals were elevated. Thus, only the IL animals clearly showed evidence of protection on challenge with CT when there was no evidence that IgG anti-CT leaked into the challenge fluids. This stood in marked contrast to the results obtained when challenge with CT followed additional surgery on the loops.

TABLE III

Challenge Experiments -- Undisturbed Chronic Loops

Exp. Group	Mean Output ± SEM (μL/cm./hr.)	Anti-CT Isotypes	Fluid anti-CT Values[1] Final Day	Fluid anti-CT Values[1] Challenge	Serum anti-CT Values[2]
IL (Series B)	97 ± 26	IgA	1.26±.28	.58±.21	.19±.12
		IgG	.02±.01	.03±.02	.19±.06
IL + SC (Series B)	151±34	IgA	.21±.06	.10±.06	.31±.11
		IgG	.04±.02	.01±.01	.51±.21
SC-Ch	171±21	IgA	.04±.01	.02±.02	.02±.01
		IgG	.21±.12	.06±.02	1.87±.22
CONTROLS (Series B)	213±18	IgA	0	0	0
		IgG	0	0	0

1 - Mean ELISA absorbance ± SEM at 1:40.

2 - Mean ELISA absorbance ± SEM at 1:40 (IgA) and 1:1600 (IgG) on final day.

D. Discussion

In these studies intra-intestinal immunization of chronically isolated (Thiry-Vella) intestinal loops with CT led to progressive increases in neutralizing capacity against CT by fluid from the loops. The neutralization test findings were paralleled by rising content of IgA anti-CT in loop fluids. After s.c. inoculation, however, loop fluids showed much less neutralizing capacity against CT and there were correspondingly smaller amounts of IgA anti-CT. Combined i.l. and s.c. inoculation gave intermediate results. The superior local (IgA) immunity obtained by the i.l. route presumably resulted from efficient stimulation of IgA precursor cells in intestinal lymphoid tissue. This conclusion is supported by the demonstration that when other antigens (2,4, dinitrophenyl-keyhole limpet hemocyanin and heat-killed Salmonella typhimurium (18,20) and live Shigella (Keren, D.F., Holt, P.S. and Collins, H.H. unpublished observations) were inoculated into T-V loops of the type used here, specific antibody, mainly IgA, also appeared rapidly in the fluid. Furthermore, in those studies serum antibody response was negligible and omission of Peyer's patches seemed to curtail the local response.

While there has long been evidence that local immunity in the intestine to Vibrio cholerae and its products could be important (21), until recently studies on cholera immunity have tended to focus strongly on systemic immunization and serum antibodies and relatively little attention has been paid to local immunity per se. In addition, while some past experimental studies have suggested that parenteral inoculation alone might be effective in achieving local immunity to CT (4,6,7,11,22), serum-derived antibodies to CT (chiefly IgG anti-CT), may have played a prominent role in many of those experiments. For example, Kaur, et al. (7) studied mucosal scrapings so that serum antibodies were surely present in their test material. Also, in earlier cross-circulation experiments where passive protection was examined (22), and in previous studies using intestinal loop assay in immunized animals (4,6), surgical manipulation could have led to breakdown of the blood-lumen barrier with subsequent leakage of serum antibody. Results obtained in the present challenge experiments emphasize that protection or neutralization tests against CT which use systemically immunized animals and recently manipulated intestine cannot distinguish between local (IgA) and systemic (chiefly IgG) anti-CT. Only when challenges were performed in undisturbed chronic T-V loops, and when no IgG anti-CT was detected in the fluids, could it be said that protection against CT was mediated primarily by local immunity.

IgG may be more prone to enter the intestinal lumen in other species. In the dog, passive protection against CT in chronic T-V loops was achieved with intravenous injections of the IgG fraction of immune serum (11). Enhanced resistance obtained with combined oral and systemic immunization in dogs (9,12) could also have been related to transmucosal leakage of IgG.

The good correlation between IgA anti-CT levels and neutralizing capacity in the loop fluids, together with the poor correlation between IgG anti-CT levels and neutralizing capacity, suggests that IgA anti-CT can neutralize at least some biologic effects of CT. Although IgM anti-CT levels were not investigated by us, rabbits normally demonstrate only small amounts of IgM in their intestinal mucosa and secretions (7,23), and in other studies using the present model only low levels of IgM antibody were thought to be formed against the antigens used (20).

Since previous studies suggested that combined intra-intestinal and parenteral immunization can enhance local immunity (9,10,12), we were initially surprised to find reduced IgA anti-CT content in loop fluids after i.l. and s.c. immunization. However, it is now evident that combined systemic and intraintestinal immunization consistently suppressed local immunity in our model system. Furthermore, results in the Series B experiments also suggest that suppression still occurs even when timing of the s.c. inoculation in relation to i.l. inoculations is varied and that the suppressive effect is sustained after final i.l. inoculation.

The suppression seen with combined i.l. and s.c. immunization can be viewed as possibly having either a toxigenic or an immunogenic basis. CT can act directly on lymphocytes to cause reduced production of immunoglobulin (24), and this toxigenic effect has been demonstrated both in vivo and in vitro. It probably proceeds via the adenyl cyclase system (24). On the other hand, if the suppression of local (IgA) immunity in our studies was immunogenic, then it presumably occurred via suppressor T-cells (25). The present data do not provide a firm choice between a toxigenic or immunogenic mechanism for suppression of IgA anti-CT in loops, but the relative non-dependence of suppression on timing of s.c. inoculations and its sustained occurrence after final i.l. inoculation favor an immunogenic mechanism. In addition, we have noted suppression of local immune response to CT after s.c. inoculation of biologically inactive toxoids of CT (see Section II). While the true significance of suppression as seen in our experiments remains to be demonstrated, the possibility should be considered that suppression of local immunity and hence suboptimal protection may occur when combined parenteral and oral vaccination are used for human beings.

II. SUPPRESSION OF LOCAL IMMUNE RESPONSE TO CHOLERA TOXIN (CT) BY SYSTEMIC IMMUNIZATION WITH TOXIN OR TOXOIDS; COMPARATIVE STUDY USING KEYHOLE LIMPET HEMOCYANIN (KLH) AND CT:

A. Introduction

In our initial studies using chronically isolated Thiry-Vella ileal loops in rabbits, we found that 3 or 4 weekly intraloop (i.l.) inoculations of cholera toxin (CT) resulted in readily detectable IgA anti-CT in loop fluids. Subcutaneous (s.c.) administration of systemic priming and booster doses of CT three weeks apart produced much lower levels of fluid IgA anti-CT. On the other hand, when both s.c. and i.l. inoculations were given the content of IgA anti-CT in fluid was below that obtained after i.l. inoculation. This finding strongly suggested a suppressive effect on the local immune response by the systemic inoculations (26, 27; see Section I).

Suppression of local immune response to CT by systemic immunization could result from either a direct toxigenic effect of CT on the IgA-producing lymphoid system, or from an indirect, presumably immunogenic, effect, perhaps via stimulation of suppressor T cells. The experiments described in this section were designed to further explore suppression of local immunity by determining whether: a) Production of IgA anti-CT in loop fluids after i.l. CT was also suppressed when cholera toxoids rather than biologically active CT were used for priming and booster systemic immunization. b) Local immune response to an unrelated antigen, keyhold limpet hemocyanin (KLH), could be similarly modified either by KLH or by CT when administered systemically.

B. Materials and Methods

1) Preparation and Care of Loops: Isolated loops of distal ileum, 20 cm. in length and containing a Peyer's patch, were prepared and maintained according to the previously described technique in outbred New Zealand white rabbits of mixed sex weighing 3-4 Kg. (14; see Section I, B-1).

2) Immunization of Animals and Specimen Preparation: Antigenic materials consisted of: a) Purified cholera toxin (Schwarz/Mann). b) Choleragenoid (28) (kindly provided by Richard Finkelstein). c) Glutaraldehyde-toxoid (29) (Wyeth lot 20201, obtained from Division of Biological Standards, NIH) in protamine sulphate and aluminum chloride adjuvant. d) Keyhole limpet hemocyanin (KLH) (Schwarz/Mann) in phosphate buffered saline (PBS). Since KLH does not dissolve completely, a suspension containing 80 mg/100 ml was stirred for several hours after which remaining undissolved material was removed by high speed centrifugation. Final concentration was determined by spectrophotometric analysis of the supernatant at 280 nm.

Table IV

IMMUNIZATION SCHEDULES

	Experimental Groups[1]	Antigen Inoculations[2] Intraloop (i.l.)	Subcutaneous (s.c.)
Intraloop CT	ILC (6)	CT 100 μg Days 0,7,14	None
	ILC + SCCD (5)	Same	Choleragenoid 30 μg Days -7 & 14
	ILC + SCGT (6)	Same	Glut. toxoid 400 μg Days -7 & 14
Intraloop KLH	ILK (6)	KLH 400 μg Days 0,7,14,21	None
	ILK + SCC (6)	Same	CT 30 μg Days -7 & 14
	ILK + SCK (5)	Same	KLH 400 μg Days -7 & 14

1 - Number of animals in parentheses.

2 - Day 0 was day of first IL inoculation.

Immunization schedules are shown in Table IV. Intraloop antigens were inoculated in 4 mls. PBS after which the distal (efferent) Silastic tube was clamped for 24 hours. Antigens for subcutaneous inoculation were dissolved in 1 ml PBS. Fluid normally accumulated in the loops and was collected by expulsion with air on the day of first i.l. inoculation and every 3-4 days thereafter. Experiments lasted 28 days from time of first i.l. inoculations. Fluid specimens were frozen at -20°C, and after thawing, mucus and any cell debris were removed by low speed centrifugation, yielding a clear supernatant used for immunoglobulin assay. Blood for serum was collected prior to first inoculation, and at weekly intervals thereafter.

3) Enzyme Linked Immunosorbent Assay (ELISA) for Antibodies: The procedures followed for anti-CT antibodies were the same as those described above (16,17; Section I-B-4). For KLH antibodies, the procedures were also the same except that KLH 1 ug/0.5 ml PBS was initially bound to the tube wall.

C. Results

1) Suppression of Local Response to CT: Mean values for IgA anti-CT in fluid rose dramatically after the third i.l. inoculation of cholera toxin in group ILC, and the elevation was sustained through day 28 (Fig. 7). By contrast, mean levels of IgA anti-CT in fluid specimens remained significantly lower than in ILC animals after day 14 in groups ILC + SCCD and ILC + SCGT. In addition, there was some suggestion that early (days 0-7)

IgA anti-CT response in the fluids was initially higher when choleragenoid was given s.c. (group ILC + SCCD) (Fig. 7). There was only a slight rise in serum IgA anti-CT in the ILC group, and no perceptible rise in that isotype against CT was found in the two groups given toxoid s.c. (Fig. 7)

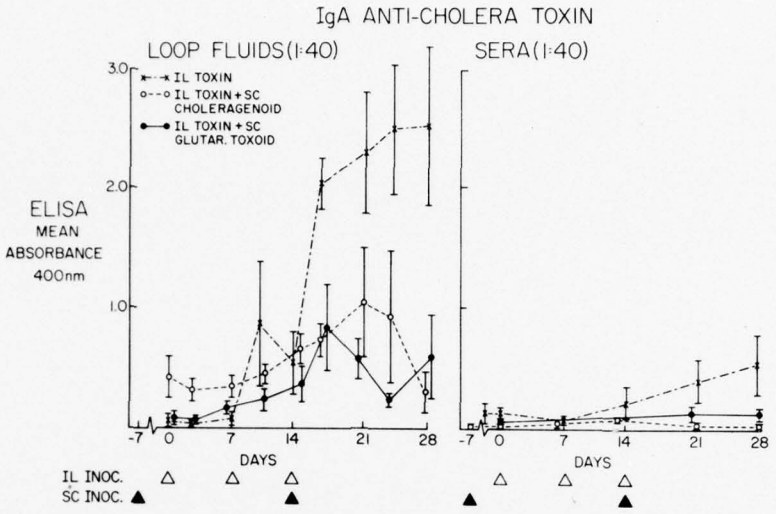


Fig. 7 IgA anti-CT by ELISA for fluids and sera in animals inoculated i.l. with CT.

IgG anti-CT in fluids rose slightly in ILC + SCGT animals but was not seen to rise in the other two groups (Fig. 8). (In fact, only two of six animals in the ILC + SCGT groups showed increases.) On the other hand, serum levels of IgG anti-CT rose markedly in the two groups that were given s.c. immunization (ILC + SCCD and ILC + SCGT) while a smaller rise of serum IgG anti-CT was noted in the ILC group (Fig. 8).

2) Modification of Local Immune Response to KLH: Local immune response to KLH by the loops showed more variation than was seen with CT in these or previous experiments, leading to a greater range of ELISA values and standard errors (Figs. 9 & 10). However, despite their preliminary nature, these data were noteworthy in several respects: a) Mean IgA anti-KLH response in loop fluid showed a gradual steady rise in the ILK animals with a peak on day 25 (Fig. 9). b) When KLH was given s.c. (ILK + SCK animals), mean values for IgA anti-KLH failed to show the expected peak. c) An earlier rise in mean values for IgA anti-KLH was observed in fluids from animals that also were inoculated with CT subcutaneously (ILK + SCC). d) Serum IgG anti-KLH showed virtually no rise in ILK animals (Fig. 10). An increase did occur, however, when CT was given s.c. along with intraloop inoculations of KLH although the values did not reach the high levels of the ILK + SCK animals.

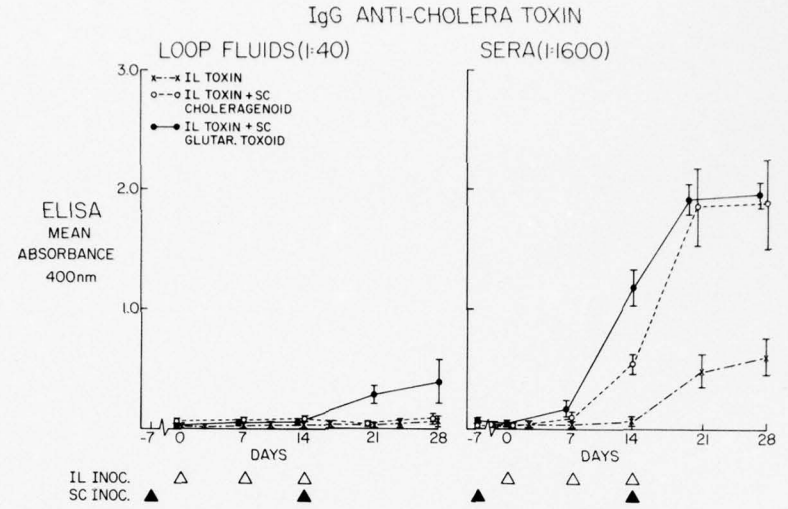


Fig. 8 IgG anti-CT by ELISA for fluids and sera in animals inoculated i.l. with CT.

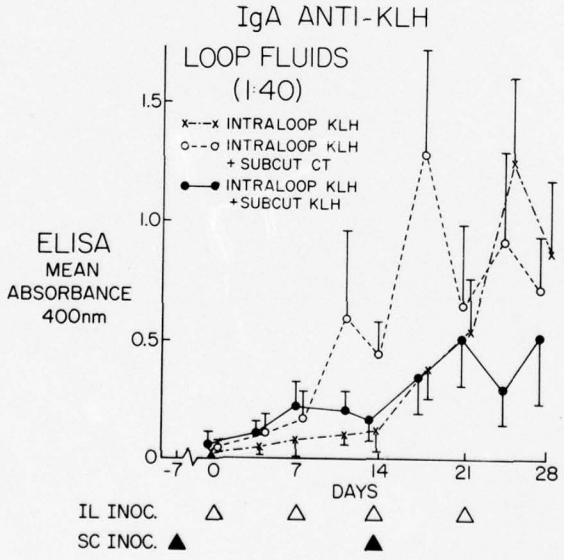


Fig. 9 IgA anti-KLH by ELISA for fluids in animals inoculated i.l. with KLH.

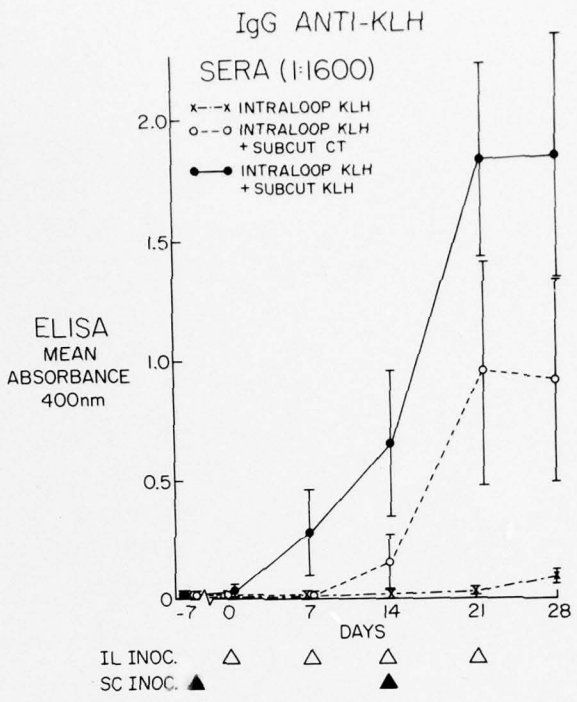


Fig. 10 IgG anti-KLH by ELISA for sera in animals inoculated i.l. with KLH.

D. Discussion

The present studies indicate that two biologically inactive but antigenically intact forms of cholera toxin (choleragenoid and glutaraldehyde toxoid) can cause suppression of local (IgA) response to cholera toxin in the intestine when administered systemically. Furthermore, the degree of suppression obtained with systemic toxoids was comparable to that noted previously (Section I) when cholera toxin was used for both the subcutaneous and intraloop inoculations. Although alternative explanations are possible, we feel these observations are clearly most consistent with the conclusion that the suppression was immunogenic rather than toxigenic in origin, and by analogy with other systems (25), suppressor T-cells were probably responsible for reducing the levels of IgA anti-CT.

While results obtained when utilizing another antigen system (KLH) to study suppression were not as clear-cut, when that antigen (KLH) was administered both locally and systemically there seemed to be relative suppression of local immune response. On the other hand, when intraloop KLH inoculation was combined with systemic administration of biologically active CT, there was no suppression and in fact the local response to KLH appeared to be enhanced. Suppression of local immune response by systemic priming appears, therefore, to be antigen-specific.

While the principle of suppression of an immune response by priming is a well established one, it has not been extensively studied in the local immune system. Pierce and Gowans (10) found enhanced production of lymphocytes containing IgA anti-CT after combined intraintestinal and peritoneal immunization with a formalinized toxoid preparation. However, in later experiments, Pierce found that under various other circumstances of systemic route and antigen preparation immunization with toxoid could cause either suppression or enhancement of the local response (unpublished observations; see Pierce NF, Proceedings of 13th Joint Conference on Cholera, United States-Japan Cooperative Medical Science Program, Atlanta, September 19-21, 1977). A variety of factors which are not well understood could influence these results. It is possible, for instance, that the intraperitoneal route can enhance local immune response in the intestine by direct stimulation of the IgA-producing system. At the same time, there is no a priori reason that suppressor T-cell - mediated suppression cannot occur with the IgA system as with IgG and IgM. The existence of such suppression is supported by the demonstration that in common variable Ig deficiency in human beings depression of the IgA response (as well as that of the other immunoglobulin isotypes) has been attributed to excessive suppressor T-cell activity (30).

The enhanced local IgA response to KLH following systemic priming with CT was not expected. We have found no evidence of immunologic cross reactivity between CT and KLH in the IgA ELISA system (unpublished data). Lyons and Friedman (31), using both in vitro and in vivo systems, found either enhanced or suppressed immune response to sheep red blood cells (SRBC) depending on timing and route of administration of CT, and they suggested that both "direct" and "indirect" effects of CT on lymphocytes can occur. Furthermore, Northrup and Fauci (32) demonstrated enhancement of systemic immune response to SRBC by CT when they gave the toxin intravenously along with the SRBC. The latter authors felt that CT may have had an adjuvant by acting as a direct toxin in a manner analogous to endotoxin or via activation of cyclic AMP. Although this may have been the case in our system, the fact that we first gave the cholera toxin at a remote site (subcutaneously) seven days before the administration of intraloop KLH makes a direct toxigenic mechanism seem less likely. The enhancement of the local immune response to KLH may also reflect a general stimulation of helper T-cells or macrophages by the CT. Although its exact mechanism is unclear, the possibility of enhancing local immune responses to various substances, or perhaps live organisms, by subcutaneous priming with small amounts of cholera toxin is intriguing. Finally, it is interesting to speculate that when CT is both the local and systemic inoculant, the overriding feature may be immunogenic suppression via specific suppressor T-cells, leading to reduced local immune response. However, when the locally introduced antigen is another substance (and under some circumstances cholera toxoid might qualify as "another substance") then a non-specific enhancing effect of cholera toxin can become apparent.

III. IgA CONTENT OF SECRETED FLUID AND INTESTINAL EPITHELIUM IN EXPERIMENTAL CHOLERA; COMPARISON WITH NET FLUID PRODUCTION AND GOBLET CELL MUCIN CONTENT.

A. Introduction

IgA is the predominant immunoglobulin in the mucosa and secretions of the gastrointestinal tract, and secretory IgA antibodies against intraluminal organisms and their products, including toxins, have been demonstrated (18). Further, IgA antibodies appear to be important in local immunity to infection, probably acting by: (a) prevention of mucosal colonization by micro-organisms through agglutination and reduction of their adherence to the mucosa; and (b) interference with uptake of microbial products (18,21). The sequence of events in the IgA system in the intestine (Fig. 11) has been delineated: synthesis of IgA by plasma cells in the lamina propria, transport into the storage within crypt epithelial cells following addition of secretory piece, and finally secretion into the lumen (18,19). Little is known, however, about influences upon function of this IgA system, including factors related to infectious diseases of the gastrointestinal tract.

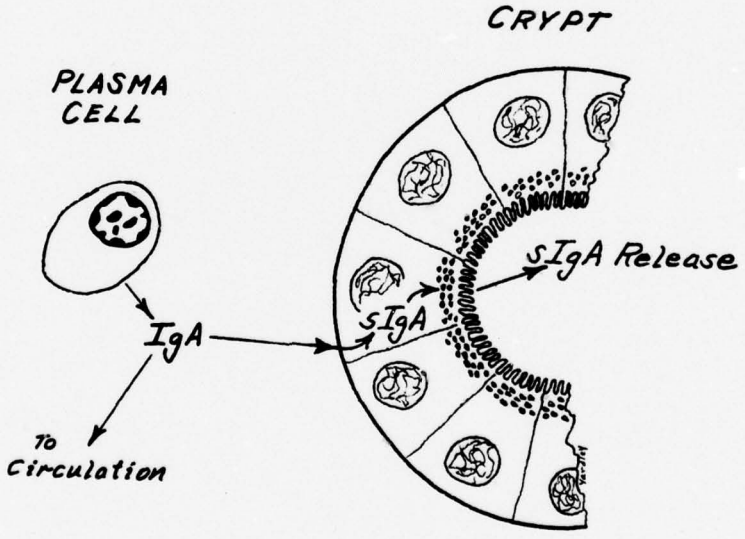


Fig. 11 IgA system in the intestinal mucosa.

Cholera, the diarrheal disease produced by Vibrio cholerae, is mediated by enterotoxin. In experimental studies, exposure of the mucosa of rabbit intestinal loops to cholera toxin (CT) causes secretion of large amounts of mucin-rich fluid. This secretion has a histologic counterpart in the depletion of mucin from epithelial goblet cells (33). Cholera fluid has also been reported to contain large amounts of secretory IgA (34). Therefore, we have studied the effects of cholera toxin upon the IgA system of the intestine. We assessed the IgA content of the secreted fluid by radioimmunoassay and of the crypt epithelium and lamina propria plasma cells by immunofluorescence in rabbit ileal loops exposed to CT, heat-inactivated CT, or saline. These findings were compared with the net fluid production and goblet cell mucin content.

B. Materials and Methods

1) Animals for Combined Output and Tissue Studies:

a) Experimental procedures: Fasted adult female New Zealand white rabbits weighing two to three kg were anesthetized with intravenous sodium pentobarbital. Two loops of distal ileum each approximately 15 to 20 cm in length were isolated with their blood supply intact, and a biopsy of normal ileum was obtained. Silastic tubing (Dow-Corning Corporation Medical Products, Midland, Michigan) with outside diameter (OD) 0.183 in. and inside diameter (ID) 0.132 in. was prepared with a circumferential ring of Silastic Medical Adhesive Silicone Type A at one end. This end was then sutured into the proximal end of each loop. Larger Silastic tubing with OD 5/16 in. and ID 3/16 in. was secured to each of the distal ends and brought out through separate stab wounds in the flanks. The loops were flushed with normal saline until the effluent was clear. The laparotomy incision was closed with towel clips around the tubing from the proximal ends of the loops. Intra-abdominal temperature was restored to 37° C and maintained throughout the experiment by a heat lamp over the animal. Hydration was maintained with intravenous 5% dextrose in normal saline.

A biopsy of each loop was taken immediately prior to the time of instillation of the test solution, designated zero time. The biopsy technique consisted of ligation of the vascular supply to the proximal one to two cm. of the loop, resection of the segment, and re-insertion of a Silastic tube. Loops were then exposed for 30 minutes to the test solutions. Additional biopsies of each loop were taken at 10, 30, 60, 180 and 300 minutes. Fluid in each loop was collected by gentle flushing with air every 30 minutes for the 300 minute duration of the experiment. The collected fluid was weighed and immediately frozen at -20°C. The Silastic tubing in the loops was clamped at both ends except during collection of fluid.

b) Test solutions: In the experimental group of nine rabbits, either the proximal or distal loop was exposed to cholera toxin in the form of 150 mg of freeze-dried, crude culture filtrate of Vibrio cholerae strain B1307 grown in 2% Bacto-peptone broth (Lot No. 001, Wyeth Laboratories, Inc., Marietta, Pennsylvania) in 4 ml bacteriostatic water for injection (Ambot Solution, Cutter Laboratories, Inc., Berkeley, California). This loop was designated the cholera loop. The other loop was exposed to the same preparation

which had been heat-inactivated at 56°C for one hour, and this loop was designated the inactive loop. To evaluate the possible effect of the cholera loop upon the adjacent inactive loop, a control group of four animals was studied. One loop, designated the inactive loop, was exposed to the heat-inactivated CT preparation as in the first experimental group of animals while the other loop, designated the PBS loop, was exposed to four ml of .01 M phosphate buffered saline at pH 7.2 (PBS).

c) Net fluid production: Rate of net fluid production from each loop in each 30 minute collection period was calculated in ul of fluid produced per hour per cm of loop. Data for the first 30 minutes were disregarded because these collections included the test solutions instilled at zero time. Cumulative net fluid production (ul/cm) from each loop throughout the remainder of the experiment was then determined. The difference in rate of and cumulative net fluid production between the two loops in each animal was also calculated for each time period.

d) Histologic and immunohistologic techniques: All biopsy specimens were mounted on chucks in Tissue-Tech II O.C.T. Compound (Lab-Tek Products, Division of Miles Laboratories, Inc., Naperville, Illinois), snap frozen in liquid nitrogen, and stored at -20° C. Frozen sections 6 um thick were later prepared in a cryostat. For assessment of goblet cell mucin, a frozen section slide from each biopsy was fixed in ethyl alcohol-acetic acid-formalin fixative (35) and stained with Alcian blue at pH 2.5 (36). A set of slides consisting of the biopsies from both loops in each animal was coded, and the coded slides were ranked by two simultaneous observers (SRH and JHY) for mucin content of goblet cells. With this system the slides were arranged in order from least to most mucin content and then assigned the corresponding position number which was used for statistical analysis.

IgA content of crypt epithelial cells and lamina propria plasma cells was studied in two sets of frozen section slides from each animal stained with fluorescein isothiocyanate conjugated (FITC) monospecific goat anti-rabbit alpha chain (provided by Dr. John J. Cebra, The Johns Hopkins University, Baltimore, Maryland). The reagent was prepared as described previously (18,19,37). In preliminary studies, serial dilutions of the reagent were tested for maximum sensitivity to differences in IgA content between the cholera and control loops, and a working dilution of 1:40 was selected. Each set of stained slides was coded. Two independent observers (SRH and DFK) ranked the slides for IgA content of the crypt epithelial cells using a Carl Zeiss Standard RA dark field microscope with halogen quartz light source, FITC primary filter (range 460-490 um), and Zeiss 50 secondary filter (Carl Zeiss Inc., New York, New York). A difference between the two observers of more than three rank positions for the same slide was resolved by re-evaluation of the discrepant slides. Position numbers were then assigned in the same manner as for goblet cell mucin. A similar attempt was made to rank IgA content of the plasma cells of the lamina propria.

e) Fluid IgA content: The frozen fluid specimens were thawed at room temperature, mixed, and assayed for radioimmunoassay for IgA. As previously described (20), the assay utilized monospecific goat anti-rabbit alpha chain adsorbed to bromacetyl cellulose (BAC) and ^{125}I labelled goat anti-rabbit Fab. A 10 ul aliquot of an appropriate dilution of each loop fluid was placed in a 10 x 75 mm glass tube containing 100 ul of 1% normal horse serum (NHS) in PBS diluent. Each specimen was run in duplicate at two dilutions. A 300 ul aliquot of a suspension of anti-alpha BAC which contained about 270 ug wet weight of the immunosorbent was added to each tube and the mixture was incubated at 37° C for one hour. One ml of cold 0.15% NHS in PBS was then added to each tube and the tubes were centrifuged at about 2230 x g in a refrigerated centrifuge to form a pellet of the BAC. The supernatant was removed and the process was repeated for two washes. The third pellet was resuspended in 100 ul of ^{125}I anti Fab which contained about 0.02 ug of the reagent with a nominal specific activity of 7 to 10 x 10^5 cpm/ug. After overnight incubation at 4° C, cold 0.15% NHS in PBS was again added to each tube and centrifugation carried out for three washes. The fourth pellet was counted for one minute in a gamma counter.

Concentration of IgA in each specimen fluid in mg/ml was determined from a standard curve of purified trypsin-resistant sIgA derived from rabbit colostrum. The standard curve was relatively linear over a range of 0.06 to 1 x 10^{-3} mg/ml of standard sIgA. The coefficient of variation for the assay was 18% at specimen IgA concentration of about 1.8 mg/ml and 13% at about 3.3 mg/ml. Fluid IgA production (ug/hr/cm) for all 30 minute intervals was calculated from the specimen IgA concentration, specimen volume, and the loop length during the time interval. Cumulative IgA production (ug/cm) for each loop in each animal throughout the experiment was also determined. The difference in IgA concentration, in rate of production, and in cumulative production between the two loops in each animal was calculated for each time period. IgA data from three animals were discarded from statistical analysis because of insufficient specimen volume from the control loops for IgA analysis in five or more of the ten 30 minute collection periods.

2) Animals for Output Studies Only: To evaluate the IgA output from cholera loops in the absence of repeated surgical manipulations and to assure adequate specimen volume for determination of fluid IgA content in all time periods, a third group of four animals was studied. The surgical procedure was similar to the previous animals except that no biopsies were taken during the course of the experiment so that the animal's abdomen was not re-entered. The experimental procedure differed from that followed previously in that following preparation of the loops, two ml of PBS were instilled into each loop at time designated -60 minutes. The PBS was left in the loops for 15 minutes, and then collected, weighed and frozen as previously. This process was repeated three times during the 60 minute control period prior to exposure of the loops to CT at zero time. Then 100 ug of purified CT (Schwarz-Mann Division of Becton, Dickinson Inc., Orangeburg, New York) in 2 ml PBS was placed in one loop, designated the cholera loop, while 2 ml of PBS alone was again placed in the other loop designated the PBS loop. After this 0 to 15 minute period, 15-minute cycles of instillation and flushing of 2 ml of PBS only were resumed in both loops until 120 minutes. For the remainder of the experiment the 2 ml of PBS were left in each loop for 30 minute intervals. The experiment was terminated at 300 minutes after instillation of CT. Net fluid production and fluid IgA content were studied for each collection period as in the previous groups of animals.

3) Statistical analysis: For net fluid production and fluid IgA data, the mean and standard error of the mean (SEM) for loops with the same test solution within each group of animals were calculated for each collection period. Mean differences between the loops within the same group of animals and SEMs were also determined. Log mean rank and SEM were calculated for goblet cell mucin content and crypt IgA content at each biopsy time. In addition, linear regression analysis of the log mean rank values from each type of loop was carried out. Tests for statistical significance included t test for significant difference between means based upon paired data (one-tailed) and evaluation of 95% confidence limits as expressed by two SEM's for mean difference from zero.

C. Results

1) Combined Output and Tissue Studies:

a) Net fluid production: In the experimental animals with cholera and inactive loops, mean rate of net fluid production by the cholera loops showed a consistent increase from 85 ul/hr/cm at 60 minutes to 442 ul/hr/cm at 210 minutes. For the remainder of the experiment it ranged between 347 and 395 ul/cm/hr. In contrast, the mean rate in the inactive loops remained relatively constant at 49 to 102 ul/cm/hr throughout the 300 minute experiment. Mean cumulative net fluid production reflected these marked differences in rate, reaching 1122 ul/cm in the cholera loops at 300 minutes but only 314 ul/cm in the inactive loops (Fig. 12). The mean rate of and mean cumulative net fluid production by the cholera loops were statistically significantly different from the inactive loops at 90 minutes ($p < .025$ for rate of and $p < .05$ for cumulative net fluid production) and onward to the end of the experiment. Further, the mean differences in rate of and cumulative net fluid production between the cholera and inactive loops were also statistically significant at greater than 95% confidence limits for the same period.

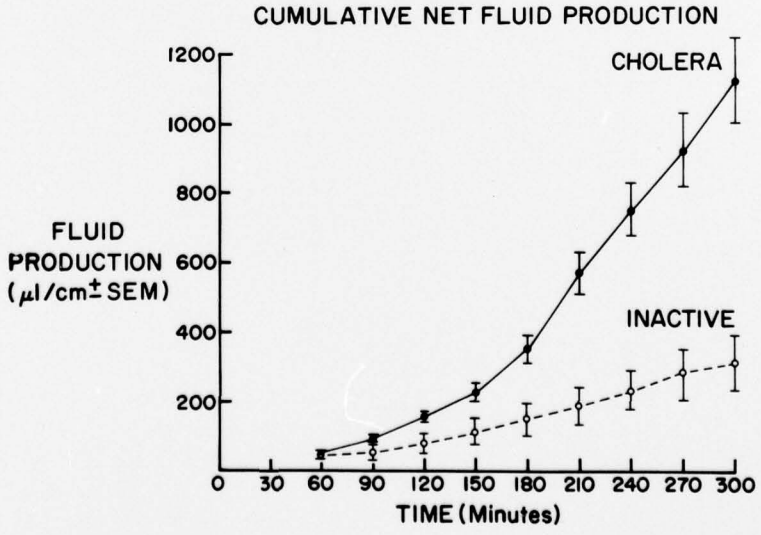


Fig. 12 Cumulative net fluid production in experimental animals with combined output and tissue studies.

In the control animals with inactive and PBS loops, the rates of and cumulative net fluid production were similar to the inactive loops in the experimental animals. Mean rate of net fluid production ranged from 46 to 134 ul/hr/cm in the inactive loops and from 41 to 110 ul/hr/cm in the PBS loops. Mean cumulative net fluid production reached 380 ul/cm in the inactive loops and 378 ul/cm in the PBS loops. There were no statistically significant differences between the loops.

b) Goblet cell mucin: In the experimental animals the mean rank of goblet cell mucin (Fig. 13) showed no consistent changes throughout the experiment in the inactive loops or during the first 60 minutes in the cholera loops. However, at 180 and 300 minutes the mucin in the cholera loops was strikingly reduced with log mean ranks of .48 and .07 respectively, compared with .82 at 0 time ($p < .0025$ at 180 minutes and $p < .0005$ at 300 minutes). In comparison with the inactive loops these reductions were also highly statistically significant ($p < .0005$). In fact, the 300 minute specimen from the cholera loop had the least mucin content in 8 of the 9 animals. Linear regression analysis (Fig. 13) was consistent with the interpretation that goblet cell mucin content remained constant in the inactive loops while it decreased throughout the experiment, beginning shortly after instillation of CT, in the cholera loops.

In the control animals, no significant changes in mean rank of goblet cell mucin occurred in either the inactive or PBS loops, and when compared, the loops showed no significant differences.

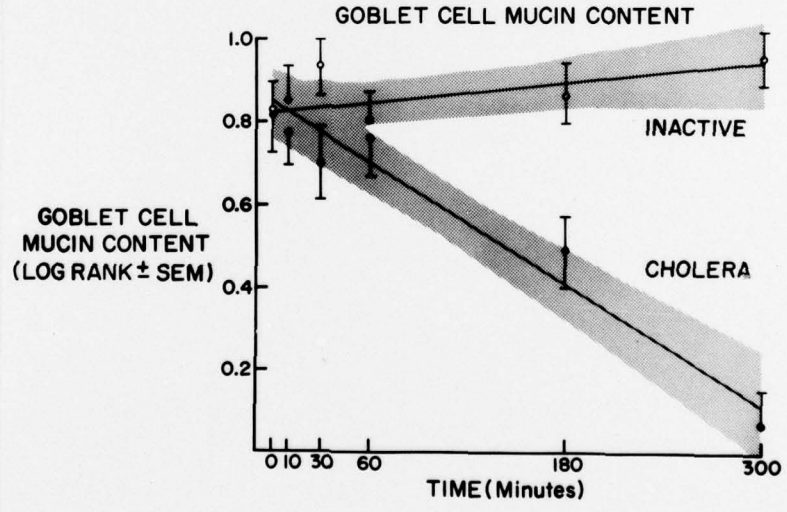


Fig. 13 Goblet cell mucin content in experimental animals with combined output and tissue studies.

c) IgA in crypt epithelial cells and plasma cells: In the experimental animals, no significant changes in the mean rank of crypt epithelial cell IgA occurred during the first 60 minutes in the cholera loops or during the first 180 minutes in the inactive loops (Fig. 14). However, crypt epithelial cell IgA was statistically significantly reduced as compared to zero time at 180 ($p < .05$), and 300 minutes ($p < .0025$) in the cholera loops and at 300 minutes ($p < .025$) in the inactive loops. The reduction in crypt IgA in the cholera loops as compared to the inactive loops (Fig. 15) was statistically significant at 60 ($p < .025$), 180 ($p < .025$), and 300 minutes ($p < .005$). Linear regression analysis (Fig. 14) was consistent with the interpretation that crypt IgA decreased from time of instillation of the test solutions in both cholera and the active loops but decreased more rapidly in the loops exposed to CT.

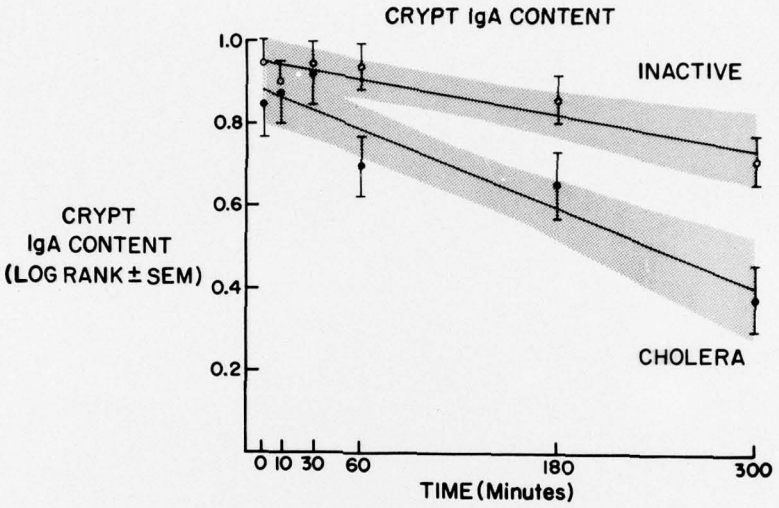


Fig. 14 Crypt epithelial cell IgA content in experimental animals with combined output and tissue studies.

In the control group mean rank of crypt IgA was decreased as compared to zero time at 180 ($< .025$) and 300 minutes ($p < .01$) in the inactive loops and at 10 ($p < .01$), 180 ($p < .025$), and 300 minutes ($p < .01$) in the PBS loops. The loops were not statistically significantly different at any time. Quantitatively, the decrease in crypt IgA content at 300 minutes was similar to that at 300 minutes in the inactive loops of the experimental animals and was less than in the cholera loops.

For IgA content of plasma cells, differences between slides sufficient to establish rank orders were not detected in either the experimental or control groups.

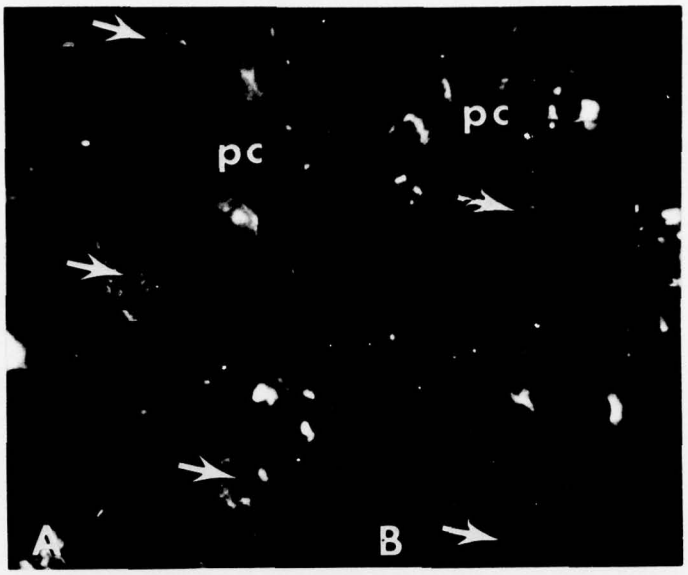


Fig. 15 Immunofluorescent demonstration of IgA in 300 minute specimens from inactive (A) and cholera (B) loops in one experimental animal. Arrows indicate crypt lumina. IgA is depleted from the crypt epithelial cells in the cholera loop. IgA in the cytoplasm of plasma cells (pc) is similar in both loops.

d) Fluid IgA concentration: In the experimental and control groups mean fluid IgA concentrations (Fig. 16) in the 30 minute specimens from cholera, inactive, and PBS loops were very low, less than 1 mg/ml, due to dilution by the test solutions instilled at 0 time. In the experimental group, from 60 minutes until the end of the experiment, mean fluid IgA concentration in the inactive loops ranged between 5.2 and 3.0 mg/ml. The mean concentration in the cholera loops was similar to the inactive loops at 60 and 90 minutes, but it fell commensurate with rising net fluid production to 2.2 mg/ml at 120 minutes, reached 0.7 mg/ml at 210 minutes, and remained less than 1 mg/ml for the remainder of the experiment. The mean fluid IgA concentration in the cholera loops was statistically significantly different from the inactive loops at 180 minutes ($p < .0025$) and onward to the end of the experiment. Further, the mean difference between the cholera and inactive loops was statistically significant at the same times.

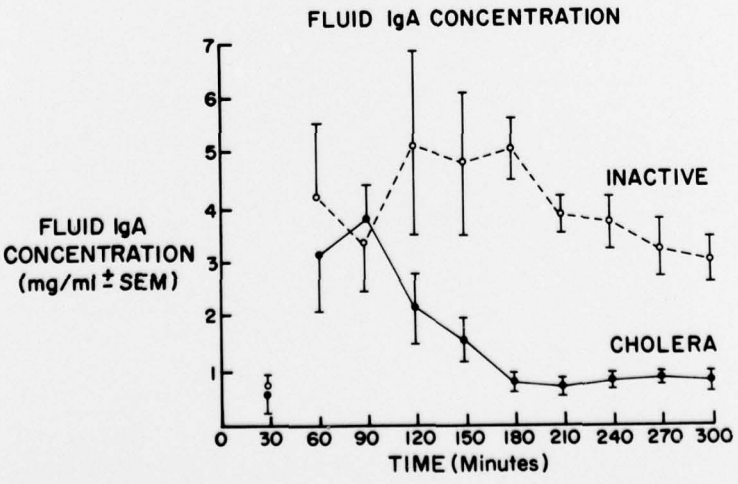


Fig. 16 Fluid IgA concentration for experimental animals with combined output and tissue studies.

In the control group, mean fluid IgA concentration was 2.9 to 6.9 mg/ml in the inactive loops and 2.4 to 5.8 mg/ml in the PBS loops. There were no statistically significant differences between the loops and the inactive loops in the experimental animals.

e) Rate of IgA production: In the experimental group, mean rate of IgA production fluctuated widely between 217 and 403 ug/hr/cm in the cholera loops and between 258 and 457 ug/hr/cm in the inactive loops. The loops showed no statistically significant differences. This finding suggested that the increased fluid production by the cholera loops (Fig. 12) was not accompanied by increased IgA production.

In the control group, mean rate of IgA production again showed great variation, from 194 to 492 ug/hr/cm in the inactive loops and 192 to 380 ug/hr/cm in the PBS loops, but there were no statistically significant differences between the loops. The rates were similar to the inactive loops in the experimental animals.

f) Cumulative IgA production: In the experimental group, mean cumulative IgA production (Fig. 17) reflected the lack of differences in rate of IgA production between the cholera and inactive loops and rose in a similar fashion in both types of loops. Mean cumulative IgA production reached 1629 ug/cm at 300 minutes in the cholera loops and 1668 ug/cm in the inactive loops.

The findings in the control group were similar to the experimental groups as mean cumulative fluid IgA production reached 1756 ug/cm at 300 minutes in the inactive loops and 1512 ug/cm in the PBS loops.

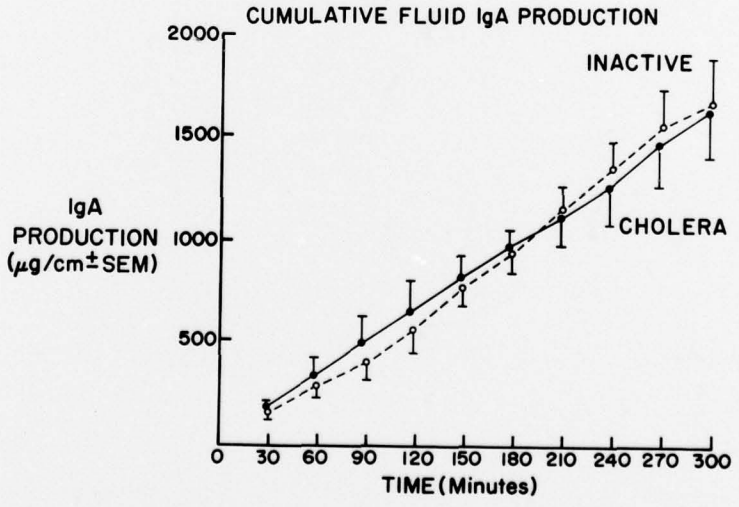


Fig. 17 Cumulative fluid IgA production in experimental animals with combined output and tissue studies.

2) Output Studies Only:

a) Net fluid production: In the third group of animals with undisturbed cholera and PBS loops, the pattern of net fluid production was different from the other groups because PBS was instilled into the loops during each collection time. Both types of loops showed net absorption, as indicated by the negative values, during the initial time periods. Mean rate of net fluid production was -152 to -12 ul/cm/hr in the cholera loops and -141 to -32 ul/cm/hr in the PBS loops between -60 and 75 minutes. From 90 minutes onward net fluid secretion occurred in the cholera loops, reaching 609 ul/cm/hr at 300 minutes, while the PBS loops showed net absorption or slight net fluid secretion. Mean rate of net fluid production by the cholera loops was statistically significantly different from the PBS loops at 105 minutes ($p < .025$), 150 minutes ($p < .05$), 210 minutes ($p < .01$), and onward to the end of the experiment. Mean difference between the cholera and PBS loops was statistically significant during the same time periods.

Mean cumulative net fluid production (Fig. 18) declined between -60 and 60 minutes in the cholera loops and between -60 and 120 minutes in the PBS loops. The cholera loops showed a plateau between 75 and 120 minutes and then a rapid increase which reached net secretion at 210 minutes. Mean cumulative net fluid production was 635 ul/cm at 300 minutes. On the other hand the PBS loops showed a plateau from 150 to 240 minutes until a slight increase occurred during the remaining two time periods. Mean cumulative net fluid production at 300 minutes was -194 ul/cm. Cumulative fluid production by the cholera loops was statistically significantly different from the PBS loops at 240 minutes ($p < .05$) and onward, and mean difference between the cholera and PBS loops was statistically significant at the same times.

b) Fluid IgA concentration: Mean fluid IgA concentration ranged from 0.8 to 1.8 mg/ml prior to instillation of CT at zero time in the cholera loops and from 1.2 to 1.9 mg/ml in the PBS loops during the same time period. During the experimental period from 0 to 300 minutes, mean fluid IgA concentration was between 1.0 and 2.7 mg/ml in the cholera loops and between 1.2 and 2.8 mg/ml in the PBS loops. Both loops showed a consistent trend of rising mean fluid IgA concentration with time, and there were no statistically significant differences between the loops. These findings were in striking contrast to the animals with combined output and tissue studies in which IgA concentration decreased markedly with rising net fluid production in the cholera loops.

c) Rate of IgA production: Mean rate of IgA production again varied widely as it had in the animals with output and tissue studies. In the cholera loops, mean rate varied from 406 to 995 ug/hr/cm between -60 and 0 time while in the PBS loops it was between 427 and 953 ug/hr/cm. Between 0 and 300 minutes, the cholera loops had mean rate of IgA production between 676 and 2354 ug/hr/cm and showed a trend of increasing values with time. During the same time mean rates of the PBS loops varied from 596 to 1127 ug/hr/cm with no trend of the fluctuating values. Because of the variability and small number of animals, the difference did not reach between the means statistical significance but did approach it ($p < .1$ at 240 and 300 minutes).

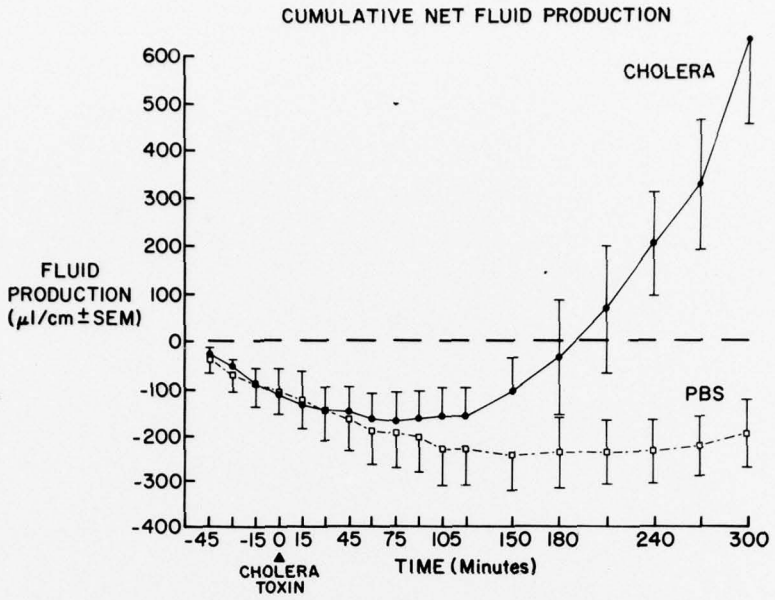


Fig. 18 Cumulative net fluid production in animals with undisturbed loops for output studies only.

However, mean difference between the cholera and PBS loops was statistically significant at 300 minutes. These results were again in contrast to the animals with output and tissue studies.

d) Cumulative IgA production: Mean cumulative IgA production (Fig. 19) for the 60 minutes preceeding instillation of the test solution containing CT was 650 ug/cm in the loops which were to receive CT in PBS and 681 ug/cm in those to receive only PBS. By the end of the experimental period at 300 minutes, the cholera loops had produced 6216 ug IgA/cm while the PBS loops had reached 4646 ug/cm. The differences between the means did not quite reach statistical significance ($p < .1$ at 300 minutes) and mean difference between the cholera and PBS loops also approached statistical significance at 300 minutes. The findings contrasted with those of the animals with output and tissue findings in that the cholera loops showed a tendency to produce more IgA than the noncholera PBS loops, and secondly that cumulative IgA output for both type of loops was three to four times that of the animals with output and tissue studies.

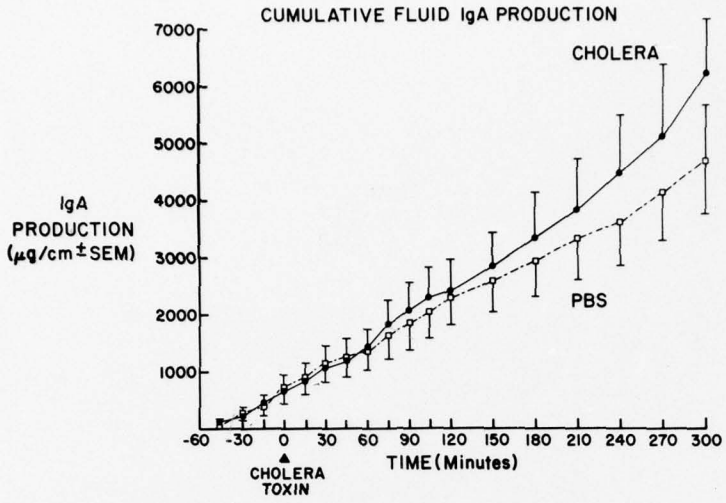


Fig. 19 Cumulative fluid IgA production in animals with undisturbed loops for output studies only.

D. Discussion

Net fluid production from the intestine represents the net effect of fluid secretion, which is stimulated by CT, and reabsorption (38). In our animals with output and tissue studies, goblet cell mucin content declined as net fluid production increased in the CT-exposed loops. On the other hand, mucin was unchanged in the inactive and PBS loops which had no stimulus to secretion. These findings confirm earlier work (33) which indicated that goblet cell mucin depletion is the morphologic counterpart of enhanced fluid secretion in this model of experimental cholera.

The relationship of crypt epithelial IgA, plasma cell IgA, fluid IgA production and net fluid production is apparently much more complex. Crypt epithelial IgA declined both in loops with CT-induced secretion and in unstimulated inactive and PBS loops, although the loss of epithelial IgA occurred earlier and was greater in the cholera loops with increased net fluid production. Plasma cell IgA showed no differences. In the fluid itself IgA concentration is dependent upon net fluid production as well as IgA secretion. In our animals with output and tissue studies in which a drainage procedure was used for collection of fluid, no increase in IgA production was found in the cholera loops as compared to inactive and PBS loops, and IgA concentration fell with increasing net fluid production in the cholera loops. By contrast, in the animals with only output studies in which a washout technique was used for fluid collection, IgA concentration did not fall with rising net fluid production in the cholera loops and fluid

IgA production appeared to be somewhat increased as compared to the PBS loops. Although a number of interpretations are possible, it may be that incomplete collection of secreted fluid with the drainage method used in the animals with output and tissue studies accounts for the failure to find an increase in fluid IgA production which would correspond to the increased loss of epithelial IgA in the cholera loops. Since epithelial IgA content is a function of uptake into the cells as well as release from them, an alternative explanation is that CT interfered with some steps leading to reaccumulation of epithelial IgA in this model. Clearly, additional studies should be carried out to sort out the various possibilities.

The effect of CT, and enterotoxins of other organisms, on the IgA system of the intestine has practical implications. Antibodies of the IgA isotype directed against components of the organisms as well as against the toxins themselves, could modify the events occurring after exposure of the host to the organism (18, 19, 21; Section I-C-4). Therefore, we were particularly interested in the epithelial and fluid IgA findings within minutes after exposure of the loops to CT as early release of antibody could represent a host defense mechanism. However, there was no evidence of such early release of IgA from the epithelium into the fluid in either of our study systems in unimmunized animals. Investigations of immunized animals would also be of interest.

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