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Local (Immunoglobulin A) Immune Response by the Intestine to Cholera Toxin and Its Partial Suppression with Combined Systemic and Intra-Intestinal Immunization

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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 CT. Fluid from the loops and sera were tested for neutralization of CT by the blueing test and for relative content of isotype-specific (immunoglobulins A [IgA] and G [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 values. The chronic and acute T-V loops showed protection against fluid production after exposure to CT in systemically and locally immunized animals. However, IgG anti-CT usually appeared in both loops; 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.

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 the protective effects of local immunity. After its release into the lumen during natural infection with *Vibrio 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 (25).

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 (2, 10-12, 14-16, 18, 19, 25). However, CT can also be presented directly to the small intestine by using chronically isolated Thiry-Vella (T-V) loops (21, 22, 24). Whereas chronic T-V loops are clearly not a completely natural in vivo system, they offer several important advantages for investigating local immune phenomena. (i) Repeated intraluminal inoculations with antigen can be performed easily so the variables of antigen preparation, dose, and administration can be readily examined. (ii) The isolated loops permit detailed serial study of intestinal secretions from undisturbed, intact mucosa without the possible variable of protein degradation by pancreatic pro-

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teases. (iii) The T-V loops permit study of other fundamental aspects of local immunity, such as the role played by Peyer's patches. (iv) 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 by intraloop (i.l.), subcutaneous (s.c.), and combined i.l.-s.c. inoculation. Sera and secreted fluid from the loops were tested for neutralization of toxin (by the blueing test) and for immunoglobulin A (IgA) and immunoglobulin G (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.

(This work was presented in part at the annual meeting of the American Gastroenterological Association, Miami Beach, Fla., 25 May 1976 [26]).

MATERIALS AND METHODS

Preparation and care of loops. With a previously described technique (17), an isolated 20-cm loop of distal ileum containing a Peyer's patch was prepared in outbred New Zealand white rabbits of mixed sex weighing 3 to 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 that no histological alterations occur in lymphoid tissue, including Peyer's patches (17).

Immunization of animals and specimen preparation. Rabbits were inoculated s.c. and i.l., or both, with purified cholera toxin (Geographic Medicine Branch, National Institute of Allergy and Infectious Diseases, Bethesda, Md., or Schwarz/Mann Div. of Becton, Dickinson & Co., Orangeburg, N.Y.). For i.l. inoculations, 100 μg of CT in 4 ml of 0.01 M neutral phosphate-buffered saline (PBS) was placed in the loop, and the distal (efferent) tube was clamped for 24 h. For s.c. inoculations, 30 μg of CT in 1 ml of PBS was used.

Two series of experiments were conducted (series A and B); the group designations and inoculation schedules are summarized in Table 1. In both series, weekly i.l. inoculations were begun 3 days after loop preparation, and s.c. inoculations were spaced 3 weeks apart. In series A, four i.l. inoculations were used, and s.c. inoculations were begun 1 week before the time for initial i.l. inoculations. 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 in that it used only three i.l. inoculations and began s.c. inoculations 3 weeks before initial i.l. inoculation. Series B thus provided study of fewer i.l. inoculations and earlier s.c. inoculations, and permitted examination of loop fluid for antibodies for a longer period after the last i.l. dose of CT.

TABLE 1. *Immunization schedules*

Experimental group[a]	Cholera toxin inoculations[b]	
	i.l.	s.c.
Series A		
i.l. (5)	days 0, 7, 14, 21	none
s.c. (6)	none	days −7 and 14
i.l. + s.c. (6)	days 0, 7, 14, 21	days −7 and 14
Controls (5)	none	none
Series B		
i.l. (7)	days 0, 7, 14	none
s.c. (8)	none	days −21 and 0
i.l. + s.c. (6)	days 0, 7, 14	days −21 and 0
Controls (6)	none	none

[a] Numbers of animals in parentheses.
[b] Day 0 was day of first i.l. inoculation.

Loop fluids were collected on the day of first i.l. inoculation and every 3 to 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 that were used for all studies. Blood for serum was collected before the first inoculation and at weekly intervals thereafter.

Skin test for neutralizing capacity against CT. The method of assay for neutralizing capacity against CT was similar to the one described by Benenson et al. (1). Sera diluted 1:10, 1:100, 1:400, and 1:1,600 and fluid specimens diluted 1:4, 1:16, 1:64, 1:256, and 1:1,024 were mixed with equal volumes of PBS with 0.02% gelatin containing 2 to 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°C for 30 min, 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 was given intravenously, 0.12 ml/100 g of body weight. Neutralization end point was the highest dilution that showed no blueing or greater than 50% reduction in average wheal diameter, or both. Geometric mean titers and their standard errors (standard error of the mean) were calculated for comparable collection times.

Materials and reagents for ELISA for antibodies to CT. The procedures for the enzyme-linked immunosorbent assay (ELISA) were modified from those developed by Engvall and Perlmann (8) and applied to CT by Holmgren and Svennerholm (13). Polystyrene tubes (type 2052, Falcon Plastics, Rutherford, N.J.) were exposed at 37°C for 3.25 h to 1 μg of purified CT contained in 0.5 ml of PBS plus 0.02% sodium azide. These CT-coated tubes were stored at 4°C; fluid was not removed 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.). Goat anti-rabbit IgG and goat anti-rabbit IgA for conjugation to alkaline phos-

phatase were provided by John Cebra and Stella Robertson. These had been prepared by immunizing goats with the Fc portion of rabbit serum IgG or with colostrum-derived secretory IgA that had been "washed" with guanidine. Goat sera were purified to their IgG fraction by ammonium sulfate precipitation followed by diethylaminoethyl-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 immunoglobulin M (IgM) molecules.

To conjugate the goat anti-rabbit IgG and anti-rabbit IgA to alkaline phosphatase, 2 mg of the goat IgG in 0.1 to 0.2 ml of 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% [wt/wt], Fisher) was added to the goat anti-Ig-phosphatase mixture at a concentration that yielded a final concentration of 0.07% glutaraldehyde. The coupling reaction was allowed to take place for 2 h at room temperature and caused the solution to become slightly cloudy. Dialysis against PBS was followed by dilution to 5 ml in tris(hydroxymethyl)-aminomethane 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 μm); 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 μg of purified rabbit IgA, IgG, or IgM (supplied by John Cebra and 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 h. Both the anti-IgA- and anti-IgG-phosphatase conjugates were found to be highly specific, showing only minimal cross-reaction with other isotypes.

ELISA test procedure. All steps were conducted at room temperature on a horizontal rotary shaker set at low speed (30 to 60 rpm), and tubes were rinsed in three changes (twice in 1 ml and once in 5 ml) of PBS-Tween, with 5 min between each step. After initial rinsing, the CT-coated tubes were exposed for 6 h to 0.5-ml samples of fluid supernatant or serum appropriately diluted in PBS-Tween. Tubes were next exposed for 16 h 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 the addition of 1 mg of nitrophenyl phosphate substrate (type 104, Sigma) in 1 ml of 0.05 M carbonate buffer (pH 9.8) containing 0.001 M magnesium chloride. The reaction was stopped at 1 h 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 1:1,600. 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 anti-CT 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 with the control specimens, were about 8%.

Challenge with CT at termination of experiments. Challenge with CT was conducted on the final day of each experiment (days 24 to 28 in series A and day 35 in series B).

In series A animals, and in the s.c. group from series B, the peritoneal cavity was reentered. 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 to 20 cm was prepared from intact intestine near the original anastomotic site in the ileum. The purpose of the acute loop was to determine whether 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 of PBS. The toxin-containing solutions were removed 0.5 h later, and fluid production by each loop was monitored for an additional 4.5 h by expelling the fluid with air at 0.5-h intervals. Fluid specimens collected from h 3 to 5 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 2 h of the experiment was calculated as microliters per centimeter per hour.

A second series of challenge experiments used four groups of animals: i.l. animals from the series B experiments, i.l. plus s.c. animals from series B, five rabbits (designated s.c.-challenge) given 30 μg of CT on days −7 and +14 and followed for 28 days in the same manner as s.c. animals from series A, and six 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 of crude cholera toxin (lot 001, Wyeth) in 3 ml of PBS for 0.5 h. Fluid was thereafter collected from the loops at 0.5-h intervals for another 5.5 h. Fluid obtained between the 2nd and 4th hours was pooled and assayed by ELISA for IgA and IgG anti-CT. Fluid outputs (in microliters per centimeter per hour) for h 4 through 6 were calculated after measuring loop lengths.

RESULTS

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 (i.l. and i.l. plus s.c. groups) than in rabbits inoculated only s.c. (s.c. group; Fig. 1). Indeed, mean titers for loop fluids never rose above 1:4 in the s.c. group. Mean titers for fluids in the i.l. plus s.c. group tended to be lower than in the i.l. group, and most of the high titers (>1:64) were in animals from the i.l. group.

Serum antitoxin titers (Fig. 1) in series A showed patterns of elevation that 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 (s.c. and i.l. plus s.c. groups). Mean serum titers for the i.l. group showed a slower rise, but from day 21 onward the serum titers were not significantly different among the three groups.

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 the tests for 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 i.l. animals in series A. On the other hand, very little IgA anti-CT was found in fluids from s.c. animals (Fig. 3). Immunization by combined i.l. and s.c. inoculation (i.l. plus s.c. group) led to mean ELISA values for IgA anti-CT in fluid that were higher than those seen with s.c. immunization, but from day 10 onward the values for IgA anti-CT were consistently lower in the i.l. plus s.c. group than in the i.l. 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 for IgA anti-CT were obtained by ELISA at all three immunization schedules.

In fluids from series B, IgA anti-CT was detectable after two i.l. inoculations of CT and was sustained for 3 weeks after the last (third) inoculation (Fig. 4). Furthermore, s.c. inoculation alone with CT, beginning 2 weeks sooner than in series A, still led to only slight elevation in mean fluid IgA anti-CT levels. However, in the i.l. plus s.c. group, suppression of IgA anti-CT was more striking than in series A, because IgA anti-CT levels rose only slightly more than in s.c. animals. As in series A, mean serum IgA anti-CT did not rise significantly in series B.

IgG anti-CT levels in series B animals were low in fluids from all experimental groups and elevated in sera from s.c. and i.l. plus s.c. animals (Fig. 5). However, levels of serum IgG anti-CT from i.l. plus s.c. animals were significantly lower than in the counterpart series A group. No reason for this finding could be determined.

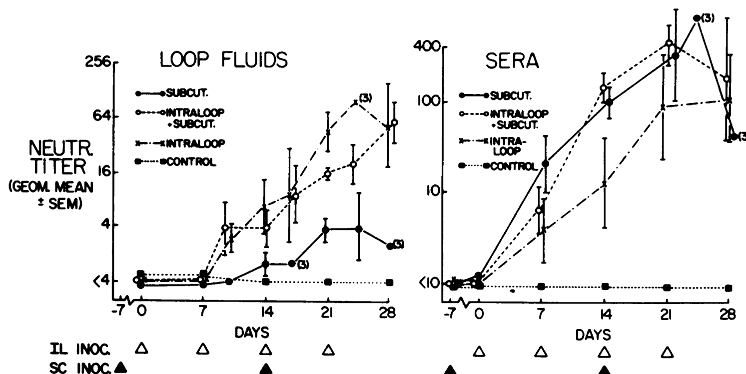

FIG. 1. *Series A experiments: capacity of fluids and sera to neutralize cholera toxin by blueing test.*

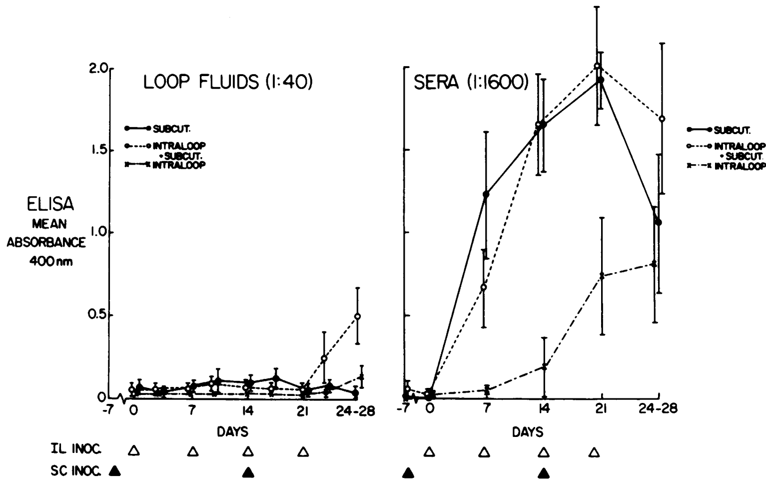

FIG. 2. *Series A experiments: Relative content of IgG anti-CT in fluids and sera as indicated by ELISA.*

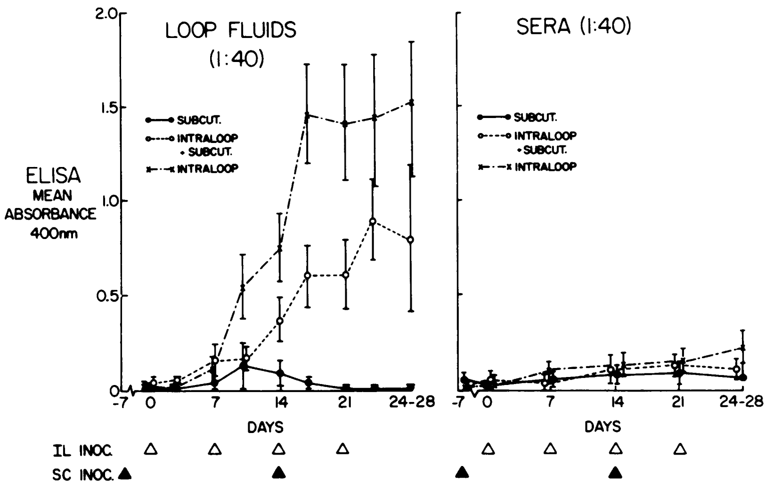

FIG. 3. *Series A experiments: Relative content of IgA anti-CT in fluids and sera as indicated by ELISA.*

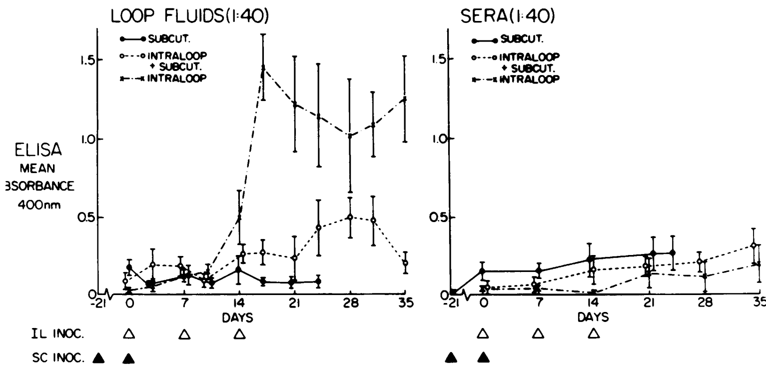

FIG. 4. *Series B experiments: Relative content of IgA anti-CT in fluids and sera as indicated by ELISA.*

Correlation of skin tests with IgA anti-CT. Individual neutralizing capacities (measured 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).

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 of crude toxin are shown in Table 2 along with ELISA values for the fluids. Irrespective of the route of immunization, levels of fluid output from both chronic and acute loops for experimental groups were below those noted for controls. This consistent pattern of reduction in output probably resulted from immunity to CT, but the reductions were not necessarily relevant to local (IgA-mediated) immunity. Whereas only i.l. plus s.c. 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.

ELISA data on challenge fluids from the acute loops in i.l. and i.l. plus s.c. 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

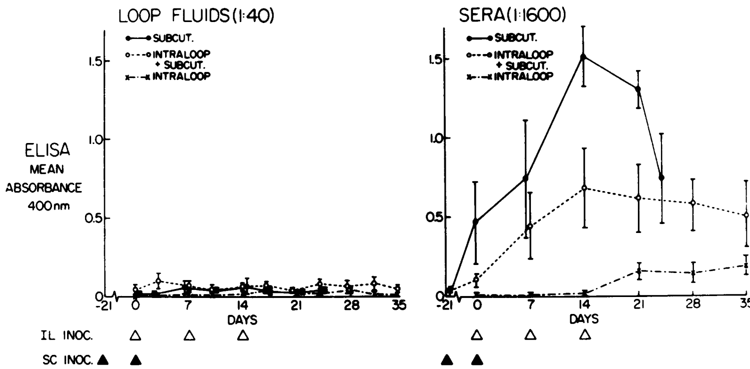

FIG. 5. *Series B experiments: Relative content of IgG anti-CT in fluids and sera as indicated by ELISA.*

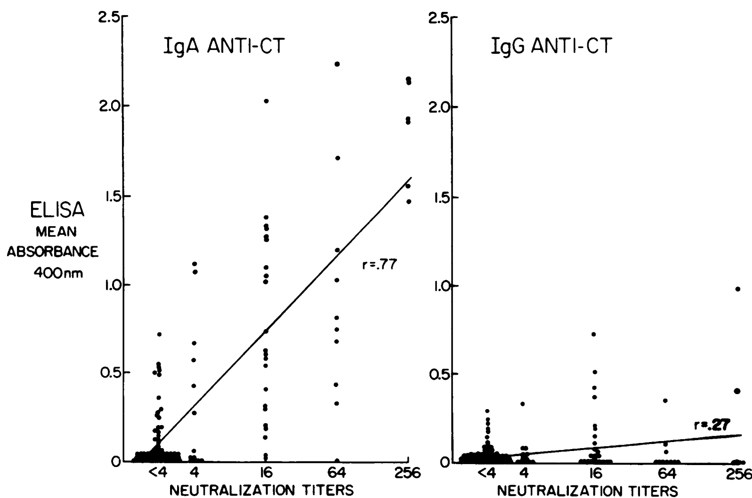

FIG. 6. *Comparison of neutralization titers with corresponding IgA anti-CT and IgG anti-CT ELISA values for all fluids in series A experiments. Absorbance values could not be directly compared because of different assay systems used. Only IgA anti-CT shows a high correlation coefficient.*

be assayed in only one i.l. animal, however, because an insufficient volume was secreted from the others.)

Table 3 shows fluid outputs and ELISA values after challenge of undisturbed chronic T-V loops with a larger amount (50 mg) of crude toxin in order to enhance output. Mean output from i.l. animals was significantly below that noted in controls ($P < 0.05$). On the other hand, i.l. plus s.c. and s.c.-challenge animals showed mean outputs that were lower than but 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 i.l. plus s.c. and s.c.-challenge groups, even though mean serum levels in those animals were elevated. Thus, only the i.l. 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.

DISCUSSION

In these studies, intra-intestinal immunization of chronically isolated (T-V) 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 the 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* [4, 5, 23] and live *Shigella* [D. F. Keren, P. S. Holt, and H. H. Collins, unpublished data]) were inoculated into T-V loops of the type used here,

TABLE 2. *Challenge experiments—chronic and acute loops*

Experimental group	Mean fluid output ± SEM[a] (μl/cm per h)		Anti-CT isotype	Fluid anti-CT[b]			Serum anti-CT[c]
	Chronic loop	Acute loop		Chronic loop (final day)	Chronic loop (challenge)	Acute loop	
i.l. (series A)	57 ± 18	37 ± 13	IgA	1.53 ± 0.36	0.88 ± 0.51	0 (n = 1)	0.23 ± 0.09
			IgG	0.15 ± 0.08	1.17 (n = 2)	0.09 (n = 1)	0.83 ± 0.35
i.l. + s.c. (series A)	70 ± 24	87 ± 37	IgA	0.96 ± 0.27	0.70 ± 0.25	0.06 ± 0.06	0.09 ± 0.05
			IgG	0.39 ± 0.14	0.58 ± 0.25	0.62 ± 0.26	1.69 ± 0.46
s.c. (series A)	73 ± 10	65 ± 17	IgA	0.01 ± 0.01	0.01 ± 0.01	0	0.14 ± 0.08
			IgG	0.03 ± 0.02	0.83 ± 0.28	0.49 ± 0.29	1.06 ± 0.43
s.c. (series B)	39 ± 13	59 ± 11	IgA	0.08 ± 0.04	0.05 ± 0.03	0.05 ± 0.02	0.26 ± 0.11
			IgG	0.04 ± 0.01	0.35 ± 0.01	0.65 ± 0.21	0.74 ± 0.28
Controls (series A)	138 ± 31	157 ± 19	IgA	0	0	0	0
			IgG	0	0	0	0

[a] SEM, standard error of the mean.
[b] Mean ELISA absorbance ± SEM at a dilution of 1:40.
[c] Mean ELISA absorbance ± SEM at dilutions of 1:40 (IgA) and 1:1,600 (IgG) on final day.

TABLE 3. *Challenge experiments—undisturbed chronic loops*

Experimental group	Mean fluid output ± SEM[a] (μl/cm per h)	Anti-CT isotype	Fluid anti-CT[b]		Serum anti-CT[c]
			Final day	Challenge	
i.l. (series B)	97 ± 26	IgA	1.26 ± 0.28	0.58 ± 0.21	0.19 ± 0.12
		IgG	0.02 ± 0.01	0.03 ± 0.02	0.19 ± 0.06
i.l. + s.c. (series B)	151 ± 34	IgA	0.21 ± 0.06	0.10 ± 0.06	0.31 ± 0.11
		IgG	0.04 ± 0.02	0.01 ± 0.01	0.51 ± 0.21
s.c.-challenge	171 ± 21	IgA	0.04 ± 0.01	0.02 ± 0.02	0.02 ± 0.01
		IgG	0.21 ± 0.12	0.06 ± 0.02	1.87 ± 0.22
Controls (series B)	215 ± 18	IgA	0	0	0
		IgG	0	0	0

[a] SEM, standard error of the mean.
[b] Mean ELISA absorbance ± SEM at a dilution of 1:40.
[c] Mean ELISA absorbance ± SEM at dilutions of 1:40 (IgA) and 1:1,600 (IgG) on final day.

specific antibody (mainly IgA) also appeared rapidly in the fluid. Furthermore, serum antibody response in those studies 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 *V. cholerae* and its products could be important (9), studies on cholera immunity have tended until recently to focus strongly on systemic immunization and serum antibodies; 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 (7, 12, 14, 15, 21), 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. (15) studied mucosal scrapings, so that serum antibodies were surely present in their test material. Also, in earlier cross-circulation experiments in which passive protection was examined (7) and in previous studies using intestinal loop assay in immunized animals (12, 14), 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 that 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 animals other than rabbits. In dogs, passive protection against CT in chronic T-V loops was achieved with intravenous injections of the IgG fraction of immune serum (21). Enhanced resistance obtained with combined oral and systemic immunization in dogs (19, 22) 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 biological effects of CT. Although IgM anti-CT levels were not investigated in the present experiment, rabbits normally demonstrate only small amounts of IgM in their intestinal mucosa and secretions (6, 15), and, in other studies using the present model, only low levels of IgM antibody were thought to be formed against the antigens used (23).

Since previous studies have suggested that combined intra-intestinal and parenteral immunization can enhance local immunity (18, 19, 22), 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 suggested that suppression occurred even when timing of the s.c. inoculation in relation to i.l. inoculations was varied and that the suppressive effect was sustained after final i.l. inoculation.

The suppression seen with combined i.l. and s.c. immunization can be viewed as having either a toxigenic or an immunogenic basis. Cholera toxin can act directly on lymphocytes to cause reduced production of immunoglobulin (3); this toxigenic effect has been demonstrated both in vivo and in vitro. It probably proceeds via the adenyl cyclase system (3). On the other hand, if the suppression of local (IgA) immunity in our studies was immunogenic, then it presumably occurred via suppressor T-cells (20). The present data do not provide a firm basis for choice between a toxigenic or immunogenic mechanism for suppression of IgA anti-CT in loops, but the relative nondependence of suppression on the 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 (unpublished data). While the true significance of the suppression 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.

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