INFECTION AND IMMUNITY, July 1978, p. 185-193
0019-9567/78/0021-0185$02.00/0

Vol. 21, No. 1
Printed in U.S.A.

Induction of a Mucosal Antitoxin Response and Its Role in Immunity to Experimental Canine Cholera

NATHANIEL F. PIERCE,* WILLIAM C. CRAY, JR., AND BIMAL K. SIRCAR†

Departments of Medicine of Baltimore City Hospitals and the Johns Hopkins University School of Medicine, Baltimore, Maryland 21224

Received for publication 27 March 1978

The induction of a jejunal antitoxin response was studied in dogs immunized with cholera toxin or toxoid. Single doses of toxoid given subcutaneously (s.c.) or of toxin given intraluminally (i.l.) were each effective in priming the mucosal immune system, whereas toxoid given i.l. was much less effective. In contrast, toxin and toxoid given i.l. were each effective as booster antigens. The local secondary response was rapid and brief, the peak occurring at about 7 days after i.l. boosting and declining by 90% after 2 more weeks. After s.c. priming and i.l. boosting with toxoid, antitoxin-containing plasma cells appeared predominantly in the portion of jejunum exposed to the i.l. booster. The appearance of antitoxin-containing plasma cells in jejunal lamina propria correlated with the amount of antitoxin recovered in jejunal washings which, in turn, correlated with protection against challenge with cholera toxin. Thus, lamina propria antitoxin-containing plasma cells appeared to be the source of protective antitoxin. However, after sequential s.c.-oral immunization with toxoid, protection against challenge with *Vibrio cholerae* far outlasted the major systemic and local antitoxin responses and was not obviously explained by either. These studies reveal methods for induction of a mucosal antitoxin response, but leave in question the mechanism of prolonged protection induced by s.c.-oral immunization of dogs.

Immunization with cholera toxoid or toxin can protect dogs and other experimental animals against intestinal challenge with cholera toxin or viable *Vibrio cholerae* (5, 10, 17, 20). This protection is presumed to reflect neutralization of cholera toxin by antitoxin before the toxin binds irreversibly to the intestinal mucosa and induces fluid secretion. For this to occur, antitoxin must be effective at the mucosal surface or within the intestinal lumen.

Intestinal antitoxin either can be serum derived, by passive transfer to the gut lumen (8, 18), or can be made by lamina propria plasma cells and actively secreted to the mucosal surface (16, 28). Serum-derived antitoxin appears to be entirely responsible for the protection of dogs after repeated parenteral immunization with cholera toxin or toxoid (20). The protective role of local secretory antitoxin and how it is best induced are less well understood. Of particular interest is the duration of protection conferred by the mucosal immune system. Two prior studies suggest that a mucosal antitoxic response is induced in dogs by repeated local exposure to cholera toxin or to a cholera toxoid with residual toxin (19, 27), but the duration of this response appeared to be brief. More recently, we have shown that subcutaneous (s.c.) priming and repeated oral boosting of dogs with purified toxoid induces relatively prolonged protection without sustained elevations in the titer of serum antitoxin (20). It was suggested that this immunization sequence also induced an antitoxin response in the lamina propria of the small bowel, which accounted for the protection. This possibility was not examined directly, however, and the mechanism by which the protection was prolonged was not explained.

We now report direct studies of the antitoxic response in canine intestinal mucosa. Several immunizing regimens have been studied, and the role of mucosal antitoxin in immunity to experimental canine cholera has been evaluated. The results show that antitoxin production in the lamina propria of the small bowel is induced by sequential parenteral-intrajejunal immunization with cholera toxoid or by repeated intraluminal (i.l.) exposure to cholera toxin. Repeated i.l. exposure to inactive toxoid was much less effective. The kinetics of the mucosal antitoxin response are described, and evidence is presented that antitoxin-producing plasma cells in jejunal lamina propria can be a source of protective secretory antitoxin. The results leave in ques-

† Present address: Cholera Research Centre, Calcutta 16, India.

tion, however, the mechanism of the prolonged protection observed after sequential s.c.-oral immunization of dogs with purified toxoid.

MATERIALS AND METHODS

Animals. Dogs were healthy mongrels weighing 8 to 20 kg. Thiry-Vella loops were made from 65 cm of jejunum beginning immediately distal to the ligament of Treitz and were allowed to heal at least 10 days before study. In some dogs, two loops were constructed to permit comparison of the immune response in adjacent segments of jejunum.

Cholera toxin and toxoids. Cholera toxin and toxoids were provided by Carl Miller, National Institute of Allergy and Infectious Diseases. Purified toxoid lots 11201, 20301, and 20401 made by Wyeth Laboratories were used. These toxoids show no reversion in vivo or in vitro to active cholera toxin. Each was made by inactivation of purified toxin (from *V. cholerae* Inaba 569B) with 200 mol of glutaraldehyde per mol of toxin at 30°C for 120 h (22). Lots 20301 and 20401 had an additional membrane filtration step before glutaraldehyde treatment to remove the last traces of bacterial somatic antigen (23). Toxoids were lyophilized with phosphate-buffered saline (pH 7.8) and 0.05 mg of thiomersal per 100 μg of toxoid, except lot 20401 which lacked thiomersal. On the day of use, toxoids were rehydrated to a concentration of 100 μg/ml. Toxoid given by the oral or i.l. route was rehydrated with sterile water. A protamine sulfate-aluminum chloride diluent was used for parenteral toxoid. It contained 3.75 mg of aluminum chloride per ml, 0.5 mg of protamine sulfate per ml, and 0.05 mg of thiomersal per ml and yielded toxoid bound completely to an aluminum phosphate gel (called precipitated toxoid [22]). Crude cholera toxin was a lyophilized culture filtrate of *V. cholerae* Inaba 569B (NIH lot 001) manufactured by Wyeth Laboratories.

Immunization. Lots 11201 and 20301 of precipitated toxoid were used for s.c. immunization. For instillation of toxoid into jejunal Thiry-Vella loops, dogs were anesthetized with pentobarbital, Foley catheters were placed in each loop orifice, the balloons were inflated with air, and one catheter was clamped. Toxoid from lot 11201 or lot 20401, given in 15 ml of isotonic glucose-electrolyte solution (21), was instilled through the other catheter which was then clamped. Catheters were removed 3 h later, when the fluid was fully absorbed. For intraduodenal instillation, a small laparotomy was performed, and toxoid in 15 ml of glucose-electrolyte solution was injected into the duodenum with a 26-gauge needle. Some dogs were given toxoid by orogastric tube as previously described (20).

Challenge with cholera toxin or with living *V. cholerae*. Dogs with Thiry-Vella jejunal loops were challenged with 125 mg of crude cholera toxin in the lumen of the loop. This dose had enterotoxic activity equivalent to 12.5 μg of purified toxin (determined by comparison of the secretory responses to graded doses of crude and purified toxins in ligated segments of rabbit small intestine [N. F. Pierce, unpublished data]). Dogs were anesthetized with pentobarbital, a Foley catheter was placed in each loop opening, and the balloon was inflated with air. Crude toxin was dissolved in 60 ml of 0.85% NaCl and instilled into the loop, and both catheters were clamped for 90 min. The catheters were then opened, the loop was flushed with 100 ml of air, and the output was measured. Thereafter fluid output was collected continuously. The total output for 7.5 h after toxin administration is reported.

Intact dogs were challenged by mouth with 10^{11} living *V. cholerae* Ogawa 395 as previously described (20); unimmunized control dogs were included in each challenge. The outcome of the challenges is described, and the degree of protection was determined as previously reported (20).

Specimen collection. Blood was obtained at the indicated intervals; serum was separated and frozen at −40°C until assayed for antitoxin. Jejunal washings were obtained and prepared for antitoxin assay as previously described (18). If toxin challenge was planned, the washing was obtained the day before challenge.

Jejunal biopsies were obtained by laparotomy under light anesthesia. A full-thickness piece of bowel was excised, trimmed to about 5 by 5 mm, washed in cold 0.01 M phosphate-buffered saline (pH 7.4), mounted in O.C.T. compound (Lab-Tek Products, Div. Miles Laboratories Inc., Naperville, Ill.), and frozen above liquid nitrogen (16). The biopsy site and abdominal incision were closed, and the dog was allowed to recover for further study.

ACC in jejunal lamina propria. Cholera antitoxin-containing cells (ACC) in jejunal lamina propria were identified by an indirect fluorescent-antibody technique using reagents and tissue fixation as reported previously (16). Single lots of fluorescein-conjugated antitoxin and purified toxoid were used as staining reagents. Adjacent microscopic fields (0.33 mm in diameter) in 5-μm-thick sections were examined for ACC in the crypt region, where they were most numerous; about 25 fields in two sections were examined per specimen. ACC frequency in these two sections was highly reproducible ($r = 0.90$). ACC frequency was expressed as number per cubic millimeter in the crypt region, 1 ACC per field equaling 2,300 ACC per mm^3.

Antitoxin titration. Two neutralization assays were used. Most sera and all jejunal washings were titrated by the rabbit gut loop assay as previously reported (15, 18). Some sera were titrated by a modification of the S49-1 mouse lymphoma cell assay described by Ruch et al. (F. E. Ruch, J. R. Murphy, L. H. Graff, and M. Field, J. Infect. Dis., in press). Results of these assays are comparable, except that the gut loop assay gives higher values when serum titers are less than 100 units per ml. Antitoxin unitage was determined by comparing each specimen with a simultaneously titrated standard serum containing 4,470 antitoxin units per ml (manufactured by Swiss Serum and Vaccine Institute and provided by Carl Miller, National Institute of Allergy and Infectious Diseases).

RESULTS

Antitoxic response in jejunal lamina propria. (i) Dogs immunized by parenteral priming and intraintestinal boosting with purified toxoid. Two groups of five to seven

dogs with jejunal Thiry-Vella loops were studied. Immunization schedules were identical, but different lots of purified toxoid were used. A 100-μg quantity of precipitated toxoid was given s.c. on day 0, followed by 250 μg of fluid toxoid given intrajejunally on days 28, 49, and 70. The titers of antitoxin in serum, the recovery of antitoxin in jejunal washings, and the frequency of ACC in jejunal lamina propria were determined at selected intervals. To avoid excessive operations, jejunal biopsies were obtained only at the outset, after i.l. boosting, and at several intervals after the final toxoid booster. The patterns of responses in the two groups were similar and are combined in Fig. 1. Several observations deserve emphasis. Serum antitoxin titers rose steadily after s.c. priming, reached a mean peak of 99 units per ml on day 56, and fell gradually thereafter. There was no consistent pattern of systemic response to the three i.l. boosters; the serum titer continued to rise after the first i.l. booster, but did not change significantly after the second or third. ACC were not detected in jejunal lamina propria at the outset or 28 days after the s.c. primer. i.l. boosting induced the appearance of ACC in lamina propria of the Thiry-Vella loop. The responses to the second and third i.l. doses were similar and greater than the response to the first ($P < 0.01$ for each). The frequency of lamina propria ACC was at a maximum 7 days after the third i.l. dose (day 77) and then fell rapidly, being only 10% of the peak level 38 days later (day 115). In contrast, the ACC response was small in jejunum which was not part of the Thiry-Vella loop and therefore not exposed to the i.l. doses of toxoid. On day 77, for example, the response was only 7% of that seen in the jejunal loop ($P < 0.001$). Antitoxin was detected in loop washings only after i.l. boosting. Antitoxin recovery from loops was consistently greater 7 days after than 21 days after i.l. boosting and correlated significantly with the frequency of ACC in lamina propria of the loop ($r = 0.58$; $P < 0.01$; Fig. 2). The scatter in the relationship between these two values may represent the combined variations in antitoxin recovery by the loop-washing technique and its measurement by the bioassay used. There was no consistent relationship between serum antitoxin titers and antitoxin recovery from jejunal loops (Fig. 1).

The relation between mucosal and systemic antitoxic responses was studied in a separate group of 20 intact dogs. The immunization schedule used was chosen because it induced a highly variable response of ACC in jejunal lamina propria. Dogs were given 50 μg of precipitated toxoid s.c. on day 0 and 250 μg of fluid

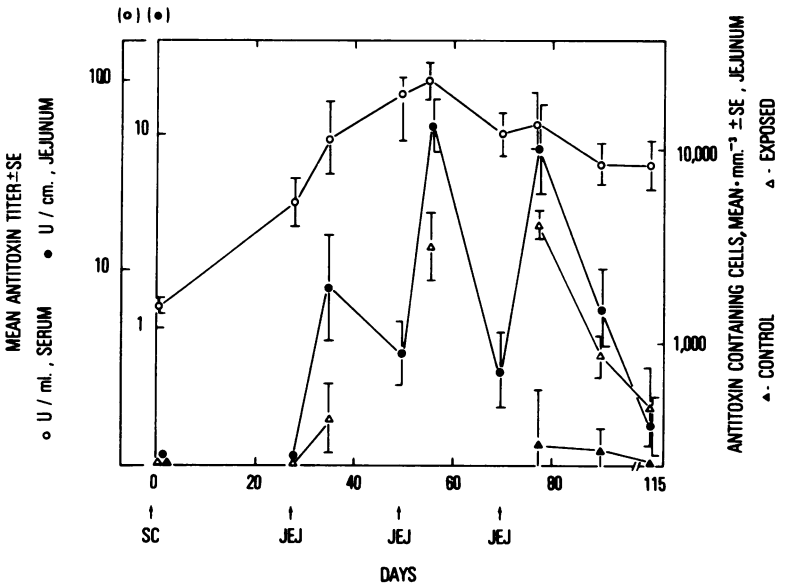

FIG. 1. *Time course of jejunal and systemic antitoxin responses in dogs immunized with purified toxoid by an s.c.-i.l. sequence. Twelve dogs were given 100 μg of toxoid s.c. on day 0 and 250 μg of toxoid i.l. in the jejunal Thiry-Vella loop on days 28, 49, and 70 (JEJ). Five received toxoid lot 20301 s.c. and lot 20401 i.l.; seven received lot 11201 s.c. and i.l. Most points represent the geometric mean of 12 determinations, but some jejunal biopsy values included as few as 5. Jejunal biopsies from exposed gut were from the Thiry-Vella loop; those from control gut were from intact jejunum not exposed to toxoid i.l. SE, Standard error.*

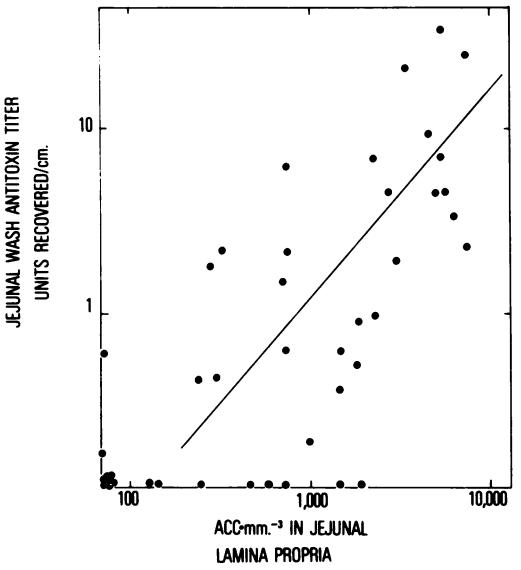

FIG. 2. *Relationship between frequency of ACC in jejunal lamina propria and recovery of antitoxin in jejunal wash fluid. The points compared are data from jejunal Thiry-Vella loop biopsies and simultaneous loop washings from the dogs described in the legend to Fig. 1. The correlation is significant ($r = 0.58$; $P < 0.01$).*

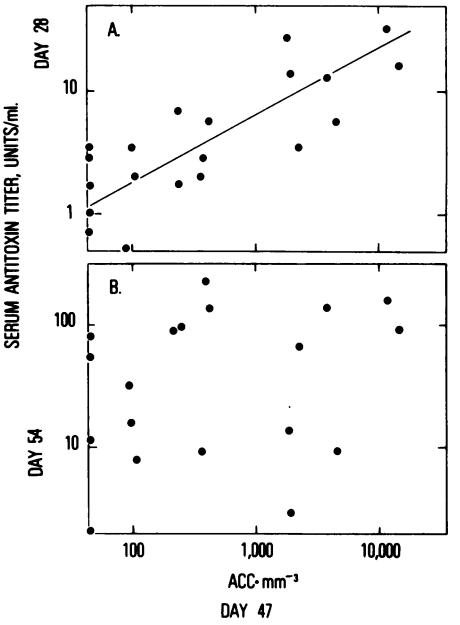

FIG. 3. *Relationship of mucosal and systemic antitoxin responses in dogs immunized by an s.c.-oral sequence. Dogs were given 50 μg of precipitated purified toxoid lot 11201 s.c. on day 0 and 250 μg of the same toxoid by intraduodenal injection on days 28 and 40. Antitoxin titers were determined by the S49-1 lymphoma cell assay. (A) Serum antitoxin titers on day 28 correlated significantly with the frequency of ACC in jejunal lamina 7 days after the second i.l. dose of toxoid ($r = 0.80$; $P < 0.01$). (B) Serum antitoxin titers on day 54 showed no correlation with ACC frequency on day 47.*

toxoid intraduodenally by laparotomy on days 28 and 40. Jejunal biopsies were obtained on day 47. Figure 3 compares the frequency of ACC in jejunal lamina propria near the peak of the secondary response (day 47) with the serum antitoxin titers induced by s.c. priming alone (day 28, Fig. 3A) and after intraduodenal boosting (day 54, Fig. 3B). In individual dogs, the magnitude of the secondary response in jejunal lamina propria correlated significantly with the serum antitoxin titer after s.c. priming ($r = 0.80$; $P < 0.01$), but showed no relation to the serum titer after intraduodenal boosting.

(ii) Dogs immunized with purified toxoid by the intestinal route. Two groups of five dogs with jejunal Thiry-Vella loops were given toxoid i.l. three times with 21-day intervals. In the first group, the toxoid was lot 11201; each dose was 250 μg. In the second group, the toxoid was lot 20401; the initial dose was 2.5 mg, and subsequent doses were 250 μg. At 7 days after the third i.l. dose of toxoid, dogs in the first group showed no change from pre-immunization serum antitoxin titers; ACC were not detected in jejunal lamina propria, nor was antitoxin detected in jejunal washings. In the second group, increasing the priming dose of toxoid 10-fold resulted in a small but detectable mucosal immune response (Table 1). The magnitude of this response was less than 10% of that seen in dogs boosted i.l. with 250-μg doses of the same toxoid after s.c. priming.

(iii) Dogs immunized by intestinal challenge with crude cholera toxin. Another group of 12 dogs, each with two jejunal Thiry-Vella loops, was challenged three times i.l. with 125 mg of crude cholera toxin. This was the equivalent, in toxic activity, of 12.5 μg of purified cholera toxin. Thus, this dose of toxin antigen was only 0.5% of the maximum dose of purified toxoid (2.5 mg) used in attempts to induce a local antitoxic response. Challenges were on days 0, 28, and 37. The systemic and secretory antitoxic responses in these dogs are shown in Table 2. At 28 days after the first challenge, there was no appreciable rise in serum antitoxin titer or in content of antitoxin in jejunal washings. By 9 days after the second challenge, however, the mean serum antitoxin titer had risen 4-fold, whereas the recovery of antitoxin from jejunal washings had risen about 30-fold above base-line levels. Antitoxin responses in proximal and distal jejunal loops on day 37 were similar.

TABLE 1. *Local and systemic antitoxin response after repeated i.l. administration of purified cholera toxoid*[a]

Day	i.l. toxoid dose	Serum antitoxin[b]	Jejunal wash antitoxin[c]	ACC in jejunal lamina propria[d]
0	2.5 mg	5.1 (4.2–6.3)	0.12	47
7				79 (69–91)
21	250 μg			
28				297 (140–629)
42	250 μg			
49		7.5 (5.4–10.5)	0.25 (0.1–0.4)	281 (150–526)

[a] Toxoid lot 20401; n = five dogs.

[b] Units per milliliter by the rabbit gut loop assay; geometric mean and range of mean ± standard error.

[c] Units of antitoxin recovered per centimeter of jejunum washed; geometric mean and range of mean ± standard error. Titers below the limit of sensitivity of the assay were given a value of 0.12 unit per cm.

[d] ACC per cubic millimeter in the crypt region; geometric mean and range of mean ± standard error. Specimens with no detectable ACC were given a value of 47 ACC/mm^3.

TABLE 2. *Antitoxin in serum and jejunal loop washings: effect of loop challenge with crude cholera toxin*[a]

Day	Serum antitoxin[b]	Jejunal wash antitoxin in[c]: Proximal loop	Jejunal wash antitoxin in[c]: Distal loop	No. of dogs studied
0	7.1 (6.0–8.6)	0.21 (0.16–0.28)	0.12[d]	12
27	7.9 (6.4–9.9)	0.21 (0.15–0.27)	0.42 (0.28–0.64)	9
36	29 (19–45)	7.1 (4.5–11)	3.1 (1.5–6.4)	8

[a] Jejunal loops were challenged with 125 mg of crude cholera toxin on days 1 and 28.

[b] Units per milliliter by the rabbit gut loop assay taken the day before challenge; geometric mean titer of antitoxin in serum and range of mean ± standard error.

[c] Units of antitoxin recovered per centimeter of jejunum washed (taken the day before challenge); geometric mean and range of mean ± standard error.

[d] Antitoxin was detected in none of these samples and was found in only 3 of 24 samples on day 0. Titers below the limit of sensitivity of the assay were given a value of 0.12 unit per cm.

Relation of mucosal antitoxin response to protection. (i) Intestinal challenge with cholera toxin. The role of secreted antitoxin in protection against intestinal challenge with cholera toxin is shown in Fig. 4. Data are from dogs described immediately above and in Table 2.

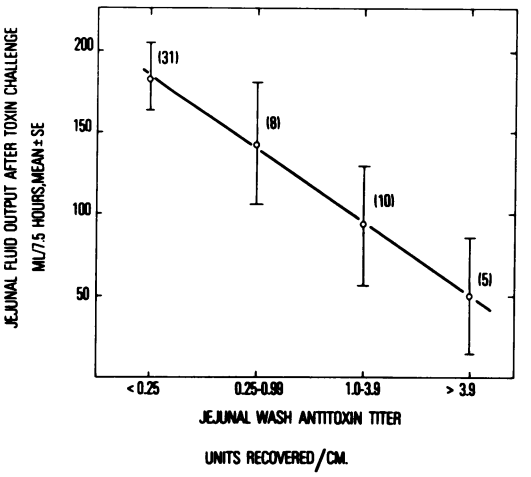

FIG. 4. *Relationship between recovery of antitoxin in jejunal washings and response to jejunal challenge with cholera toxin in normal and previously challenged dogs. Washings were obtained 1 day before loop challenge with 125 mg of crude cholera toxin. Challenges were on days 0, 28, and 37. The number of samples contributing to each mean is shown in parentheses. Twelve dogs were studied, and each had two jejunal Thiry-Vella loops. SE, Standard error.*

Results of each challenge are included, except for two challenges (involving four loops) which were technically unsatisfactory. There was an obvious inverse linear relationship between the prechallenge antitoxin recovery from the loop and the amount of fluid secreted in response to toxin challenge.

(ii) Oral challenge with living *V. cholerae*. Six dogs were immunized with 50 μg of precipitated toxin s.c. on day 0, followed by 10 50-μg doses of toxoid by orogastric tube between days 28 and 40. Multiple jejunal biopsies were obtained, and the dogs were challenged by mouth with living *V. cholerae* on day 92. Results are shown in Fig. 5. Also shown, for comparison, are previously reported serological and protection data from identically immunized dogs which were challenged at other intervals (20). After immunization, the mean serum antitoxin titer rose almost fivefold (34 units per ml) by day 42 and then fell, being less than twice the preimmunization level after day 100. The frequency of ACC in jejunal lamina propria was greatest 4 days after completing oral boosting (1,500 ± 400/mm^3) and fell rapidly thereafter. ACC were almost undetectable 26 days later (66 ± 12/mm^3, day 70). Nevertheless, significant protection against oral challenge with living *V. cholerae* lasted at least until day 288 (20), and protection averaged 62% in the 35 dogs challenged between days 169 and 288 ($P < 0.001$), when significant

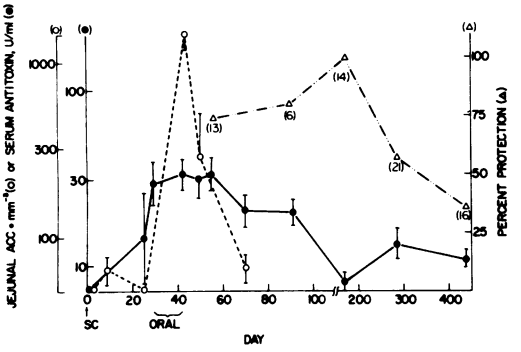

FIG. 5. *Relationship of mucosal and systemic antitoxin responses to protection of dogs immunized by an s.c.-oral sequence. Dogs were given 50 μg of precipitated purified toxoid lot 11201 s.c. on day 0 and 50 μg of the same toxoid by orogastric tube on 10 occasions between days 28 and 40. Challenge was with 10^{11} living V. cholerae Ogawa 395 given by orogastric tube. Percent protection expresses protection against severe or lethal disease when compared with concurrently challenged controls. Numbers in parentheses indicate number of immunized dogs challenged. Each jejunal biopsy value is derived from 6 to 9 dogs; mean serum antitoxin titers are derived from 6 to 21 dogs. The vertical bars indicate the range of the geometric mean ± the standard error. All data on protection, except at day 92, were reported previously (20) and are included here for summary purposes.*

antitoxic responses in serum or jejunal lamina propria were essentially undetectable.

The possibility that *V. cholerae* challenge induced a very rapid, protective mucosal antitoxic response in immunized dogs was also examined in the six dogs described above. Jejunal biopsies were obtained on day 70, dogs were challenged with live *V. cholerae* on day 92, and biopsies were obtained again on day 93 or 94. Results are shown in Table 3. These dogs showed protection similar to that in the larger series summarized in Fig. 5. Jejunal biopsies taken 18 to 42 h after challenge showed modest increases in the frequency of lamina propria ACC, which averaged 2.6-fold ($P < 0.05$).

TABLE 3. *ACC frequency in jejunal lamina propria before and after challenge of immunized dogs with living V. cholerae*[a]

Dog	ACC/mm³ in jejunal biopsy[b] on day:			Challenge outcome on day 92[c]
	70	93	94	
1	131	411		Severe diarrhea
2	75	47		Well
3	47	187		Mild diarrhea
4	79		341	Well
5	47		126	Well
6	47		180	Well
Geometric mean	66	174[d]		
Range of mean ± standard error	55–78	127–239		

[a] The s.c.-oral immunization with purified toxoid lot 11201 is described in the text. The last oral toxoid dose was on day 40.

[b] Specimens with no ACC seen were given a value of 47/mm³. Three control dogs biopsied at 42 h after challenge had no detectable ACC in jejunal lamina propria.

[c] Severity of diarrhea. Among unimmunized control dogs, four died and one had severe diarrhea. Immunized dogs had 80% protection against severe or lethal diarrhea; $P = 0.08$ by Fisher's exact two-tailed test.

[d] Significantly different than value on day 70; $P < 0.05$.

DISCUSSION

Two immunizing regimens induced mucosal antitoxin: (i) s.c. priming and i.l. boosting with purified toxoid and (ii) repeated i.l. doses of crude toxin. The former also protected dogs for at least 8 months against challenge with living *V. cholerae* in the absence of a sustained systemic antitoxic response (20). The latter extends earlier observations (27) showing a simple way to induce mucosal antitoxin using only i.l. antigen.

How parenteral antigen induces mucosal priming is not clear, but it also occurs in rats given cholera toxoid intraperitoneally with Freund adjuvant (16). Most plasma cells in gut lamina propria produce immunoglobulin A. They come from precursors in Peyer's patches and arrive via the systemic circulation after the patch is stimulated with antigen (3, 7, 16). Stimulation is usually by antigen sampled from the gut lumen (14), but this study and earlier studies (16, 20) show that parenteral antigen may also be effective. Possibly, parenteral antigen sensitizes lymphocytes which migrate to Peyer's patches to facilitate a mucosal response. Alternatively, parenteral antigen may reach Peyer's patches and exert a direct priming effect. The observation that mucosal priming by parenteral toxoid parallels systemic priming (Fig. 3) does not favor either possibility, but does suggest that parenteral toxoid primes for systemic and mucosal responses by a common mechanism. Although parenteral priming occurs, the mucosal immune system cannot be both primed and boosted with parenteral toxoid. After parenteral priming, i.l. toxoid was required for a mucosal booster response (16, 20), which agrees with the reported failure of repeated injections of killed polio vaccine to induce a mucosal immune response in humans (12).

The i.l. route was also effective for mucosal priming, especially with crude cholera toxin. Inactivated toxoid given in a 200-fold-greater dose was much less effective. The same differences are seen when toxin and toxoid are used for i.l. priming in rats (N. F. Pierce, J. Exp. Med., in

press). This may reflect destruction of antigenic determinants on toxin during the preparation of the toxoid, but this is probably not the entire explanation, because the same toxoid is an effective primer when given s.c. and when used as an i.l. booster. The priming effect of i.l. toxin may come from two molecular features which are lost in the preparation of the toxoid. The first is avid binding of toxin to GM_1 ganglioside in most cell membranes, including lymphocytes (9), which may enhance trapping of absorbed toxin by lymphoid cells in unprimed Peyer's patches. The second is activation of membrane-bound adenyl cyclase which may enhance the immune response in lymphoid tissue (1); cholera toxin has been shown to act as an adjuvant in a systemic immune response (11). The toxoids used in this study showed neither residual toxic activity nor reversion to active toxin in vivo (22). Our prior report that i.l. purified toxoid primed for an antitoxic response in canine jejunum is probably explained by residual toxin and further in vivo reversion to toxin demonstrated in the toxoid used in that study (19).

After effective priming, crude toxin and inactive toxoid were each effective as i.l. boosters of the mucosal response. The greater effectiveness of purified toxoid as an i.l. booster than as an i.l. primer also is seen in rats (Pierce, J. Exp. Med., in press) and may reflect enhanced trapping of toxoid by previously sensitized lymphocytes in Peyer's patches. The reason for the greater mucosal response to a second i.l. toxoid dose than to the first, after s.c. priming, is unclear. Possibly, the first i.l. booster further expanded the clone(s) of Peyer's patch lymphocytes participating in the immune response.

Most of the antitoxin present in jejunal washings after i.l. immunization or sequential s.c.-i.l. immunization came from plasma cells in the jejunal lamina propria rather than from serum. This conclusion is based on three observations. First, the antitoxic response in serum did not parallel that in jejunal washings (Fig. 1), suggesting that jejunal antitoxin was not serum derived. Second, the serum titers were insufficient to contribute appreciably to the titers in jejunal washings. A previous study with identical assay methods has shown a ratio of serum antitoxin units per milliliter to jejunal antitoxin units recovered per centimeter exceeding 100:1 in dogs transfused with hyperimmune serum (18). Thus, at the peak of the local response, less than 10% of the antitoxin in jejunal washings was serum derived, although this portion would be greater at the nadir of the local response (Fig. 1). Third, there was a significant correlation between the frequency of ACC in jejunal lamina propria and the recovery of antitoxin in jejunal washings. The isotype of secretory antitoxin was not determined, but was presumed to be mostly secretory immunoglobulin A because that is the major secretory immunoglobulin in dogs (24). Similar immunization of rats also induces a jejunal antitoxic response which is mostly immunoglobulin A (16). Failure of serum antitoxin to correlate with the mucosal response after i.l. boosting (Fig. 3B) shows that the serum antitoxin response is not a reliable correlate of the local response, at least when parenteral priming is used. It also suggests a random relationship at the time of i.l. boosting between the amounts of absorbed toxoid trapped in Peyer's patches and the amounts reaching previously primed systemic lymphoid tissue.

The rapid and brief secretory antitoxin responses seen in this study were similar in timing to those seen in other studies of the enteric mucosal immune system in which nonreplicating antigens were used (4, 16, 25). Day 7 after boosting was used to measure the peak of the response because earlier studies showed almost no secretory antitoxic response for 3 to 4 days and a sharp peak of secretory antitoxin between days 4 and 8 (16, 19).

This study also confirms earlier reports that a specific mucosal immune response is focused at the site of antigen exposure (13, 16), the response in boosted segments of jejunum being 14-fold greater than that in nonboosted portions. The lower response in nonexposed jejunum may represent dissemination of a portion of the cellular response from the site of boosting to nonexposed mucosal surfaces (6, 25). Whether this difference was due to selective homing of antitoxin-committed imunoblasts to antigen-exposed sites or to antigen-driven activation or division of sensitized cells after their arrival is not certain. This observation suggests, however, that optimal prophylaxis of mucosal infections by local immunization may require delivery of the antigen to the mucosal surface to be protected.

Finally, this study shows a protective role for secretory antitoxin. Protection against i.l. challenge with a bolus of cholera toxin correlated closely with the amount of antitoxin present in prechallenge jejunal washings. This, along with the evidence that secretory antitoxin derived from lamina propria ACC, provides a direct quantitative link between the presence of ACC in jejunal lamina propria and antitoxic protection.The duration of ACC in the lamina propria, however, was brief and thus not clearly responsible for the prolonged protection induced by s.c.-oral immunization; ACC were almost undetectable 30 days after oral boosting, but significant protection lasted for at least 8 months.

Serum antitoxin titers were also insufficient to explain this protection, because protection declines rapidly when mean serum titers fall below 100 units per ml (20). The possibility that ACC in jejunal lamina propria were protective in numbers undetectable by our assay cannot be excluded. However, the number detectable on day 70 (66/mm^3) was insufficient to yield measurable antitoxin (Fig. 2) and, thus, would not likely protect against toxin challenge (Fig. 4). It is also uncertain that protection was due to a very rapid local secondary response which aborted the disease within its 6- to 12-h incubation period (26) because the frequency of ACC in jejunal lamina propria had risen only 2.6-fold at 18 to 42 h after challenge. In other studies, a detectable antitoxic response in jejunal lamina propria or jejunal washings was not seen until at least 4 days after boosting (16, 19). It remains possible that protection induced by s.c.-oral immunization with toxoid was due to an immune response other than, or in addition to, the production and secretion of antitoxin. The mechanism of the prolonged protection against rechallenge seen in volunteers convalescent from cholera is also unknown (2). Although the role of the secretory immune response in prolonged prophylaxis of experimental cholera is unclear, the timing of the mucosal booster response described in this and previous reports (16, 19) suggests that it may be largely responsible for early termination of established mucosal infection.

ACKNOWLEDGMENTS

This work was supported by Public Health Service contract N01 AI 02051 from the National Institute of Allergy and Infectious Diseases. B.K.S. was supported by a grant from the World Health Organization.

We thank Douglas Penvose, Pamela Engel, and Patricia Scribner for excellent technical assistance and acknowledge the research facilities provided by the Gerontology Research Center of the National Institute of Aging under its Guest Scientist Program.

LITERATURE CITED

1. **Braun, W., M. Ishizuka, R. Winchurch, and D. Webb.** 1971. On the role of cyclic AMP in immune responses. Ann. N.Y. Acad. Sci. **185**:417–422.
2. **Cash, R. A., S. I. Music, J. P. Libonati, J. P. Craig, N. F. Pierce, and R. B. Hornick.** 1974. Response of man to infection with *Vibrio cholerae*. II. Protection from illness afforded by previous disease and vaccine. J. Infect. Dis. **130**:325–333.
3. **Craig, S. W., and J. J. Cebra.** 1971. Peyer's patches: an enriched source of precursors for IgA-producing immunocytes in the rabbit. J. Exp. Med. **134**:188–200.
4. **Freter, R., and E. J. Gangarosa.** 1963. Oral immunization and production of coproantibody in human volunteers. J. Immunol. **91**:724–729.
5. **Fujita, K., and R. A. Finkelstein.** 1972. Antitoxic immunity in experimental cholera: comparison of immunity induced perorally and parenterally in mice. J. Infect. Dis. **125**:647–655.
6. **Goldblum, R. M., S. Ahlstedt, B. Carlsson, L. Å. Hanson, V. Jodal, G. Liden-Janson, and A. Sohl-Akerlund.** 1975. Antibody-forming cells in human colostrum after oral immunization. Nature (London) **257**:797–798.
7. **Gowans, J. L., and E. J. Knight.** 1964. The route of recirculation of lymphocytes in the rat. Proc. R. Soc. London Ser. B **159**:257–282.
8. **Holmgren, J., Å. Andersson, G. Wallerstrom, and Ö. Ouchterlony.** 1972. Experimental studies on cholera immunization. II. Evidence for protective antitoxic immunity mediated by serum antibodies as well as local antibodies. Infect. Immun. **5**:662–667.
9. **Holmgren, J., L. Lindholm, and I. Lönnroth.** 1974. Interaction of cholera toxin and toxin derivatives with lymphocytes. I. Binding properties and interference with lectin-induced cellular stimulation. J. Exp. Med. **139**:801–819.
10. **Holmgren, J., A.-M. Svennerholm, Ö. Ouchterlony, Å. Andersson, G. Wallerstrom, and U. Westerberg-Berndtsson.** 1975. Antitoxic immunity in experimental cholera: protection, and serum and local antibody responses in rabbits after enteral and parenteral immunization. Infect. Immun. **12**:1331–1340.
11. **Northrup, R. S., and A. S. Fauci.** 1972. Adjuvant effect of cholera enterotoxin on the immune response of the mouse to sheep red blood cells. J. Infect. Dis. **125**:672–673.
12. **Ogra, P. L.** 1969. The secretory immunoglobulin system of the gastrointestinal tract, p. 259–279. *In* D. H. Dayton, P. A. Small, R. M. Chanock, H. E. Kaufman, and T. B. Tomasi (ed.), The secretory immunoglobulin system. U.S. Government Printing Office, Washington, D.C.
13. **Ogra, P. L., and D. T. Karzon.** 1969. Distribution of poliovirus antibody in serum, nasopharynx and alimentary tract following segmental immunization of lower alimentary tract with polio vaccine. J. Immunol. **120**:1423–1430.
14. **Owen, R. L.** 1977. Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study. Gastroenterology **72**:440–451.
15. **Pierce, N. F., J. F. Banwell, R. B. Sack, R. C. Mitra, and A. Mondal.** 1970. Magnitude and duration of antitoxin response to human infection with *Vibrio cholerae*. J. Infect. Dis. **121**(Suppl.):S111–S113.
16. **Pierce, N. F., and J. L. Gowans.** 1975. Cellular kinetics of the intestinal immune response to cholera toxoid in rats. J. Exp. Med. **142**:1550–1563.
17. **Pierce, N. F., E. A. Kaniecki, and R. S. Northrup.** 1972. Antitoxic protection against experimental cholera. J. Infect. Dis. **126**:606–616.
18. **Pierce, N. F., and H. Y. Reynolds.** 1974. Immunity to experimental cholera. I. Protective effect of humoral IgG antitoxin demonstrated by passive immunization. J. Immunol. **113**:1017–1023.
19. **Pierce, N. F., and H. Y. Reynolds.** 1975. Immunity to experimental cholera. II. Secretory and humoral antitoxin response to local and systemic toxoid administration. J. Infect. Dis. **131**:383–389.
20. **Pierce, N. F., R. B. Sack, and B. K. Sircar.** 1977. Immunity to experimental cholera. III. Enhanced duration of protection after sequential parenteral-oral toxoid administration to dogs. J. Infect. Dis. **135**:888–896.
21. **Pierce, N. F., and C. K. Wallace.** 1972. Stimulation of jejunal secretion by a crude *Escherichia coli* enterotoxin. Gastroenterology **63**:439–448.
22. **Rappaport, R. S., G. Bonde, T. McCann, B. A. Rubin, and H. Tint.** 1974. Development of a purified cholera toxoid. II. Preparation of a stable, antigenic toxoid by reaction of purified toxin with glutaraldehyde. Infect. Immun. **9**:304–317.

23. **Rappaport, R. S., W. A. Pierzchala, G. Bonde, T. McCann, and B. A. Rubin.** 1976. Development of a purified cholera toxoid. III. Refinements in purification of toxin and methods for the determination of residual somatic antigen. Infect. Immun. **14**:687–693.
24. **Reynolds, H. Y., and J. S. Johnson.** 1970. Canine immunoglobulins. IV. Coproimmunoglobulins. J. Immunol. **104**:888–895.
25. **Robertson, S. M., and J. J. Cebra.** 1976. A model for local immunity. Ric. Clin. Lab. **6**(Suppl. 3):105–119.
26. **Sack, R. B., and C. C. J. Carpenter.** 1969. Experimental canine cholera. I. Development of the model. J. Infect. Dis. **119**:138–149.
27. **Sack, R. B., J. Johnson, N. F. Pierce, D. F. Keren, and J. H. Yardley.** 1976. Challenge of dogs with live enterotoxigenic *Escherichia coli* and effects of repeated challenges on fluid secretion in jejunal Thiry-Vella loops. J. Infect. Dis. **134**:15–24.
28. **Svennerholm, A.-M., and J. Holmgren.** 1977. Immunoglobulin and specific-antibody synthesis in vitro by enteral and nonenteral lymphoid tissues after subcutaneous cholera immunization. Infect. Immun. **15**:360–369.