

CELLULAR KINETICS OF THE INTESTINAL IMMUNE RESPONSE TO CHOLERA TOXOID IN RATS*

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The intestinal lamina propria contains large numbers of plasma cells, most of which produce IgA. The antibodies secreted by these cells are thought to play a major role in protecting the mucosal surface of the gut against pathogenic microorganisms or their toxic products. The source of these cells, the effect of the route of antigen administration upon their appearance, and the factors that determine their distribution along the gut are incompletely understood.

Earlier studies in the rat have shown that plasma cells in the lamina propria are derived, at least in part, from large lymphocytes found in thoracic duct lymph (1,2) and that about half of these already contain internal IgA when freshly isolated from the lymph (3,4). The IgA-containing TDL may originate in Peyer's patches (5).

The present studies were undertaken to clarify further the importance of lymph and blood-borne lymphocytes as the source of plasma cells in the lamina propria of the gut. By observing the specific cellular response to a protein antigen it has been possible to confirm that most of the lamina propria plasma cells are derived from cells found earlier in thoracic duct lymph. It has also been possible to demonstrate immunologic memory in the mucosal immune system of the gut, to determine the effect of the route of antigen administration upon primary and secondary responses in this system, and to determine the effect of intestinal exposure to antigen upon the distribution of specific antibody-containing plasma cells in the lamina propria. Cholera toxoid was chosen for these studies because it is an antigen to which the rats had no prior exposure, because it should ultimately be possible to correlate the presence of antitoxin-containing cells (ACC)¹ in the lamina propria with a direct measure of protection against the local effects of the parent antigen (cholera toxin), and because gut immunization with this antigen is of potential practical importance.

Materials and Methods

Rats. Rats were males or females of the inbred PVG/c strain (hooded, Ag-B5) which were maintained in a specific pathogen-free unit until removed for immunization.

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¹ *Abbreviations used in this paper:* ACC, antitoxin-containing cells; FCA, Freund's complete adjuvant; PBS, phosphate-buffered saline; TDL, thoracic duct lymphocytes.

Toxoid Immunization. Cholera toxoid was prepared from a culture filtrate of *Vibrio cholerae*, Inaba, 569B by Dr. R. O. Thomson, Wellcome Research Laboratories, Beckenham, Kent. Two preparations were employed, a crude toxoid and an immunopurified toxoid. Crude toxoid was precipitated by ammonium sulfate (50 kg per 100 liters) from the filtrate of a formalin-treated culture medium, redissolved in water, dialyzed against water, and lyophilized. For immunopurification the culture filtrate was passed down a column of Sepharose (Pharmacia [Great Britain] Ltd., London) coupled with equine cholera antitoxin previously polymerized with gluteraldehyde. After washing, the absorbed toxoid was eluted with 0.1 N HCl, neutralized, filter sterilized, and incubated at 37°C for 21 days. Formalin (0.2%) was again added, followed by incubation for 21 days (37°C), dialysis, and filter sterilization. The latter formalin treatment was to stabilize the toxoid and prevent reversion to active toxin.

Purified toxoid containing about 25,000 antitoxin combining U (6) per ml and about 3 mg of protein per ml was used in all studies in which toxoid was given intraperitoneally (i.p.) or intraintraintestinally. Crude toxoid containing about 100 antitoxin combining U per mg was used only for oral immunization. The doses of crude toxoid are indicated in the text as the equivalent amounts of purified toxoid. For oral immunization, drinking water was replaced by crude toxoid dissolved in 0.15 M NaCl plus terramycin (1 mg/ml). Rats were individually caged to permit measurement of oral intake, and any toxoid not taken after 48 h was discarded and replaced. For intraintraintestinal immunization, a small laparotomy was performed and 0.3 ml of immunopurified toxoid (1 mg) injected into the lumen of the gut with a No. 26 needle, care being taken to avoid peritoneal contamination with toxoid. For i.p. immunization, 100 µg of toxoid in 0.2 ml 0.15 M NaCl was injected with or without Freund's complete adjuvant (FCA).

Antitoxin Assay. The antitoxin content of inactivated sera was measured by agglutination of formalinized sheep erythrocytes sensitized with purified cholera toxin, National Institutes of Health lot 1071, supplied by Dr. John Seal, National Institute of Allergy and Infectious Diseases (7). A control standard serum manufactured by the Swiss Serum and Vaccine Institute (Lot No. EC3 (A-2/67)-B, antitoxin content 4,470 U/ml; antitoxin unit defined in reference 8) gave a titer of 1:1,280 in this assay.

Purified Antibodies Labeled with Fluorescein and ¹²⁵I. Purified antitoxin was prepared from sera of hyperimmunized rabbits by affinity chromatography on CnBr-activated Sepharose-4B (Pharmacia Fine Chemicals) coupled with purified toxoid (9). Fluorescein isothiocyanate (Nordic Laboratories, Maidenhead, England) was coupled to purified antitoxin (10) and the conjugate stored in small aliquots at -20°C. Rabbit F(ab')₂ antirat IgA, IgG₂, and IgM were prepared by Dr. A. F. Williams (11). These were iodinated with ¹²⁵I at 5 µCi/µg (11) and used within 4 wk.

ACC in Thoracic Duct Lymph. Thoracic duct lymph was collected at 4°C as previously described (1). Individual collections did not exceed 12 h, but drainage was continued in some rats for up to 7 days. Cell counts in lymph were made with a Coulter Counter, model Fn (Coulter Electronics Ltd., Dunstable, England). Smears were prepared from washed thoracic duct lymphocytes (TDL) which had been resuspended in fetal calf serum. After fixing in 95% ethanol for 60 min at 4°C they were incubated sequentially with purified cholera toxoid (100 µg/ml in phosphate-buffered saline [PBS]) and fluorescein-conjugated antitoxin (51 µg/ml), each for 30 min at 20°C, and washed in PBS for 2 h.

Fluorescent cells were examined under dark ground with a Zeiss fluorescence microscope illuminated with an HBO 200 mercury vapor lamp (Carl Zeiss, Oberkochen, West Germany). The proportion of fluorescent cells was derived from a total count of about 3,000 lymphocytes made by switching to a tungsten lamp. No fluorescent lymphocytes were seen among TDL from nonimmunized rats stained as above or among TDL from immunized rats in which toxoid was omitted from the staining sequence or in which the smear was incubated with unconjugated antitoxin before incubation with fluorescein-conjugated antitoxin.

ACC in Gut Lamina Propria. Segments of proximal jejunum, distal ileum (within 5 cm of the ileo-cecal valve), and midtransverse colon, 1 cm in length, were obtained from rats under ether anesthesia. 5-µm frozen sections were mounted on glass slides previously subbed with chrome alum/gelatin, fixed with methanol for 3-5 min at 20°C, air dried, and gently washed with PBS. The tissue was then incubated with purified toxoid (1,000 µg/ml in PBS) for 30 min, washed and incubated with fluorescein-conjugated antitoxin for 30 min, washed in PBS for 2 h, and air dried (all at 20°C).

Counts of fluorescent cells in the lamina propria were made by examining adjacent fields in the

base of the mucosa. About 20-25 fields could be examined in each transverse section of gut. The density of ACC in the lamina propria was calculated from the volume of tissue examined, the depth of tissue being 5 μm and the field diameter 220 μm . Thus, a mean of one fluorescing cell per field equalled 5,260 cells/ mm^3 in the region of the mucosa examined. No fluorescent cells were seen in sections of gut from nonimmunized rats stained as above or in sections from immunized rats in which toxoid was omitted from the staining sequence.

Ig Class of Antibody in ACC. The Ig class of antibody in ACC was determined by combined incubation of TDL smears or gut sections with fluorescein-conjugated antitoxin and ^{125}I -labeled rabbit F(ab')₂ antirat Ig. The anti-Ig was diluted 1:10-20 with DAB (Dulbecco A and B balanced salt solution), and unlabeled anti-Ig was added to maintain a protein concentration of 8 $\mu\text{g}/\text{ml}$. TDL smears or gut sections were incubated with one drop each of diluted ^{125}I -labeled anti-Ig and purified toxoid (30 min, 20°C), washed with PBS, and stained with fluorescein-conjugated antitoxin as above. After a final wash, the slides were again fixed in 95% ethanol for 30 min at 20°C. For autoradiography, the slides were dipped in Ilford K2 emulsion (Ilford Ltd., Ilford, Essex, England) diluted 2:1 with water and developed after exposure for 20 h. The preparations were examined both for fluorescing cells (using UV light) and cells with autoradiographic labeling (7-100 grains/cell, using tungsten light and a dark field condenser). Cell counts continued until at least 100 fluorescing cells were counted on each slide (TDL smears) or until all fluorescing cells had been counted (gut sections).

Statistics. Statistics were evaluated by Student's *t* test.

Results

Appearance of ACC in Thoracic Duct Lymph and Gut Lamina Propria

PRIMARY I.P. OR INTRAIESTINAL IMMUNIZATION. When rats were given a single dose of 100 μg purified toxoid i.p. or 1 mg purified toxoid intraduodenally, no ACC were seen among TDL examined 1-5 days later or in small bowel mucosa examined 4-14 days later. When 100 μg purified toxoid was given in FCA i.p., few ACC were identified among TDL collected 15-21 days later (Fig. 1). However, beginning 19 days after immunization and lasting at least until day 90, ACC were present in the lamina propria, predominantly that of the colon. A few were seen in the ileum and virtually none in the jejunum (Fig. 2). These doses of antigen, routes of administration, and examination intervals served as controls for subsequent studies on the effect of boosting in previously immunized rats.

INTRAIESTINAL OR I.P. BOOSTING OF PREVIOUSLY PRIMED RATS. To study secondary cellular responses in the intestinal immune system rats were given either intraduodenal or i.p. toxoid boosters 14 days after either i.p. or oral toxoid priming. The most striking response was observed in rats primed with an i.p. dose of toxoid in FCA and boosted intraduodenally (Fig. 1). ACC were consistently present among TDL collected 50-100 h after the intraduodenal boost reaching an average peak of about 200,000 ACC/h at 76 h. The ACC were almost entirely large lymphocytes with homogeneous cytoplasmic fluorescence (Fig. 3). About 60% were intensely fluorescent. Only rarely did small lymphocytes or plasmacytoid cells contain antitoxin. The number of ACC per hour among TDL was related to the size of the booster dose of toxoid: rats given 100 μg intraduodenally had a mean peak response of 60,000 ACC/h (2 rats), while those given crude toxoid to drink (equivalent to 5 mg purified toxoid) averaged 580,000 ACC/h (15 rats). The number of ACC per hour among TDL was not affected by prolonged lymph drainage, the peak response being similar in rats cannulated at the time of intraduodenal boosting or 1-3 days later (Fig. 1).

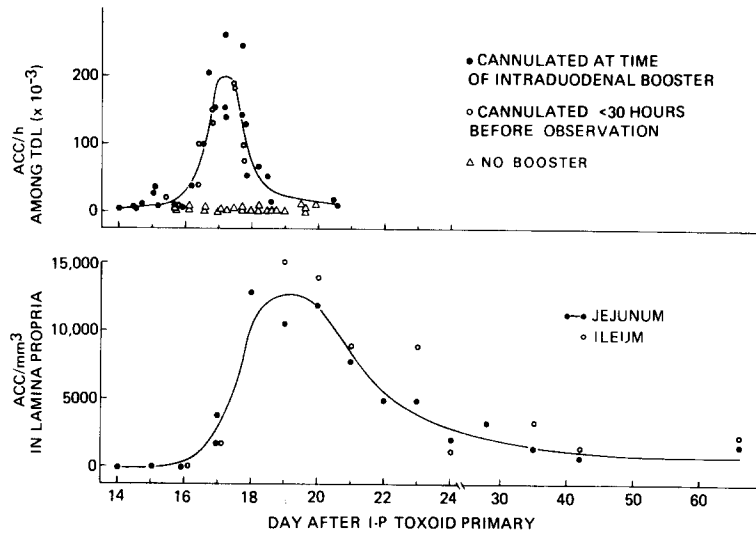


FIG. 1. Appearance of ACC in lymph and small intestine after intraduodenal boosting of i.p. primed rats. Rats were given 100 μg toxoid in FCA, i.p., on day 0 and 1 mg toxoid intraduodenally on day 14. Each point is a single observation, except for ACC in jejunum and ileum on day 19 where each point is the mean of five observations. Data on ACC among TDL is from 11 rats (3 not boosted, 7 cannulated at time of intraduodenal boosting, and 4 cannulated 1–3 days after boosting <30 hours before the observation). Data on ACC in gut is from 20 rats.

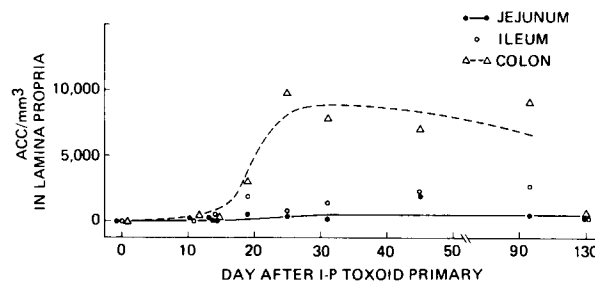


FIG. 2. Appearance of ACC in gut after i.p. priming with toxoid in adjuvant. 11 rats received 100 μg toxoid in FCA, i.p., on day 0 and were killed serially.

In the mucosa of the small intestine ACC appeared on the 3rd day after boosting, peaked between the 4th and 6th days, and then declined steadily to approach counts seen in nonboosted animals by the 10th day. Morphologically the ACC were plasma cells and were found in greatest numbers in the basal mucosa among the crypts and in smaller numbers in the lower half of the villi. Few were found in the tips of the villi and none in Peyer's patches. The density of ACC in lamina propria was somewhat greater in ileum than jejunum. No secondary response to the intraduodenal booster was observed in the colon (Table I). When the interval between i.p. priming and intraduodenal boosting was extended to 77–105 days, the magnitude of the secondary cellular response in the small intestine was reduced by about 65% (Table II).

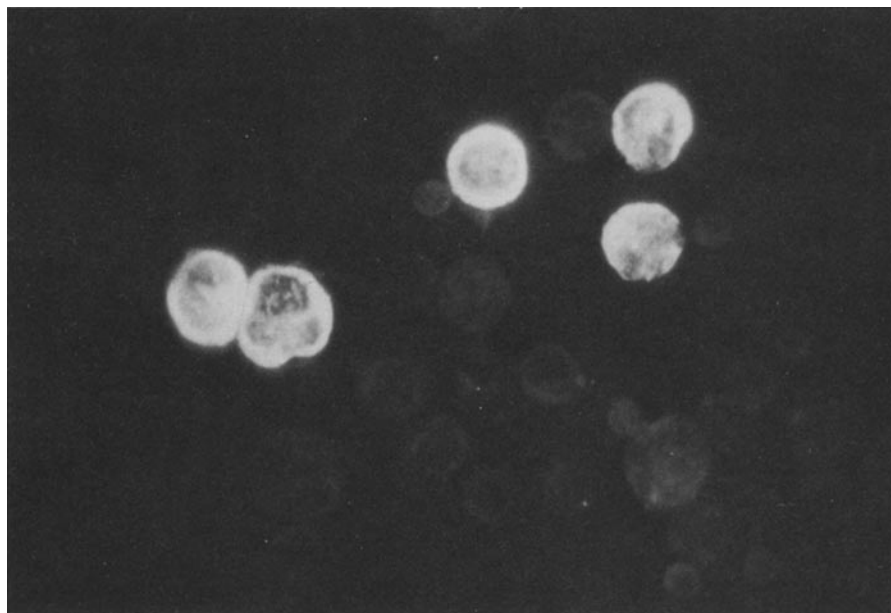


FIG. 3. Antitoxin-containing cells in thoracic duct lymph. Rats were given 100 μ g toxoid in FCA i.p. on day 0 and drank 5 mg toxoid on day 14. Lymph was collected 84–96 h after stopping oral toxoid. Smear of TDL shows five brightly fluorescent ACC (\times 760).

When i.p. priming was followed in 14 days by an i.p. boost different results were obtained. ACC were identified among TDL, but the counts were much lower and none were brightly fluorescent (Fig. 4). In the small intestine and colon there was no increase in ACC above that seen at the same interval after parenteral priming alone (Fig. 4).

Rats given 1 mg toxoid intraduodenally on days 0 and 14 had no ACC in the small gut on days 18, 19, and 20 and very few among TDL between days 14 and 20. Attempts were therefore made to achieve oral priming with larger doses of toxoid. Rats were given crude toxoid to drink until they had taken the equivalent of 25–40 mg of purified toxoid over a period of 8–16 days. An intraduodenal or i.p. booster was given 14 days after ending oral administration. Fig. 5 shows that rats boosted intraduodenally developed a mean peak of 80,000 ACC/h among TDL about 76 h after boosting. About 75% of ACC were intensely fluorescent. The response to this boost was less than that observed in rats primed i.p. and showed considerable variation from rat to rat. In the gut, ACC occurred predominantly in the jejunum, with smaller numbers in the ileum and none in the colon (Fig. 5). The magnitude of this response also varied considerably from rat to rat, the mean peak jejunal response being about half of that observed in animals primed by the i.p. route. Oral priming alone gave no ACC among TDL, very few in the small gut, and none in the colon.

When orally-primed rats were challenged i.p. 14 days after the end of priming, a small response of ACC was observed among TDL and in the small intestine (Fig. 6). The TDL response of about 30,000 ACC/h peaked about 84 h after

TABLE I
Effect of Site of Intestinal Boosting and of Thoracic Duct Drainage on Distribution of ACC in Gut of Primarily Immunized Rats

Immunization		No. rats	<i>t</i> test	Density of ACC in lamina propria‡		
Primary	Site of secondary injection*			Jejunum	Ileum	Colon
<i>mean/mm³ × 10⁻³ ± SE</i>						
100 µg toxoid in FCA i.p.	1. None	6	—	0.7 ± 0.1	2.0 ± 0.5	3.1 ± 1.1
	2. Duodenum	5-7	<i>P</i> vs. 1	10.3 ± 2.0 <0.005	14.9 ± 2.1 <0.001	5.7 ± 1.3 NS
	3. Ileum	6-7	<i>P</i> vs. 1	5.3 ± 0.8 <0.001	20.8 ± 3.7 <0.001	8.5 ± 2.0 <0.05
	4. Colon	6-8	<i>P</i> vs. 1	0.6 ± 0.3 NS	3.4 ± 1.4 NS	16.7 ± 3.0 <0.005
	5. Duodenum and thoracic duct drainage§	6-7	<i>P</i> vs. 2 <i>P</i> vs. 1	2.0 ± 0.6 <0.005 NS	2.8 ± 0.6 <0.001 NS	7.2 ± 1.0 <0.05 <0.05

NS, not significant.

* 1 mg cholera toxoid 14 days after primary.

‡ Observations on day 19.

§ Thoracic duct drainage 14-19 days after primary.

TABLE II
Effect of Interval between Primary and Secondary Immunization with Cholera Toxoid on ACC Response in the Small Intestine

Immunization		No. rats	Time of ACC assay	Density of ACC in lamina propria	
Primary	Site of secondary injection			Jejunum	Ileum
<i>days after primary</i>					
<i>mean/mm³ × 10⁻³ ± SE</i>					
100 µg toxoid in FCA i.p.	1. None	6	19	0.7 ± 0.1	2.0 ± 0.5
	2. None	2	96-131	0.3	1.5
	3. Duodenum*	6	19	10.3 ± 2.0	14.9 ± 2.1
	4. Duodenum*	6	77-110	2.8 ± 0.8‡	4.5 ± 1.4§

* 1 mg cholera toxoid 5 days before ACC assay.

‡ *P* vs. 1: <0.05; *P* vs. 3: <0.01.

§ *P* vs. 1: <0.2; *P* vs. 3: <0.005.

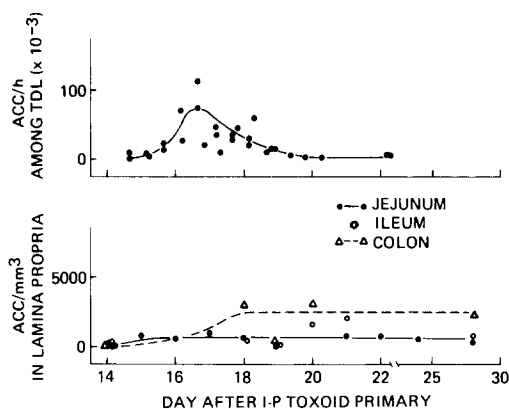


FIG. 4. Appearance of ACC in lymph and gut after i.p. boosting of i.p. primed rats. Rats were given 100 μg toxoid in FCA i.p. on day 0 and 100 μg plain toxoid i.p. on day 14. Data on ACC among TDL is from 3 rats, on lamina propria from 15 rats.

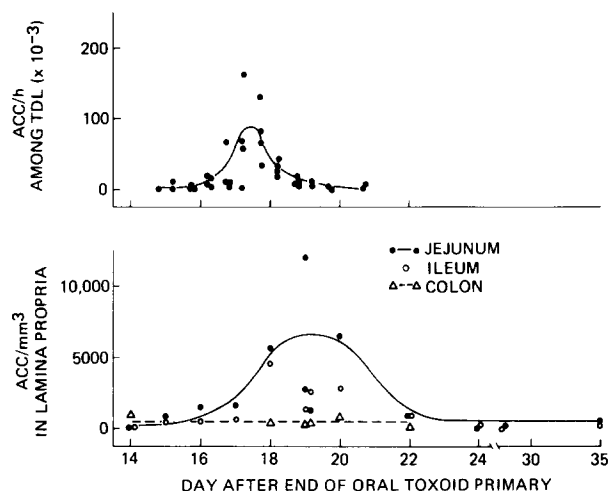


FIG. 5. Appearance of ACC in lymph and intestine after intraduodenal boosting of orally primed rats. Rats drank 25–40 mg of toxoid over 8–16 days. 14 days after the end of oral toxoid they received 1 mg of toxoid intraduodenally. Data on ACC among TDL are from 4 rats, on lamina propria ACC from 13 rats.

boosting. About 65% of these ACC were intensely fluorescent. ACC were seen in the jejunum and ileum, but very few appeared in the colon. This response clearly differed from animals immunized entirely by the i.p. route, in which intensely fluorescent cells were absent among TDL, and in which there was no secondary response of ACC in gut lamina propria.

Influence of Regional Intestinal Challenge on Distribution of ACC in Gut Lamina Propria. In rats primed with i.p. toxoid, the region of the gut into which a booster dose of toxoid was injected had a marked influence upon the distribution of ACC in the lamina propria at the peak of the secondary response (Table I). After injections into the duodenum, terminal ileum, or ascending colon the greatest density of ACC in the lamina propria always occurred at, or

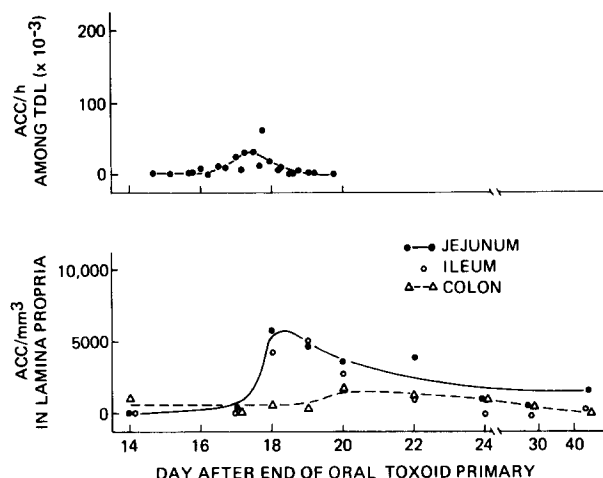


FIG. 6. Appearance of ACC in lymph and intestine after i.p. boosting of orally primed rats. Rats were primed orally by drinking 25–40 mg of toxoid in 8–16 days. 14 days after the end of oral toxoid they received 100 μ g toxoid, i.p., without adjuvant. Data on ACC among TDL from 4 rats, on lamina propria ACC from 9 rats.

distal to, the injection site. Thus, after colonic injection there was a marked response of ACC in the transverse colon, but no significant increase in ACC in the small intestine. Conversely, after duodenal injection there was a marked response of ACC in jejunum and ileum, but no significant response in the colon. After ileal injection significant increases in ACC density were observed throughout the gut, but the greatest response was clearly in the terminal ileum.

Effect of Draining Thoracic Duct Lymph Upon the Appearance of ACC in Gut Lamina Propria. To test the idea that the ACC that arose in the lamina propria after immunization with toxoid were derived from lymph-borne precursors, continuous drainage of cells, and lymph from the thoracic duct was instituted in primed rats (toxoid in FCA i.p.) at the time of intraduodenal boosting. This procedure virtually abolished the ACC response in the small intestine on examination 5 days after boosting (Table I). A small increment of ACC was observed in the colon.

Gut Localization of Transfused ACC from Thoracic Duct Lymph. Washed TDL from immunized rats were given intravenously to normal rats to determine whether they included cells that would appear as ACC in the lamina propria of the intestine. TDL were collected 2¹/₂–4 days after oral boosting from a panel of donor rats which had been primed i.p. 14 days earlier. Cells were pooled, washed once in PBS, resuspended in PBS, and injected i.v. into two pairs of littermate recipients. One recipient in each pair was given oral toxoid from the time of the cell transfer until death to determine whether intestinal toxoid increased the density of lamina propria ACC, possibly by recruiting more ACC precursors or by inducing their division within the lamina propria. Table III shows that the recipients had large numbers of ACC in the lamina propria, especially in that of the small intestine, when killed 5–6 h after cell transfer. The density of ACC in the small gut was four to fivefold greater than in the colon. The administration

of toxoid orally during and after cell transfer did not increase the density of ACC in any portion of the gut.

Serum Antitoxin Titers in Immunized Rats. Oral priming with or without intraduodenal or i.p. boosting yielded little or no serum antibody (Fig. 7). In contrast, rats that had been primed i.p. developed moderate titers by day 22 and a significantly increased titer after either intraduodenal or i.p. boosting.

Ig Class of Antitoxin Within ACC in TDL and Gut Lamina Propria. The Ig class of the antitoxin within TDL from immunized rats and the proportion of all Ig-containing cells in lymph which were synthesizing antitoxin are set out in

TABLE III
Appearance of ACC in Gut of Rats Given TDL from Specifically-Immunized Donors

Donor TDL i.v. ($\times 10^{-7}$)		Recipients			
Total	ACC	Treatment	*ACC in gut		
			Jejunum	Ileum	Colon
			<i>mean/mm³ $\times 10^{-3}$</i>		
125	2.9	None	18.0	14.5	3.5
		Oral toxoid	12.0	12.0	2.5

Donor rats primed with 100 μ g toxoid in FCA i.p. and boosted orally with 5 mg toxoid 14 days later. Each recipient given two i.v. injections of cells collected from thoracic duct of donors during periods 60–84 h and 84–96 h, respectively, after boosting. Recipients killed 5–6 h after second injection.

* Mean response of two rats given toxoid in drinking water (3–5 mg) from time of first cell transfer to killing and of two rats given none.

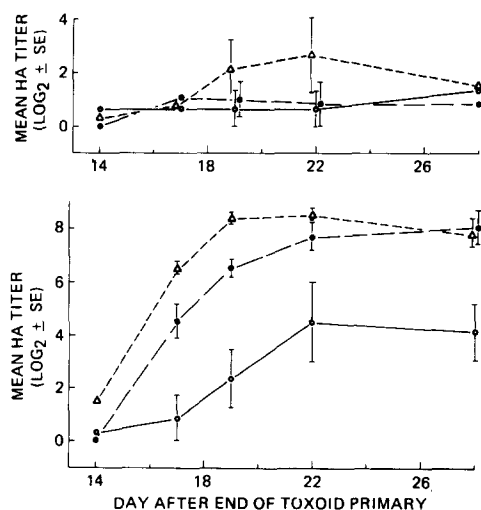


FIG. 7. Serum antitoxin titers in rats immunized with cholera toxoid. Rats were primed with toxoid either orally (upper) or i.p. (lower) as described in the text. 14 days after priming they received 100 μ g toxoid, i.p., without adjuvant (Δ); 1 mg toxoid, intraduodenally (\bullet); or nothing (\circ). Each point is the mean from six rats.

Table IV. Consistent with previous reports (3,4) IgA was the predominant internal Ig in rat TDL.

The Ig class of internal antitoxin in TDL was influenced by the route of immunization. When immunization was entirely by the intestinal route it was virtually all IgA and at the peak of the response about 17% of all IgA-containing cells were ACC (Table IV). In rats primed i.p. and boosted intraintestinally it was still predominantly IgA, but 9% of ACC contained IgG₂ (Table IV). In two additional rats that had been primed and boosted i.p. the small number of ACC that appeared in thoracic duct lymph (Fig. 4) contained predominantly IgG₂ (73%), while only 16% contained IgA; at the peak of this response 46% of all IgG₂-containing cells in the lymph were ACC.

In the lamina propria of the ileum IgA was also the predominant class of Ig in ACC. In two rats primed i.p. with toxoid in FCA and boosted intraduodenally, IgA, IgG₂, or IgM were present in 76, 6, and 5% of ACC, respectively, when tissue was examined 5 days after boosting.

Discussion

The aim of the present work was to study the development of immunity to cholera toxin in the rat as assessed by the appearance of cells containing cholera antitoxin in the lamina propria of the intestine. The first problem was to devise immunization procedures which would provoke the appearance in the gut of large numbers of ACC. The second problem was to determine the origin of these cells, particularly the extent to which they may be derived from migrating large lymphocytes which enter the blood by way of the thoracic duct. It was not established that the density of ACC in the lamina propria is a direct measure of the resistance of the intestine to challenge with cholera toxin, although this is a reasonable assumption.

Very few ACC appeared in the small intestine after single administrations of cholera toxoid either i.p. in FCA or orally. However, when animals that had been primed i.p. were subsequently given toxoid orally or by intraintestinal injection large numbers of ACC, in which the immunoglobulin was almost exclusively IgA, appeared rapidly in the thoracic duct lymph and, shortly afterwards, in the lamina propria of the small intestine. In contrast, an i.p.

TABLE IV
Frequency of TDL with Internal Ig and Cholera Antitoxin

Immunization		No. rats	Class of Ig in ACC. % ACC with internal:			% Ig-containing cells which are also ACC		
Primary	Secondary		IgA	IgG ₂	IgM	IgA	IgG ₂	IgM
100 µg toxoid in FCA i.p.	Oral (5 mg) or intraduodenal (1 mg) toxoid 14 days after priming	5	82 (76-88)	9 (6-14)	0	11 (9-14)	16 (11-28)	0
25-40 mg toxoid in drinking water over 8-16 days		2	100 (99, 100)	0.5 (1, 0)	0	17 (16, 18)	0.6 (0, 1.2)	0

Values are mean and range in TDL 3-4 days after boosting. Cells collected during first day after cannulation of thoracic duct.

boost given after i.p. priming gave no ACC response in the gut, which accords with other reports that parenteral immunization gives a poor secretory IgA response (12,13). Oral priming was relatively inefficient, requiring the prolonged administration of large amounts of antigen, possibly reflecting the limited capacity for antigen trapping in Peyer's patches (14) or the failure of effective quantities of antigen to reach the gut-associated lymphoid tissue. The vigorous ACC response observed after intrainestinal challenge indicates that i.p. or prolonged oral administration of toxoid can establish immunological memory in the secretory IgA system of the gut.

The secondary ACC response among TDL was detectable 2 days after intrainestinal boosting and rose rapidly during the next day to a maximum of about 200,000 ACC/h; at the height of the response about 1% of all TDL were ACC of which more than 80% contained IgA. The time-course of this cellular response was similar to that seen in the lymph from peripheral lymph nodes after regional immunization with other antigens (15), although the predominant class of cell-contained Ig was different: IgA in TDL and predominantly IgG and IgM in cells in the lymph from peripheral lymph nodes (16).

Earlier studies have shown that plasma cells in the lamina propria of the gut are derived, at least partly, from nonrecirculating precursors among the large lymphocytes in thoracic duct lymph (1,2,4), which probably originate in Peyer's patches or mesenteric lymph nodes (5,17). The observation in the present study that thoracic duct drainage, started at the time of intrainestinal boosting, profoundly depressed the ACC response in the gut suggests that lymphocytes in the thoracic duct are the major source of these cells. In addition, the importance of the intestinal route for efficient boosting by antigen is strong evidence that these ACC arise in gut lymphoid tissue drained by the thoracic duct, that is, in the Peyer's patches or mesenteric lymph nodes. During the secondary response up to 17% of TDL with internal IgA also contained antitoxin. Although it seems likely that these ACC among TDL are the cells that were subsequently identified in the lamina propria it is possible that non-ACC in lymph also contributed to the response by completing their differentiation after migration into the lamina propria. It has been shown in nonimmunized rats that about 20% of TDL that carry surface IgA lack internal IgA (3).

The migration of large lymphocytes into embryonic and neonatal gut (18,19,4) suggests that selective homing does not depend on the presence of bacterial antigens in the gut. Similarly, after the transfer of TDL from immune donors, large numbers of ACC accumulated in the gut of recipients that had not been exposed to toxoid. Although specific antigen is not necessary for the localization of IgA-containing cells in the gut the present study showed that it can exert a significant effect upon their distribution along it. After intrainestinal boosting the greatest density of ACC consistently occurred at, or distal to, the boosted site irrespective of whether this was proximal or distal small bowel or colon. This was not due to antigen-induced activation or multiplication of precursors within the lamina propria because, firstly, rats fed toxoid during and after the transfer of immune TDL showed no greater numbers of ACC in the lamina propria than recipients not fed toxoid, and secondly, the concentration of ACC at the boosted site appeared to occur at the expense of ACC in more distant regions of the gut.

Previous studies have shown that [³H]thymidine labeled large TDL home predominantly to the small gut, the large gut receiving only a small fraction of all gut-localizing cells (1,2). The only conditions in the present study in which the ACC density in the large gut exceeded that in the small was after i.p. priming alone and in rats given a colonic booster after i.p. priming. The possibility that the large and small gut differ in the mechanisms by which plasma cells are generated or localized cannot be excluded.

The results of this study have practical implications for inducing optimal mucosal immunity to soluble nonreplicating protein antigens. They are consistent with previous studies that suggest that parenteral antigen administration does not induce mucosal immunity (12), apart from the contribution of antibody derived from the serum (20). While mucosal immunization can be accomplished entirely by the local route it is clear that i.p. priming followed by local boosting is a more efficient regime, and this may also be true when priming is by the subcutaneous or intramuscular routes. Parenteral priming has the additional advantage that it prepares the whole gut, and probably other mucosal surfaces (13), for a secondary response to a local booster. Recent studies have shown that a regime of subcutaneous priming and oral boosting with cholera toxoid leads to prolonged protection. Dogs immunized in this way were protected against oral challenge with viable *V. cholerae* for at least 8 mo. This protection occurred in the absence of any lasting elevation in serum antitoxin titer and was of much longer duration than that induced when priming and boosting were done entirely by the subcutaneous or oral routes.²

Summary

The aims of this study were (a) to find a regime of immunization with cholera toxoid in rats which would establish a high density of antitoxin containing cells (ACC) in the lamina propria of the intestine and (b) to determine the origin of the ACC. The best cellular response was achieved by a single i.p. dose of toxoid in FCA followed by an intrainestinal boost 2 wk later. ACC appeared in the thoracic duct lymph 2 days after boosting, reaching a peak of about 200,000 ACC/h at 3–4 days. This was followed by the appearance of large numbers of ACC in the intestine. The i.p. dose of toxoid by itself gave rise to very few ACC in the gut or thoracic duct lymph, but it had clearly primed the gut immune system for a secondary response. Priming was also achieved by the prolonged oral intake of toxoid. The importance of the intestinal route for boosting was shown by the failure of i.p. challenge to give an ACC response in the intestine after i.p. priming and the small response it provoked after oral priming. ACC among thoracic duct lymphocytes (TDL) and in the lamina propria contained predominantly IgA. Two observations indicated that the major source of the lamina propria ACC was from cells that emerged in the thoracic duct lymph after intrainestinal challenge. Firstly, the establishment of a thoracic duct fistula immediately before challenge prevented the appearance of ACC in the intestine. Secondly, many ACC appeared in the intestine of normal rats after the injection of TDL rich in ACC. Although homing of ACC precursors to the gut

² Pierce, N. F. Unpublished studies.

was not antigen-dependent, the distribution of ACC in the lamina propria was considerably influenced by the site of the intestinal challenge, the density of ACC being greatest at or distal to the site of injection of toxoid into the lumen of the gut.

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References

1. Gowans, J. L., and E. J. Knight. 1964. The route of recirculation of lymphocytes in the rat. *Proc. R. Soc. Lond. B. Biol. Sci.* 159:257.
2. Hall, J. G., D. M. Parry, and M. E. Smith. 1972. The distribution and differentiation of lymph-borne immunoblasts after intravenous injection into syngeneic recipients. *Cell Tissue Kinet.* 5:269.
3. Williams, A. F., and J. L. Gowans. 1975. The presence of IgA on the surface of the rat thoracic duct lymphocytes which contain internal IgA. *J. Exp. Med.* 141:335.
4. Guy-Grand, D., C. Griscelli, and P. Vassalli. 1974. The gut-associated lymphoid system: nature, and properties of the large dividing cells. *Eur. J. Immunol.* 4:435.
5. 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.* 134:18.
6. Craig, J. P. 1971. Cholera toxins. In *Microbial Toxins*. S. Kadis, T. C. Montie, and S. J. Ajl, editors. Academic Press, Inc., New York. 2a:189.
7. Hochstein, H. D., J. C. Feeley, and W. E. DeWitt. 1970. Titration of cholera antitoxin in human sera by microhemagglutination with formalinized erythrocytes. *Appl. Microbiol.* 19:742.
8. Craig, J. P., E. R. Eichner, and R. B. Hornick. 1972. Cutaneous responses to cholera skin toxin in man. I. Responses in unimmunized American males. *J. Infect. Dis.* 125:203.
9. Jensenius, J. C., and A. F. Williams. 1974. The binding of antiimmunoglobulin antibodies to rat thymocytes and thoracic duct lymphocytes. *Eur. J. Immunol.* 4:91.
10. The, T. H., and T. E. W. Feltkamp. 1970. Conjugation of fluorescein isothiocyanate to antibodies. I. Experiments on the conditions of conjugation. *Immunology.* 18:865.
11. Jensenius, J. C., and A. F. Williams. 1974. Total immunoglobulin of rat thymocytes and thoracic duct lymphocytes. *Eur. J. Immunol.* 4:98.
12. Ogra, P. 1971. The secretory immunoglobulin system of the gastrointestinal tract. In *The Secretory Immunologic System*. D. H. Dayton, Jr., P. A. Small, Jr., R. M. Chanock, H. E. Kaufman, and T. B. Tomasi, Jr., editors. U. S. Government Printing Office, Washington, D. C. 259.
13. Newcomb, R. W., K. Ishizaka, and B. L. DeVald. 1969. Human IgG and IgA diphtheria antitoxins in serum, nasal fluids, and saliva. *J. Immunol.* 103:215.
14. Bienenstock, J., and J. Dolezel. 1971. Peyer's patches: lack of specific antibody-containing cells after oral and parenteral immunization. *J. Immunol.* 106:938.
15. Hall, J. G., and B. Morris. 1963. The lymph-borne cells of the immune response. *Q. J. Exp. Physiol. Cogn. Med. Sci.* 48:235.
16. Beh, K. J., and A. K. Lascelles. 1974. Class specificity of intracellular and surface immunoglobulins of cells in popliteal and intestinal lymph from sheep. *Aust. J. Exp. Biol. Med. Sci.* 52:505.
17. Griscelli, C., P. Vassalli, and R. T. McCluskey. 1969. The distribution of large

- dividing lymph node cells in syngeneic recipient rats after intravenous injection. *J. Exp. Med.* 130:1427.
18. Parrott, D. M. V., and A. Ferguson. 1974. Selective migration of lymphocytes within the mouse small intestine. *Immunology.* 26:571.
 19. Halsted, T. E., and J. G. Hall. 1972. The homing of lymph-borne immunoblasts to the small gut of neonatal rats. *Transplantation (Baltimore).* 14:339.
 20. 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.