INFECTION AND IMMUNITY, Apr. 1980, p. 17-23
0019-9567/80/04-0017/07$02.00/0

Vol. 28, No. 1

Antitoxic Cholera Immunity in Mice: Influence of Antigen Deposition on Antitoxin-Containing Cells and Protective Immunity in Different Parts of the Intestine

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The importance of the mode of antigen presentation (intravenous, oral, or enteral restricted to the lower ileum) in the development of a local immune response and immunological memory for such a response in different parts of the intestine was studied in mice. Cholera toxin was used as antigen and the immune response was assayed by determining both the number of specific antitoxin-containing cells in the lamina propria and protection against experimental cholera. The results showed that all of these routes of antigen presentation could induce significant memory along the entire small intestine. In contrast, the actual production of antitoxin-containing cells or protective immune response elicited by booster immunization was restricted to those parts of the intestine that were directly exposed to antigen; i.e., lower ileum boosting resulted in immunity in the distal ileum but not in the proximal jejunum, whereas oral or intravenous boosting gave a response in both jejunum and ileum. Protection correlated closely with the number of antitoxin-containing cells in the lamina propria (correlation coefficient, 0.88); ≥4,000 antitoxin-containing cells per mm^3 conferred solid immunity to cholera toxin-induced diarrhea. The total number of immunoglobulin-containing cells in intestines was not significantly influenced by the specific immunizations. There were four times as many of these cells in the upper jejunum (167,000 cells per mm^3) as in the lower ileum, but the proportions of immunoglobulin A-containing cells (80 to 85%), immunoglobulin M-containing cells (14 to 20%), and immunoglobulin G-containing cells (0.4 to 0.9%) were similar in various parts of the intestine. The results indicate a differential dependence on local tissue antigen for the intestinal antibody-secreting cells and their memory cell precursors.

Rodent intestines contain numerous plasma cells, the majority of which secrete immunoglobulin of the immunoglobulin A (IgA) class (4, 5). In mice most of these IgA-containing cells are found in the lamina propria, are continually renewed, and have a life span of approximately 4.7 days (14). These cells emanate from Peyer's patches, enter the thoracic duct as large lymphocytes, and then recirculate to the intestinal lamina propria and possibly also to other mucosal surfaces (6, 11, 21).

The reason for this circulatory odyssey is unknown. In contrast to the original hypothesis of Gowans and Knight (6), evidence has accumulated which indicates that the striking ability of these blast cells to home back to the gut does not seem to be mediated primarily by antigen. Thus, it has been shown that homing of lymphocytes to the intestine occurs in fetal sheep in utero (7), in the guts of unsuckled, Caesarian-derived neonatal rats (8), and in sterile, heterotopic transplants of fetal intestine (16). On the other hand, several studies with viral antigens clearly have demonstrated that local vaccination gives rise to a greater local IgA response than if the vaccine is administered parenterally (1); furthermore, the results of Ogra and Karzon (18) suggested that the IgA response to local polio immunization in the distal colon was essentially confined to the antigen-exposed part of the gastrointestinal tract. Husband and Gowans (10) recently demonstrated that the intestinal development of specific antibody-containing cells (ACC) after immunization with cholera toxin probably results from antigen-dependent as well as antigen-independent processes. They found that after a segmented booster immunization, there appeared in thoracic duct lymph-specific ACC, which were randomly distributed in the lamina propria of the entire intestine, but survived and proliferated only in the intestinal segment where antigen was present.

This paper describes the distribution of the local mucosal immune response in different

parts of the small intestines of mice after various immunization routes for priming or boosting, using cholera toxin as the antigen.

MATERIALS AND METHODS

Animals. Inbred strain C57Bl/6J (H-2^b) mice 6 to 12 weeks old were used. The animals were carefully matched with regard to age and sex within each experiment.

Immunizations. Groups of mice were given varying numbers of intravenous (i.v.) or oral (p.o.) immunizations with purified cholera toxin. The immunization procedures have been described in detail previously (13). Restricted immunization of the lower ileum was done under ether anesthesia via a small laparotomy in the lower part of abdomen. About 5 cm from the entrance of the ileum into the ascending colon, 0.2 ml of toxin diluted in phosphate-buffered saline was injected directly into the ileal lumen; retrograde reflux was prevented by occlusion of the intestine proximal to the injection site for 3 to 5 min. Mice injected i.v. or into the distal ileum with phosphate-buffered saline or mice given bicarbonate–phosphate-buffered saline solution p.o. served as controls and were analyzed concurrently with the immunized animals. We used three or four initial immunizations for building up a local immunological memory in the intestine, and after the actual antibody response resulting from this priming had vanished (12, 20), a single booster immunization was given for eliciting the local antibody response under study.

Determination of specific ACC and different classes of immunoglobulin-containing cells. Specific ACC and immunoglobulin-containing cells of the IgA, IgG, and IgM classes in the lamina propria were determined microscopically by using peroxidase-labeled reagents.

Horseradish peroxidase (type VI; Sigma Chemical Co., St. Louis, Mo.) was conjugated to the immunoglobulin fractions of antisera specific for mouse IgA, IgG2B, and IgM (Meloy Laboratories, Springfield, Va.) (17). Conjugation of peroxidase to anti-cholera toxin immunglobulin and control studies of the binding specificities of the conjugates were done as described previously (12, 17).

Intestinal specimens (length, 5 mm) were embedded in paraffin, cut into 5-μm transverse sections, deparaffinated in xylol, and incubated with Triton X-100–1,4′-aminotriazole in order to eliminate endogenous peroxidase activity (11). ACC were developed by incubating the sections with cholera toxin (1 μg/ml), followed by anti-cholera toxin peroxidase conjugate; the immunoglobulin-containing cells of the various classes were developed by incubating the specimens with the appropriate peroxidase-conjugated anti-immunoglobulin. Stained cells were examined after incubation with 3′3′-diaminobenzidine and hydrogen peroxide. The optimal reagent concentrations were determined by chessboard titrations (12).

ACC and immunoglobulin-containing cells were determined for at least 10 full transverse sections of intestine from each of three animals to reduce errors due to an uneven distribution of positive cells in the lamina propria. The tissue surface area of each such section was approximately 1.5 mm^2; the lamina propria was roughly 25% of this. The average ACC diameter was around 10 μm. When a transverse section of the gut was prepared, the ACC located immediately above or below the upper section surface were cut and therefore could be stained. It is evident that ACC outside the range of ± 10 μm on both sides of the upper section surface were not cut and therefore were not stained. The number of ACC per cubic millimeter of intestinal tissue was thus equal to the number of ACC per section (area, 1.5 mm^2) multiplied by 1,000/20 = 50 (which was the number of ACC per 1-mm thickness of the gut section) and divided by 1.5. The number of ACC per cubic millimeter of lamina propria was therefore obtained by multiplying the number of ACC per section by 133([4 × 50]/1.5 = 133). This calculation means that all stained areas were counted, even those approaching the resolving power of the microscope (in our case, about 1 μm). The thickness of the layer included in the calculations was ± 10 μm on both sides of the upper section surface. However, even if only structures ≥ 5 μm are counted, a layer of $\pm$ 9.3 μm on both sides of the section surface is taken into consideration, which implies a value for the above factor of 143 instead of 133. Thus, no great error in the estimation of ACC per cubic millimeter of lamina propria is introduced if the thickness of the counting layer is set at 20 μm (i.e., $\pm$ 10 μm).

The mean numbers and standard errors of ACC and immunoglobulin-containing cells were calculated by conventional statistical methods, using the section as the unit of calculation. The use of the section and not the animal as the unit for the statistical calculations was justified by the fact that when the data were subjected to analysis of variance, they did not reveal any significant between-animal variance. All values given are based on at least two separate experiments.

Protection tests. Protective immunity against intestinal challenge with cholera toxin was studied by using a ligated loop assay (13). One loop was tied as far proximally as possible (i.e., 4 to 6 cm below the pylorus), and the other loop was tied as far distally as possible on the small intestine. The proximal and distal loops were each injected with 2.5 μg of cholera toxin in a volume of 0.2 ml. After 5 h the length of each loop was measured, and the loop with the accumulated fluid was weighed. The protective effect of immunization (i.e., the inhibition of cholera toxin-induced fluid accumulation) was estimated by comparing the fluid response per centimeter for control and immunized mice. At least two experiments for each immunization schedule were done, using five-animal groups. The significance of differences in fluid accumulation between immunized and unimmunized animals was calculated by Student's t test.

RESULTS

Immunoglobulin-containing cells of different classes in intestine. Numerous immunoglobulin-containing cells were present in sections from all parts of the small intestines of unimmunized mice. Most cells were found in the lamina propria, both in the subepithelial villus area and in between the glandular crypts. The

overwhelming majority contained IgA (Table 1), and there were four times as many of these cells in the proximal part of the jejunum as in the distal part of the small intestine.

IgM- and IgG-containing cells were few compared with IgA-containing cells. Their distribution along the small intestine was similar to that of IgA-containing cells in the sense that there were 5 times more IgM-containing cells and 10 times more IgG-containing cells in the proximal jejunum than in the distal ileum (Table 1). The ratio of IgA-containing to IgM-containing cells was approximately 5:1 in all parts of the intestine, whereas the ratios of IgA-containing to IgG-containing cells were 100:1 in the proximal part, 1,000:1 in the middle part, and 500:1 in the distal part (Table 1).

The numbers of immunoglobulin-containing cells of all classes did not differ significantly between control and cholera toxin-immunized animals in the various parts of the intestine after any of the immunization procedures performed.

TABLE 1. *Distribution of different classes of immunoglobulin-containing cells and ACC in the small intestines of normal mice*

Cells displaying specific staining for:	No. of cells/mm³ of lamina propria		
	High jejunum	Middle jejunum	Low ileum
IgA	137,309 ± 5,182[a]	66,234 ± 3,110	34,899 ± 2,970
IgM	27,850 ± 3,016	15,399 ± 1,278	5,812 ± 797
IgG[b]	1,476 ± 366	438 ± 138	159 ± 66
ACC	0	0	0

[a] Mean ± standard error of the mean of 30 to 40 sections from three animals.

[b] The number of IgG-containing cells was calculated by multiplying the observed number of IgG2B cells by three, since IgG1-, IgG2A-, and IgG2B-producing cells have shown very little variation in different lymphoid tissues of mice (unpublished data).

Distribution of ACC in different parts of the intestine after immunization. Experiments were undertaken to study the development of cholera toxin-specific intestinal ACC after immunizations by different routes, with special reference to whether direct antigen exposure is needed for stimulation of an ACC response or memory for such a response. The experimental approach was to immunize directly into the distal ileum to obtain a restricted lower intestine antigen exposure (the exposed segment contained two to four macroscopically visible Peyer's patches) and to give the antigen either p.o. or i.v. to expose the whole small intestine from the mucosal or serosal side, respectively. After various combinations of these immunization procedures, the numbers of ACC in the proximal and distal small intestine were determined.

Table 2 shows that p.o. priming and p.o. boosting induced a high number of ACC in the proximal as well as the distal intestine, whereas priming and boosting in the lower ileum gave rise to a significant ACC number only in the distal part of intestine. The number of ACC in the upper intestine after priming in the lower ileum was significantly increased only when this priming was followed by a p.o. or i.v. booster. The amount of antigen in the booster dose was of importance because proximally as well as distally many more ACC were formed after an i.v. booster with 10 μg than with 1 μg of cholera toxin, when the priming immunization consisted of four antigen administrations into the lower ileum.

TABLE 2. *Numbers of specific ACC after various immunization schedules and their proportions of the total number of immunoglobulin-containing cells in the small intestine*

Immunization[a]				High jejunum ACC		Lower ileum ACC	
Priming		Booster					
No. of times	Type	No. of times	Type	No.[b]	% Of total immunoglobulin-containing cells	No.[b]	% Of total immunoglobulin-containing cells
3	p.o.	1	p.o.	6,919 ± 778	4.2	6,517 ± 770	16.0
3	LI	1	LI	359 ± 172	0.2	13,140 ± 799	32.2
3	LI	1	p.o.	1,011 ± 132	0.6	3,378 ± 355	8.3
4	LI	1	i.v.	133 ± 40	0.1	1,530 ± 224	3.8
4	LI	1	i.v. (10 μg)	1,028 ± 127	0.6	2,803 ± 262	6.9

[a] When not stated otherwise, all i.v. doses were with 1 μg of cholera toxin and all p.o. or lower ileum (LI) doses were with 5 μg of cholera toxin.

[b] The priming consisted of three or four immunizations with intervals of 10 days between the two first immunizations and 6 days between subsequent ones. The booster was given 10 days after the last priming immunization, when essentially all residual immunity after priming had vanished (12, 20). ACC were measured as number of cells per cubic millimeter of lamina propria 4 days after boosting (mean ± standard error of the mean of at least 60 sections from six mice in two experiments).

Table 2 also shows that the specific ACC usually comprised only a small fraction of the total number of immunoglobulin-containing cells, especially in the proximal jejunum, where they did not exceed 5%. However, after exclusive immunization in the lower ileum, the ACC comprised about 30% of the total immunoglobulin-containing cells in the distal intestine. Interestingly, the high number of specific ACC did not increase the total number of IgA-, IgG-, or IgM-containing cells ($P > 0.20$ for all classes, as determined on 30 sections from three animals in each group).

No specific ACC were found in the intestines from control animals (Table 1).

Protective immunity and its correlation with immunization schedule and ACC. The fluid secretory response to intestinal challenge with cholera toxin was studied in the proximal and distal parts of the small intestine after the various immunization schedules (Table 3).

An initial set of experiments was done to evaluate the effect of generalized and restricted enteral immunizations. It was found that p.o. priming and p.o. boosting gave full protection in the proximal as well as distal part, whereas p.o. priming followed by a booster in the lower ileum gave full protection in the distal loop but no protection in the proximal loop. The latter pattern was also seen when priming in the lower ileum was followed by a booster in the lower ileum. In contrast, protection in the distal part was associated with about 50% protection in the upper intestine when priming in the lower ileum was followed by a p.o. booster.

It was then of interest to combine the parenteral route with restricted (lower ileum) and generalized (p.o.) enteral immunization. Although an i.v. booster dose of 1 μg of cholera toxin only gave protection in the distal loop after priming in the lower ileum, a 10-μg i.v. booster gave protection in the proximal loop as well as the distal loop. After i.v. priming, p.o. boosting induced protective immunity in the proximal intestine as well as the distal intestine. This was in contrast to boosting in the lower ileum, which gave protection only in the distal part of the intestine. However, slightly better protection with the latter combination of immunizations could be achieved by increasing the amount of antigen in the i.v. priming to a total of 17 μg of cholera toxin instead of 4 μg. There was a good correlation between ACC number and the protective effect, i.e. the degree of inhibition of cholera toxin-induced fluid secretion in the loop assay, after various immunizations (Fig. 1). Half-maximal protection was obtained when the ACC density in the lamina propria was about 2,000 cells per mm^3, whereas ≥4,000 ACC per mm^3 gave full protection and ≤250 ACC per mm^3 gave no detectable protection against the toxin challenge.

DISCUSSION

The aim of this study was to define some of the factors relating to the presentation of antigen

TABLE 3. *Effects of various routes of immunization on resistance to cholera toxin-induced fluid secretion in the proximal and distal parts of the small intestine*

Immunization[a]				Proximal jejunum		Distal ileum	
Priming		Booster		% Inhibition of fluid output[b]	P^b	% Inhibition of fluid output	P
No. of times	Type	No. of times	Type				
3	p.o.	1	p.o.	91 ± 7	<0.01	96 ± 6	<0.01
3	p.o.	1	LI[c]	20 ± 11	NS	109 ± 7	<0.05
3	LI[c]	1	LI	−4 ± 9	NS	98 ± 6	<0.02
3	LI	1	p.o.	57 ± 8	<0.05	106 ± 3	<0.02
4	LI	1	i.v.	3 ± 12	NS	69 ± 11	<0.01
4	LI	1	i.v. (10 μg)	36 ± 4	<0.05	56 ± 8	<0.02
4	i.v.	1	p.o.	49 ± 6	<0.05	44 ± 6	<0.02
4	i.v.	1	LI	9 ± 9	NS	30 ± 4	<0.02
4	i.v. (1 + 2 + 4 + 10 μg)	1	LI	8 ± 10	NS	48 ± 7	<0.01

[a] For immunization schedules, see Table 2. Inhibition of fluid secretion (protective immunity) was determined 4 days after the booster immunization.

[b] The intestinal loops were each challenged with 2.5 μg of cholera toxin. In immunized mice this challenge induced fluid accumulations of 134 ± 7 mg/cm in the proximal loop and 87 ± 7 mg/cm in the distal loop. Percent inhibition of fluid output in the immunized animals was calculated in relation to the mean value for the concurrently tested control group; each value represents the mean ± standard error of the mean of 10 to 15 immunized animals from two to three experiments. P values were calculated by comparing the fluid outputs in the immunized and control animals. NS, Not significant ($P > 0.20$).

[c] LI, Lower ileum.

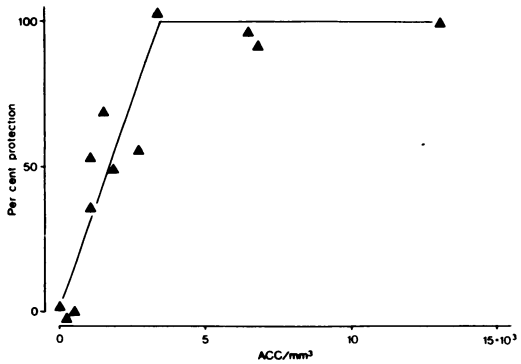

FIG. 1. *Correlation between protective immunity (determined as percent inhibition of cholera toxin-induced fluid secretion) and number of ACC in lamina propria. The data plotted are from Tables 1 through 3. Regression analysis indicated a highly significant correlation (correlation coefficient, 0.88).*

that are important for the development of a local immune response in different parts of the intestine. This was done by varying the routes and doses of administration of the antigen (cholera toxin) for priming and boosting, respectively. Whereas p.o. and i.v. immunizations probably stimulate the entire length of the small intestine from the mucosal and serosal sides, respectively, introduction of the antigen into the lower ileum should essentially exclude the upper part of the small intestine from direct antigen contact. The mucosal immune response was assayed by determining either the number of specific ACC in the lamina propria or the protective immunity against experimental cholera in different parts of the intestine 4 days after booster immunization, i.e. at a time when the protective immunity was maximal (12, 13). Immunization in the lower ileum does not exclude the antigen from reaching a number of macroscopically visible Peyer's patches. This is of importance since Peyer's patches contain B lymphocytes rich in precursors for the plasma cells which repopulate the intestinal lamina propria after antigen exposure.

The results show that it is possible by either p.o. or i.v. immunization to generate an immunological preparedness in all parts of the intestine for an anamnestic-type response to p.o. booster. Our previous studies have shown that more than 90% of the ACC and essentially all of the protective immunity after these immunization schedules represent the actual response to the booster immunization and that the magnitude of this response is critically dependent on the earlier priming immunizations (12, 20). Thus, there seem to be memory cells for mucosal ACC and protective immunity, which have a longer life span than ACC plasma cells themselves. The present results indicate that such memory cells are distributed along the whole intestine after either p.o. or i.v. immunization. Our data also show that even when priming is restricted to the lower ileum, it is possible by either p.o. or i.v. boosting to elicit significant ACC and protective immune responses in the upper jejunum, albeit of a lower magnitude than those attained in the distal ileum. This suggests that the distribution and persistence of memory cells in the intestine, which probably originate from Peyer's patches (5, 7, 8, 10), do not depend critically on the presence of local antigen. On the other hand, our results indicate that the actual response of ACC and protective immunity, which is different from memory, is restricted to the directly antigen-exposed parts of the intestine. Irrespective of the route of priming (i.v., p.o., or lower ileum), a booster immunization in the lower ileum elicited an immune response in the lower ileum but no response in the proximal jejunum. In contrast to these results, Cebra et al. (2), using two Thiry-Vella loops in rabbits, described that the IgA antibody responses were similar in the antigen-stimulated loops and the unstimulated control loops. However, our results agree with the recent findings of Husband and Gowans (10), who demonstrated that in rats after intraperitoneal priming, a booster deposition of antigen in either of two separated intestinal segments gave rise to an ACC response only in the antigen-exposed segment. Their data further suggested that the antigen did not per se dictate the migration and initial distribution, but that it determined the ultimate distribution of ACC in the lamina propria by stimulating local ACC proliferation. In the absence of local antigen, the few initial ACC disappeared within 12 h. Our results with an exclusively ileal immune response after ileal boosting are likely explained by such antigen-dependent cell proliferation. If this explanation is correct, our results after p.o. or i.v. boosting support the hypothesis that either route of immunization is capable of delivering antigen to the intestine, which can mediate the antigen-driven local proliferation of ACC (10). Husband and Gowans did not study the distribution and fate of memory cells within the intestine. Our results suggest not only that the initial distribution of these cells is independent of local antigen but also that the memory cells, which are different from the ACC, survive in the absence of antigen. The data do not distinguish between the possibility that the memory cells have repopulated the various Peyer's patches along the intestine and the possibility that they are distributed in the lamina propria. In the study by Husband and Gowans, the surgical removal of Peyer's patches from an intestinal segment essentially obliterated the generation of

ACC into the thoracic duct lymph after challenge with antigen. It did not, however, decrease the number of ACC in lamina propria. This was interpreted as an ability of the antigen to stimulate proliferation of simultaneously injected thoracic duct lymph ACC (10, 19), but it could probably equally well represent local antigen-driven proliferation of memory cells already present in the lamina propria from the intraperitoneal priming. More recent studies by Husband et al. (9) have shown that in intraperitoneally primed sheep the ACC response in lamina propria after intestinal boosting was not decreased by cannulation and drainage of the thoracic duct lymph. This supports the possibility of antigen-induced local proliferation of memory cells into ACC in the lamina propria. The recent studies of Mayrhofer and Fisher (15) also support the idea of cell proliferation within the intestinal lamina propria; such proliferation would also be consistent with our previous finding of significant clustering of the ACC in the lamina propria (12).

The influence of the various immunizations on the total number of immunoglobulin-containing cells of different classes was also studied. The majority of the immunoglobulin-containing cells, irrespective of immunoglobulin class, were found in the proximal intestine; this is in agreement with earlier studies in other species (3, 4, 10). This might reflect the greater load of dietary antigens in this part of the intestine and suggests that these antigens maintain a greater number of intestinal immunoglobulin-containing cells than the commensal gut flora, which are heaviest in the distal small intestine. In no instance did the immunization with cholera toxin give rise to any significant difference in either the number or the distribution of immunoglobulin-containing cells in the lamina propria. This was not surprising as far as the cells in the upper part of the intestine were concerned, since the immunoglobulin-containing cells were so many that the specific ACC never exceeded 5% of their number. However, in the lower part of the small intestine, the ACC occasionally exceeded 30% of the total number of immunoglobulin-containing cells without giving rise to any significant increase in the total IgA-, IgG-, or IgM-containing cells. This finding raises the possibility of the existence of hitherto unknown regulatory mechanisms for maintaining a relatively constant number of mature plasma cells in the gut.

Our results show a very good correlation between protective immunity and intestinal ACC, confirming previous findings in mice (12). Although we did not identify the immunoglobulin class of the specific ACC, most of the latter cells were probably of the IgA-containing type, both because the ACC often outnumbered the total IgM- and IgG-containing cells, and because our earlier studies indicated that only locally synthesized IgA antibodies mediate protection against experimental cholera in mice (20).

ACKNOWLEDGMENTS

We are grateful to Lena Ekman, Ulla Svedin, and Amelie Lundgren for skilful technical assistance.

This work was supported by grants 16X-3382 and 12X-2543 from the Swedish Medical Research Council and by a grant from the Faculty of Medicine, University of Göteborg, Göteborg, Sweden.

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