THE ROLE OF ANTIGEN FORM AND FUNCTION IN THE PRIMARY AND SECONDARY INTESTINAL IMMUNE RESPONSES TO CHOLERA TOXIN AND TOXOID IN RATS*

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IgA plasma cells in the intestinal lamina propria are derived largely from precursors in Peyer's patches (1). Absorption of antigen and its interaction with Peyer's patch lymphoid tissue are probably the initial events leading to a mucosal IgA response. Induction of a local IgA response to an orally administered soluble protein in unsensitized animals has usually required prolonged feeding of large amounts of antigen (2, 3). Conditions which might improve the antigenicity of proteins at mucosal surfaces have not been described. In specific, variations in antigen form or function which might enhance either its absorption or its interaction with lymphoid cells have not been studied. Definition of features which improve the immunogenicity of locally applied antigens would give insight into the mechanisms of antigen processing by intestinal lymphoid tissue, permit the design of more detailed studies of the mucosal immune system, and guide the development of materials for oral immunization.

This report describes quantitative studies of the mucosal antitoxin response in rats after enteric administration of different forms of cholera toxin and toxoid, protein antigens which differ primarily in their ability to bind to cell membranes and to activate membrane-bound adenyl cyclase. The results suggest that these two features markedly affect the ability of these antigens to induce primary or secondary type responses in the intestinal immune system when given locally. Crude antigen preparations are also shown to be more effective as mucosal immunogens than are purified materials. Simple, two-dose regimens of local antigen which provoke a mucosal immune response are demonstrated, and immunologic memory induced by a single local dose of antigen is described.

Materials and Methods

Rats. Rats were females of the inbred Wistar-Lewis strain supplied by Charles River Breeding Laboratories (Wilmington, Mass.), and they were housed in a conventional rodent colony.

Toxin and Toxoid Preparations. Purified cholera toxin (choleragen) was NIH lot 1071, made by Finkelstein and LoSpalutto (4). Crude cholera toxin was NIH lot 001, made by Wyeth Laboratories, Marietta, Pa. according to the method of Craig (5), except that it was not dialyzed before lyophilization. Both were provided by Dr. C. E. Miller, National Institute of Allergy and

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TABLE I
Comparison of Toxin and Toxoid Preparations

Preparation	BD_4/mg*	Lf/mg‡	BD_4/Lf§
Purified toxin	8.5×10^7	1,039	8.2×10^4
Crude toxin	3.2×10^3	–	–
Purified B subunit	2.1×10^3	1,631	1.3
Purified toxoid	1.8×10^2	450	0.4
Crude toxoid	0.8×10^3	21	38

* A measure of toxic activity. One BD_4 is the amount of material which produces a blue lesion with a diameter of 4 mm after intradermal injection in rabbits. The lesion size is measured 22–24 h after injection and 1 h after intravenous administration of Pontamine Sky Blue dye. Assays were performed by Dr. J. P. Craig, Downstate Medical Center, Brooklyn, N. Y. (6, 7).

‡ Lf units are a measure of B subunit content of the material based on flocculation with antibody to B subunit. These were determined by Dr. R. O. Thomson, Wellcome Research Laboratories (for purified toxoid and crude toxoid), or Dr. R. A. Finkelstein (for purified toxin and B subunit, from ref. 8). The assay method and unitage of a standard equine anti-B subunit serum have been described elsewhere (8).

§ Toxic activity relative to content of B subunit antigen.

Infectious Diseases, Bethesda, Md. Purified B subunit of cholera toxin (choleragenoid, lot GB76) was a gift of Dr. Finkelstein, University of Texas, Southwestern Medical School, Dallas, Texas (4). Purified cholera toxoid and crude toxoid were prepared by Dr. R. O. Thomson, Wellcome Research Laboratories, Beckenham, England. Purified toxoid was lot Px377c, inactivated with formalin and immunopurified as previously described (3). Crude toxoid was lot VT2177c. It was prepared from a formalin-treated culture filtrate of *Vibrio cholerae*, Inaba 569B, by precipitation with ammonium sulfate (50 kg per 100 liters), dialysis against water, reprecipitation with 2.1 M potassium phosphate pH 8.0, dialysis against water, and lyophilization. The toxic activity and B subunit content of these preparations are summarized in Table I. Residual toxic activity in purified toxoid was negligible (0.0005% of purified toxin, based on BD_4/Lf). It was only threefold greater in purified B subunit (0.0016%), but was 100-fold greater in crude toxoid (0.05%).

Immunization. Toxin or toxoid preparations were given intraduodenally (i.d.)[1] or intragastrically (i.g.). For i.d. immunization, purified antigens were given in 0.5 ml of 0.05 M borate-buffered saline with 0.02% gelatin, pH 7.4; crude antigens were given in 0.5 ml of 0.15 M NaCl. Injection was directly into the duodenal lumen as described elsewhere (3). For i.g. immunization, crude antigens were dissolved in 0.5 ml of 0.2 M $NaHCO_3$ and given through a thin polyvinyl orogastric tube after an overnight fast. None of the immunizing regimens used induced diarrhea.

Antitoxin-Containing Cells (ACC) in Intestinal Lamina Propria. The methods used to identify and count ACC in small bowel lamina propria were exactly as previously reported (3). In brief, pieces of intestine were frozen over liquid nitrogen and 5-μm thick sections were cut on a cryostat and fixed in methanol. ACC were identified by an indirect fluorescent antibody technique which involved sequential staining of tissue sections with purified toxoid followed by an immunopurified fluorescein-conjugated rabbit antitoxin. Their appearance is shown in Fig. 1. The frequency of ACC is expressed as the number per mm^3 in the basal region of the lamina propria.

Comparison of Antigen Effectiveness for i.d. Priming and Boosting. Few ACC appeared in intestinal lamina propria after a single i.d. injection of most antigens tested. Therefore, the efficacy of priming by different antigen forms and doses was judged by measuring the frequency of ACC in lamina propria 5 days after a constant effective i.d. booster. Previous studies had shown the booster response to be maximal at this interval (3). Similarly, the efficacy of different antigen forms and doses for i.d. boosting was judged by measuring the response 5 days after boosting of rats given a constant effective i.d. primer.

Antitoxin Assay. Serum antitoxin was measured by a neutralization assay using S49-1 mouse

[1] *Abbreviations used in this paper*: ACC, antitoxin-containing cells; FCA, Freund's complete adjuvant; i.d., intraduodenal; i.g. intragastric; i.p., intraperitoneal.

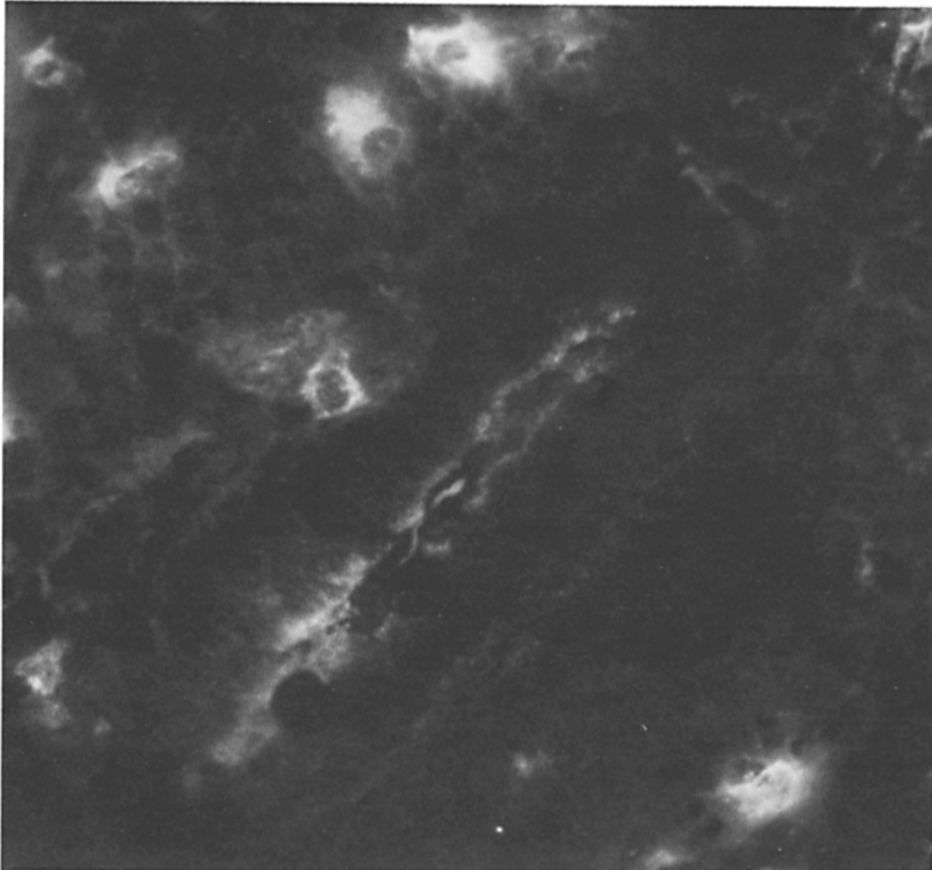

FIG. 1. Antitoxin-containing plasma cells in jejunal lamina propria. A rat was given 40 mg crude toxoid plus 1.5 mg crude toxin i.d. on day 0, and 40 mg crude toxoid i.d. on day 113. Jejunal biopsy was on day 118. Several brightly fluorescent plasma cells are seen near a tangentially cut crypt. Antitoxin is also visible in the apical portion of crypt epithelial cells in a pattern typical of secretory IgA (9). × 640.

lymphoma cells grown in 96-well tissue culture plates. The technique was that described by Ruch et al. (10), with minor modifications. It is based on evidence that cholera toxin, in picogram amounts, inhibits division of S49-1 lymphoma cells, and that this effect is prevented by preincubation of toxin with antitoxin. All assays were performed in duplicate. Results are expressed in antitoxin units/ml determined by comparison with a simultaneously assayed standard antitoxin (Swiss Serum and Vaccine Institute, lot EC3 [A-2/67]-B, 4470 U/ml) provided by Dr. C. E. Miller.

Statistics. Statistical analysis was by Student's t test applied to the difference in geometric mean responses.

Results

i.d. Priming of the Mucosal Immune Response

DOSE-RESPONSE TO DIFFERENT FORMS OF TOXIN AND TOXOID. When rats were given 1 mg of purified toxoid i.d. followed in 14 days by 40 mg of crude toxoid by the same route, no ACC were seen in jejunal lamina propria examined 5 days

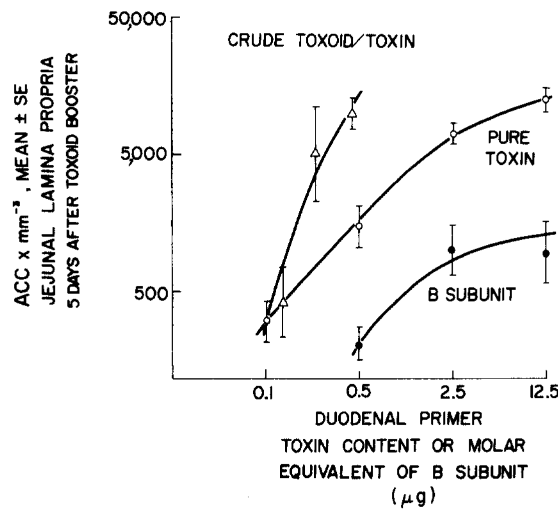

FIG. 2. Dose-response to i.d. priming with purified toxin, B subunit, or crude cholera toxoid plus crude toxin. Priming (i.d.) was on day 0, boosting was on day 14 with 40 mg crude toxoid; jejunal biopsies were taken 5 days later. Each point is the geometric mean of data from at least eight rats. The dose of purified toxoid is given in μg. The dose of B subunit is the molar equivalent of the toxin dose, e.g. 8.3 μg B subunit is the molar equivalent of 12.5 μg toxin. The dose of crude toxoid plus crude toxin was plotted according to its total content of active toxin. Three doses of crude toxoid were used 8, 19, and 40 mg, each with the addition of 1.5 mg crude toxin.

later. In contrast, initial i.d. doses of purified toxin, purified B subunit, or crude toxoid combined with crude toxin gave evidence of local priming which was dose-dependent. This was easily seen by examining the response after a constant i.d. booster dose of crude toxoid (Fig. 2). Unboosted rats examined at the same interval after priming with these materials (day 19) had mean responses which were only 2–17% as large, and unprimed rats examined 5 days after this booster dose had no detectable response (Table II). On a molar basis, purified toxin was about 10-fold more effective than its B subunit, priming being detected with as little as 100 ng of toxin. When compared with respect to its toxin content, crude toxoid combined with crude toxin was more effective than purified toxin and gave a steeper dose-response curve (Fig. 2).

MUCOSAL PRIMING WITH TOXOID-TOXIN COMBINATIONS. In the comparison above, crude toxoid was combined with crude toxin for local priming because the combination was more effective than the individual components. Evidence of this enhancement is summarized in Table III. The priming efficacy of 40 mg of crude toxoid was enhanced five to sevenfold by the addition of 1.5 mg crude toxin ($P < 0.01$). The response to this combination was greater than the sum of the separate priming effects of these materials; combining an equivalent amount of purified toxin (150 ng) with crude toxoid was equally effective ($P < 0.05$). In contrast, addition of an equimolar amount of purified B subunit (100 ng) had no enhancing effect.

These results suggested that toxin enhanced the priming efficacy of toxoid, or vice versa. This possibility was studied using combinations of purified toxoid and purified toxin for i.d. priming. The results, shown in Fig. 3, demonstrate

TABLE II
Immune Response in Rat Jejunum after Local Exposure to Cholera Toxoid and/or Toxin

i.d. Immunization, day 0	ACC Frequency in jejunal lamina propria*		
	Day 5	Day 19 not boosted	Day 19 boosted, day 14‡
Crude toxoid (40 mg) and crude toxin (1.5 mg)	156 (117–207)	231 (138–381)	9,900 (7,560–12,950)
Purified toxin (12.5 μg)	115 –	700 (466–1,051)	13,100 (11,120–15,430)
Purified B subunit (8.3 μg)	144 (132–158)	162 (130–199)	935 (541–1,620)
Purified toxoid (1 mg)	115 –	115 –	115 –
Crude toxoid (40 mg)	115 –	NT	NT

* ACC × mm^{-3} in jejunal lamina propria 5 or 19 days after i.d. injection of the indicated material; a single injection was given unless stated otherwise; geometric mean and range of ± SE. Each mean was derived from 5 to 16 rats. For calculation of means, biopsies with no detectable ACC were given a value of 115 × mm^{-3}.
‡ Booster dose was 40 mg crude toxoid, i.d.
NT, not tested.

TABLE III
Effect of Added Toxin or B Subunit on Mucosal Priming by Crude Cholera Toxoid

Primary i.d. immunization	Number of rats	ACC Frequency in jejunal lamina propria after i.d. boosting*	P‡
Crude toxoid alone (40 mg)	9	1,500 (800–2,800)	–
plus crude toxin (1.5 mg)	11	9,900 (7,600–12,900)	<0.01
plus purified toxin (150 ng)	9	7,400 (5,400–10,400)	<0.05
plus purified B subunit (100 ng)	13	2,700 (1,700–4,300)	NS§
Crude toxin alone (1.5 mg)	5	409 (339–496)	NS

* i.d. boosting was with 40 mg crude toxoid on day 14. Jejunal biopsies were taken 5 days later. ACC frequency is given as geometric mean ACC × mm^{-3} and range of ± SE.
‡ Versus results in rats primed with crude toxoid alone.
§ NS, not significant.

that varying amounts of purified toxin combined with 1-mg doses of purified toxoid were no more effective as local primers than were the doses of purified toxin given alone.

Boosting (i.d.) after Local Priming

DOSE-RESPONSE TO DIFFERENT FORMS OF TOXIN AND TOXOID. After i.d. priming with an effective dose of purified toxin (12.5 μg), booster responses were

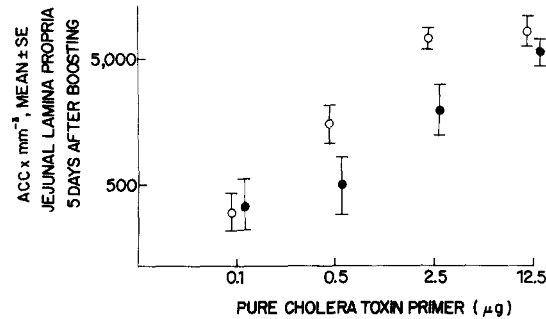

FIG. 3. Dose-response to i.d. priming with purified toxin with or without the addition of purified toxoid. Rats were primed i.d. with purified toxin with or without 1 mg purified toxoid on day 0. Boosting was with 40 mg crude toxoid i.d. on day 14 and jejunal biopsies were obtained 5 days later. Each point is the geometric mean of data from 8 rats. ○, primed with toxin only; ●, primed with toxin plus 1 mg pure toxoid.

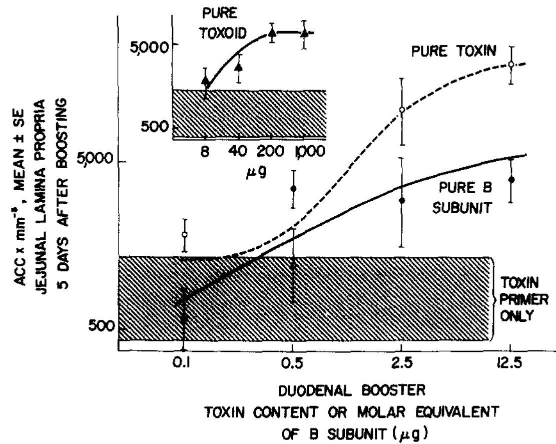

FIG. 4. Dose-response to i.d. boosting with purified toxin, B subunit, or purified toxoid. Rats were primed i.d. with 12.5 μg purified toxin on day 0. Boosting (i.d.) was on day 14 and jejunal biopsies were obtained 5 days later. Each point is the geometric mean of data from eight rats. The hatched bar is range of mean ±SE from jejunal biopsies taken 19 days after i.d. priming, no booster given.

elicited by i.d. doses of purified toxin or B subunit (Fig. 4). Their relative effectiveness for i.d. boosting was similar to that seen for priming, toxin again being about 10-fold more effective on a molar basis. In contrast, purified toxoid, which was ineffective as an i.d. primer in a dose of 1 mg, was an effective i.d. booster; a significant booster effect was seen with a dose as low as 40 μg. None of these materials induced appreciable responses after 5 days in unprimed rats (Table II).

Duration of Local Memory After i.d. Priming. The duration of local memory was determined by giving an i.d. booster of crude toxoid at varying intervals after i.d. priming with crude toxoid plus crude toxin, and counting the jejunal ACC which appeared after boosting. Maximum booster responses were not seen until 2 wk after i.d. priming, and they were of similar size with booster intervals

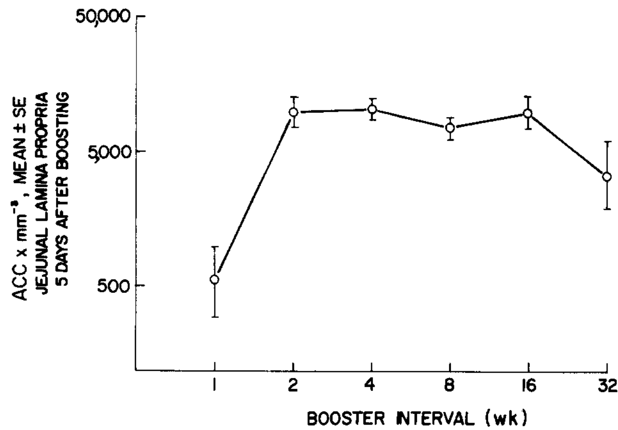

FIG. 5. Duration of local memory after i.d. priming. Rats were primed i.d. with 40 mg crude toxoid plus 1.5 mg crude toxin on day 0. Boosting was with 40 mg crude toxoid i.d. at the indicated interval. Each point is the geometric mean of data from at least eight rats.

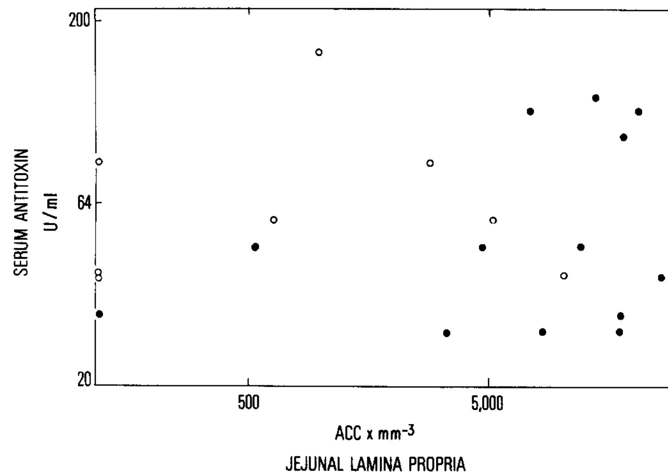

FIG. 6. Relation of mucosal and systemic antitoxin responses after local immunization. Rats were primed i.g. with 40 mg crude toxoid plus 1.5 mg crude toxin (●), or with 12.5 μg purified toxin (○). Boosting was on day 14 with 40 mg crude toxoid i.g. Jejunal biopsy for ACC frequency, and serum for antitoxin titer, were obtained 5 days later.

of as long as 16 wk. With a booster interval of 32 wk, a diminished and more variable booster response was seen (Fig. 5).

Relation of Mucosal and Systemic Antitoxin Responses After Local Immunization. Oral or i.d. immunization induced modest but detectable antitoxin responses in serum. For example, in nonimmunized rats, the serum titer was 15 ± 1 U/ml (geometric mean ± standard error), whereas rats given crude toxoid (40 mg) plus crude toxin (1.5 mg) by mouth, followed in 14 days with crude toxoid by mouth (40 mg) had titers of 63 ± 9 and 51 ± 8 U/ml on days 14 and 19, respectively. There was, however, no correlation between serum antitoxin titers and the frequency of ACC in jejunal lamina propria of individual rats 5 days after local boosting. This includes rats immunized as above or primed i.g. with

TABLE IV
Comparison of Mucosal and Systemic Antitoxin Responses after Local Immunization with Purified Cholera Toxoid

	Serum antitoxin U/ml, mean ± SE*	ACC in jejunal lamina propria*
1. Immunized 1 mg pure toxoid i.d., days 0 and 14	63 ± 22 n = 5	None seen n = 15
2. Not immunized	15 ± 1 n = 28 $P < 0.001$	None seen n = 10

* Determined 5 days after i.d. boosting.

purified toxin and boosted as above (Fig. 6). In addition, rats given two i.d. doses of purified toxoid (1 mg each) developed a significant fourfold elevation in serum antitoxin ($P < 0.001$) with no detectable ACC in jejunal lamina propria (Table IV).

Discussion

We have previously described an IgA antitoxic response in the intestinal mucosa of rats given purified toxoid by a parenteral-intestinal sequence or fed crude toxoid for a prolonged period (3). The present study shows that a vigorous mucosal antitoxin response can also be induced in rat intestine by a simple two-dose local immunizing regimen, provided that the initial dose contains either active holotoxin or its B subunit. The first and second doses appear to induce local primary and secondary type responses, respectively. As discussed below, the marked differences in the effectiveness of cholera toxin, its B subunit, or cholera toxoid as local antigens appear to result from established differences in the properties of these proteins. While all three are antigenic, sharing the major B subunit antigen of cholera toxin, only cholera toxin and the B subunit bind avidly to ubiquitous GM_1 ganglioside receptors on cell membranes (11), and only the holotoxin activates membrane-bound adenyl cyclase (12).

Purified toxoid was ineffective as a local primary antigen, which agrees with our earlier studies in rats and dogs (3, 13). This was not simply due to destruction of antigenicity during toxoiding, since the same material primed the mucosal immune system in rats when given i.p. with (Freund's complete adjuvant (FCA) (3) and in dogs when given subcutaneously (13), and was also effective as a local booster in previously primed rats and dogs (3, 13). Nor was this failure due to the lack of absorption of the toxoid since repeated local doses provoked a systemic antitoxin response. Possibly, purified toxoid was a poor local primer because it could not bind to ganglioside receptors on the membranes of Peyer's patch cells and was thus poorly "trapped". Ferritin, another nonadherent protein (14), is also a poor mucosal antigen in unprimed mice; prolonged feeding of large amounts is needed to induce a local IgA response (2). Poor antigen trapping in unprimed animals may reflect a paucity of macrophages in Peyer's patches, which has been demonstrated in mice (15). The idea that membrane-binding facilitates local priming is supported by the observed greater

priming efficacy of the B subunit which binds avidly to GM_1 ganglioside in cell membranes. On the other hand, this property of membrane adherence by a nonimmune mechanism may give less advantage in local boosting of previously primed animals. This is suggested by the much better performance of purified toxoid as a local booster which may reflect an enhanced ability to bind absorbed antigen to antibody-like surface receptors on an expanded population of sensitized lymphocytes in Peyer's patches.

Cholera toxin was the most effective purified antigen studied, being about 10-fold more effective on a molar basis than its B subunit, both as a local primer and booster. This difference was not likely the result of increased absorption of toxin from the intestinal lumen or its increased trapping in Peyer's patches, since these antigens have similar membrane-binding properties. Cholera toxin, however, also activates membrane-bound adenyl cyclase, thus increasing intracellular levels of cAMP (16). Agents which increase cAMP levels in lymphocytes, as cholera toxin does (17, 18), have marked effects upon a wide variety of lymphocyte-mediated activities (19), including antibody production (20). Cholera toxin has already been shown to both enhance and suppress systemic antibody production when given parenterally, the effect depending upon dose and time of administration in relation to specific immunization (18, 21). These actions are thought to be mediated by its effect upon levels of cAMP in lymphoid cells (18). It is likely that the greater efficacy of cholera toxin than B subunit both as a local primer and booster also reflects this pharmacologic effect on lymphocyte (or macrophage) function. Thus cholera toxin may be a self-adjuvanting molecule possessing two features, membrane-binding and adenyl cyclase activation, which markedly enhance its effect as a local immunogen.

A crude cholera toxoid with residual toxin also gave local priming, and its effectiveness was increased five to sevenfold by the addition of small amounts of crude or purified toxin, but not by equimolar amounts of B subunit. The combination of crude toxoid with residual and added toxin gave a greater local priming than could be explained by its content of toxin alone or by the sum of the separate effects of the crude toxoid and the added crude toxin. These results suggest that the local priming effect of toxin is enhanced by other components of crude toxoid or, conversely, that holotoxin enhances the responses to toxoid forms in the crude toxoid. In either case, the role of holotoxin appears largely dependent upon its ability to activate adenyl cyclase rather than its membrane-binding property, since purified B subunit was ineffective. The simple possibility that toxin and toxoid act in a synergistic manner appears unlikely, however, since this could not be shown with combinations of purified toxoid and toxin. It is possible that other materials or antigen forms in crude toxoid account for its enhanced activity when combined with active toxin.

The duration of local immunologic memory was studied in rats given a single i.d. dose of crude toxoid plus crude toxin. The results differed appreciably from those previously reported for rats primed with purified toxoid plus FCA i.p. (3). Local memory after i.d. priming was undiminished for 4 mo and only modestly diminished at 8 mo. In contrast, local memory present 2 wk after i.p. priming was reduced by 70% when the booster interval was extended to 3 mo (3). We have now shown that this decline in memory is due to the emergence of antigen-

specific suppression of the local immune response as the dominant effect of i.p. immunization (N. F. Pierce, unpublished observations); the mechanism of this suppression is being studied. Irrespective of the mechanism, the present study shows that local memory can last longer than previously reported, possibly because the local primer used was less effective in provoking a late suppressive effect.

Results of this study are of potential importance in the development and evaluation of effective oral vaccines. They show that a simple two-dose oral regimen using a soluble protein can induce a vigorous mucosal immune response in the small bowel. They also emphasize the difficulty in local priming of the mucosal immune system and suggest two specific properties for antigens, membrane-binding, and adenyl cyclase activation, which may enhance their performance as local immunogens raising the possibility that proteins for oral immunization should be selected for, or modified to have, one or both of these properties. The lack of correlation of systemic and mucosal immune responses in individual animals emphasizes the compartmentalization of the systemic and mucosal immune systems and the practical difficulties in assessing the local response to oral antigens, especially in humans in whom access to intestinal tissue or fluid is limited. And finally, although the role of immunologic memory in defense of the intestine by the IgA system is uncertain, its presence is confirmed and it is shown to be relatively prolonged after effective local priming.

Summary

This report describes studies of the mucosal antitoxic response in rats after enteric administration of several forms of cholera toxin or toxoid, proteins which differ primarily in their ability to bind to cell membranes and activate cellular adenyl cyclase. These two characteristics appeared to markedly enhance the local primary response to these antigens. A single dose of toxoid lacking these features was ineffective in local priming even though it was absorbed and induced a systemic immune response. Single dose mucosal priming occurred only with preparations which bind to cell membranes and was enhanced by those which also activate cellular adenyl cyclase. In contrast, single-dose mucosal boosting was best accomplished by materials with these properties but was also seen with a toxoid lacking both of these functions. The property of membrane binding appears to be most advantageous in mucosal priming, perhaps by increasing effective trapping of absorbed antigen in unprimed mucosal lymphoid tissue, whereas the ability to activate adenyl cyclase appears to enhance primary and secondary type responses about equally. Combinations of crude toxoid and toxin were also more effective in mucosal priming than purified materials, a finding which is unexplained. A single dose of this combination induced mucosal priming which was fully developed in 2 wk, undiminished after 4 mo, and only modestly diminished after 8 mo, thus demonstrating relatively prolonged memory in the IgA mucosal immune system. Effective two-dose local immunizing regimens were developed, and it was shown that there was no correlation between the mucosal and systemic secondary antitoxin responses provoked by these regimens.

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