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Intestinal Immune Response to Cholera Toxin: Dependence on Route and Dosage of Antigen for Priming and Boosting

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The influence in immunization with cholera toxin of the route and antigen dose on intestinal antibody formation and protective immunity against experimental cholera was studied in mice. Administration by either the intravenous or oral route induced effective priming as well as boosting of mucosal immunity, with the effects on intestinal immunoglobulin A antitoxin synthesis and protective antitoxic immunity showing excellent concordance. A strong antigen dose dependence was found for both priming and boosting of the local immunity, irrespective of route. Very efficient high-dose priming did, however, partially decrease the dose dependence of the booster response and, conversely, a high booster dose partly overcame the relative inefficiency of low-dose priming. The results suggest that the amount of antigen reaching the immunocompetent cells in the gut rather than the route of administration per se determines the mucosal immunizing effect.

In a recent study we demonstrated that in mice, after immunization with cholera toxin, there was a close correlation between immune protection against experimental cholera and the intestinal formation of immunoglobulin A (IgA) antitoxin. Antibodies produced by extraintestinal lymphoid tissues did not contribute to the protective immunity (12).

The optimal conditions for inducing a local IgA antibody response in the gut are still incompletely understood. Previous studies in mice have indicated that oral (p.o.) administration of cholera toxin is more effective than immunization by the parenteral route (7). Pierce and Gowans (9) found that parenteral priming followed by intraintestinal boosting was more effective than a strictly enteral immunization regimen in inducing antitoxin-containing cells in the small intestine of rats; on the other hand, parenteral boosting after enteral priming was less effective than two enteral immunizations. In humans, a single subcutaneous whole-cell cholera vaccination has been shown to give rise to significant antibody formation in naturally primed individuals but little, if any, such response in persons who had only received subcutaneous priming (10, 11). One possible explanation for these conflicting data on the relative effectiveness of various routes of antigen administration might be the different doses of antigen employed. Considerably larger amounts of antigen have generally been used for enteral than for parenteral immunizations (2, 7, 8).

The aim of the present study was to analyze in further detail the influence of the administration route and the dose of cholera toxin antigen on intestinal antitoxin synthesis and on protective immunity against experimental cholera. It was found that both repeated enteral and parenteral immunizations as well as a combination of the two routes in sequence effectively stimulated local IgA antibody formation and immune protection in the intestine. Furthermore, the results suggest that the amount of antigen that reaches the immunocompetent cells in the gut, irrespective of administration route, is of major importance for the magnitude of the local response obtained.

MATERIALS AND METHODS

Animals. Inbred C57BL/6J($H2^q$) mice of both sexes that were between 9 and 12 weeks of age at the onset of immunization were used.

Immunization. Groups of 6 to 10 mice each were immunized p.o. or intravenously (i.v.) with purified cholera toxin (Schwarz/Mann, Orangeburg, N.Y.). Priming consisted of either four p.o. or four i.v. immunizations: the initial two immunizations were given 10 days apart, and the subsequent ones were every 6th day. Ten days after the last priming dose, a single i.v. or p.o. booster immunization was given.

The p.o. immunizations were done by instillation of various doses of the antigen, dissolved in 0.5 ml of phosphate-buffered saline supplemented with 3% (wt/vol) $NaHCO_3$, into the stomach; the i.v. immunizations were done by injecting different amounts of the antigen, dissolved in 0.1 ml of phosphate-buffered saline, into a tail vein (7). Mice given phosphate-buffered saline-bicarbonate solution p.o. or phosphate-buffered saline i.v. served as controls.

Protection tests. Protective immunity against intestinal challenge with purified cholera toxin was analyzed 4 days after the booster immunization by use of the ligated loop assay previously described (7). At

least five mice from each immunization group and an equal-sized control group were tested on each occasion. In each animal two 5- to 6-cm long loops were ligated on the middle of the small intestine into which were injected graded doses of purified cholera toxin in alternating positions. The fluid accumulation (milligrams of fluid per centimeter of loop) was measured 4 h after challenge, and the toxin dose giving rise to half-maximal fluid accumulation (the 50% effective dose) was estimated by interpolation. The protective effect of immunization—the protection factor—was determined as the ratio between the 50% effective dose for the immunized animals and that for the concurrently tested control animals (7, 12).

Intestinal antibody formation. Local antibody formation in the intestine was determined essentially as described earlier (12) by letting intestinal tissue specimens synthesize protein in vitro and then determining the newly formed antitoxin. Four days after the booster immunization, the small intestine of at least three animals from each group was quickly excised. Minced, carefully washed intestinal tissue pieces, taken as representative for the entire length of the small intestine, were incubated at 37°C for 18 h in a tissue culture tube containing freshly prepared Eagle medium (pH 7.2) supplemented with 5% heat-inactivated normal rabbit serum and 200 IU of penicillin-streptomycin per ml. After completion of incubation, specific antitoxin of the IgA and IgG classes was determined in the tissue culture medium by means of the enzyme-linked immunosorbent assay as described (12), with cholera toxin used as solid-phase antigen and alkaline phosphatase-conjugated anti-mouse IgA or anti-IgG immunoglobulin used for class-specific antibody detection (4).

Serum antibodies. Sera were prepared from blood collected by eye puncture 10 days after immunization by the regimens used for priming. Samples were pooled group-wise, heat inactivated at 56°C for 30 min, and analyzed for IgG and IgA anti-cholera toxin antibodies by means of the enzyme-linked immunosorbent assay.

RESULTS

Influence of route of immunization. The ability of various routes of immunization with cholera toxin to induce a mucosal immune response was studied. Priming by p.o. administration of 5 μg of cholera toxin four times, followed by a single 5-μg p.o. booster 10 days later, gave rise to a highly protective immune response in the mice against intestinal challenge with cholera toxin (Table 1) and confirmed earlier findings (7). Priming and boosting by the i.v. route with doses of cholera toxin similar to those used for the p.o. immunization also resulted in significant, although lower, protection (the gradual increase of the antigen doses was done to avoid toxic effects on the animals).

Sequential combination of the two immunization routes was also highly protective. Thus, p.o. priming followed by parenteral boosting, or vice versa, gave rise to protection similar to that achieved with the "pure" enteral immunization regimen (Table 1).

The mucosal immune response was also measured in terms of antibody synthesis by tissue-cultured intestine from the immunized mice. Table 1 shows that the pure enteral or parenteral immunization regimens as well as a combination of the two routes gave rise to significant formation of specific IgA antitoxin. IgG antitoxin was also found in the tissue culture medium but did not correlate with protection; the highest values were found after the strictly parenteral immunization scheme, which gave less protection than the other regimens (Table 1).

Influence of immunization dose. The influence of the antigen amount used for priming and boosting was also studied. As shown in Table 2, p.o. priming and p.o. boosting with a 10-fold lower dose than the previously used optimal one was ineffective; the intestinal IgA and IgG formation as well as the resistance to experimental cholera did not exceed the values observed in nonimmunized control animals. When only the priming immunization doses were reduced, however, and the booster antigen dose remained high, low but significant intestinal IgA formation as well as protective immunity were induced (Table 2).

TABLE 1. *Mucosal immunity induced by various routes of immunization with purified cholera toxin*

Immunization				Protection factor[a]	Intestinal antibody formation	
Priming		Boosting				
Route	Dose (μg)	Route	Dose (μg)		IgA	IgG
p.o.	5 + 5 + 5 + 5	p.o.	5	8.0	205	45
i.v.	1 + 2 + 4 + 10	i.v.	10	3.4	105	310
p.o.	5 + 5 + 5 + 5	i.v.	10	8.0	NT	NT
i.v.	1 + 2 + 4 + 10	p.o.	5	8.0	265	70
	Controls			1.0	20	<10

[a] Ratio between 50% effective challenge dose values in immunized and concurrently tested control animals.
[b] Enzyme-linked immunosorbent assay antitoxin titers in tissue culture medium; note that IgA and IgG antibodies were measured with different conjugates and thus are not comparable (the IgA detection system was less sensitive than the IgG system). NT, not tested.

TABLE 2. *Antigen dose-dependence for priming and boosting the mucosal immune system*

Immunization[a]				Protection factor[b]	Intestinal antibody formation[c]	
Priming		Boosting				
Route	Dose (μg)	Route	Dose (μg)		IgA	IgG
p.o.	0.5 + 0.5 + 0.5 + 0.5	p.o.	0.5	1.0	15	<10
		p.o.	5	1.9	25	<10
i.v.	1 + 1 + 1 + 1	i.v.	1	1.0	15	70
		p.o.	0.5	1.0	20	15
		i.v.	10	1.8	45	100
		p.o.	5	2.5	165	30
p.o.	5 + 5 + 5 + 5	p.o.	0.5	2.8	35	15
		i.v.	1	5.3	75	20

[a] With purified cholera toxin.
[b] Ratio between 50% effective challenge dose values in immunized and concurrently tested control animals.
[c] Enzyme-linked immunosorbent assay antitoxin titers in tissue culture medium; note that IgA and IgG antibodies were measured with different conjugates and thus are not comparable (the IgA detection system was less sensitive than the IgG system).

Parenteral priming with a low dose of antigen followed by a low i.v. or p.o. booster dose induced neither protection nor intestinal IgA antibody formation. However, similar to the situation in the p.o.-primed mice, it was possible to elicit a significant mucosal immune response by using a high antigen dose for boosting. Both the i.v. and the p.o. routes of boosting then gave rise to some protective immunity as well as local IgA antitoxin synthesis (Table 2).

Finally, the effect of decreasing the booster dose was tested in mice that had received p.o. priming with high doses of antigen. As shown in Table 2, both p.o. and i.v. boosting with antigen doses that were too low to be effective in animals primed with low doses were found to elicit a substantial protective immune response as well as significant intestinal synthesis of IgA antitoxin.

Correlation between protection and *in vitro* antibody synthesis. We previously showed a close correlation between the immune protection in vivo and the in vitro intestinal synthesis of IgA antitoxin after pure enteral and parenteral immunization regimens (12). This correlation was confirmed when the present values of protection were plotted against the IgA antitoxin synthesis titers and the plots were subjected to regression analysis. A highly significant correlation was found ($r = 0.86$). A similar plot between protection and IgG antitoxin did not show a significant correlation.

Role of serum antibodies. The levels of serum antibody attained after p.o. or i.v. priming may have an important effect on the amount of antigen available to the intestinal lymphoid tissues during booster immunization, especially after the i.v. route. This might explain the lower protection and intestinal IgA antibody formation in mice primed and boosted i.v. (see Tables 1 and 2). Serum antitoxin titers were therefore determined in groups of mice 10 days after the animals had received the same immunizations as used for the priming of the previously analyzed groups, i.e., on the day for boosting in the other experiments. As shown in Table 3 the i.v. immunizations induced much higher serum antibody titers than did corresponding p.o. doses of antigen.

DISCUSSION

The present study was performed to test the influence of the administration route and dose of cholera toxin antigen on the local antitoxin antibody response in mouse intestine. Various routes and doses of cholera toxin were combined to allow evaluation of the route and dose dependence of both the actual (booster) response and the memory for mucosal immunity.

The results indicate that both p.o. and i.v. administration of cholera toxin per se can build up a memory as well as boost a mucosal immune response against experimental cholera in mice.

TABLE 3. *Serum antibody titers at the time for boosting*

Immunization		Antitoxin titer[a]	
Route	Dose (μg)	IgG	IgA
p.o.	5 + 5 + 5 + 5	4,000	800
i.v.	1 + 2 + 4 + 10	100,000	100
p.o.	0.5 + 0.5 + 0.5 + 0.5	800	125
i.v.	1 + 1 + 1 + 1	120,000	<10

[a] Determined with the enzyme-linked immunosorbent assay on serum samples collected 10 days after immunization.

Thus, both i.v. and p.o. immunization with a relatively high dose of cholera toxin (5 to 10 times higher than that needed for a maximal systemic antibody response [7]) gave very good priming for a subsequent p.o. booster immunization. Similarly, a high dose of antigen was about equally effective when given i.v. or p.o. in boosting a mucosal immune response in mice primed by the p.o. route. In the light of these data, the lesser IgA and protective responses that were seen after the strictly parenteral immunization regimen are probably explained by the presence of high levels of antibodies in serum at the time for the booster immunization (7, 12), which have a greater blocking effect on i.v.- than on p.o.-administered boosting antigen. This interpretation is supported by serum antibody determinations which showed that on the day for boosting animals primed by the i.v. route had 25 to 150 times higher serum antitoxin titers than the p.o.-primed mice.

Our results show a strong influence of the dose of antigen used for priming on the local immune response induced by the booster immunization. A 5- to 10-fold increase in either the p.o. or i.v. priming dose resulted in a sharp increment in the response attained after boosting. We interpret this effect to indicate a clear dose dependence of immunological memory for local antibody formation. Our previous studies of the time course of mucosal immunity have shown that antibody formation following each of the priming immunizations is transient so that after 10 days there is very little residual IgA antibody synthesis (<15% of the levels attained 4 days after boosting [11]), there are only few antitoxin-containing cells (6), and there is no detectable protection (7, 12). These findings argue against the possibility that the observed antigen dose dependence during priming simply reflects recruitment of more antibody-forming cells persisting through the booster response.

Also, the booster response in the intestine was highly dependent on the antigen dose. Boosting with a low dose of cholera toxin resulted in only partial immunity and then only in animals which had been primed with a high antigen dose. This suggests that memory cells induced by low antigen doses probably had a relatively low affinity for cholera toxin.

In accordance with other studies (2, 7–9), it was found that stimulation of a significant local immune response in the gut requires larger amounts of antigen than does stimulation of a systemic response. Still, in comparison with other protein antigens such as ferritin (1) or Formalin cholera toxoid (8), the amount of cholera toxin that is needed to stimulate a mucosal immune response is remarkably small, especially with regard to the p.o. route of administration. For these other antigens p.o. administration of milligram amounts has been needed to obtain a local immune response, whereas a few micrograms of cholera toxin is sufficient (7, 9, 12). The high immunogenicity of orally administered cholera toxin is probably due to two different properties of the antigen: its high-affinity binding to the intestinal mucosa and its ability to stimulate adenylate cyclase activity (3, 8). Thus, a cholera toxoid with intact binding capacity to the GM1 ganglioside receptors along the intestine, such as the newly developed B-subunit immunogen (5), should be considerably more effective in inducing a mucosal immune response than most other protein antigens, including Formalin cholera toxoid (8), which lacks such binding capacity. In support of this, Pierce (8) showed that enterally administered cholera toxin and B subunit were both capable of stimulating a mucosal immune response in contrast to toxoids lacking membrane-binding activity.

The finding that similar antigen amounts are needed for effective parenteral and enteral immunization might have direct practical implications. If this is true also for humans, oral administration of cholera toxoid should be preferred both because it will cause less discomfort for the patients and because this administration regimen will require less extensive purification of the antigen.

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