INFECTION AND IMMUNITY, Jan. 1984, p. 341–346
0019-9567/84/010341-06$02.00/0

Vol. 43, No. 1

Induction of Optimal Mucosal Antibody Responses: Effects of Age, Immunization Route(s), and Dosing Schedule in Rats

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Received 4 August 1983/Accepted 11 October 1983

The antitoxin response in intestinal mucosa was studied in rats immunized either intestinally or by combined parenteral and intestinal dosing with cholera toxin or cholera toxoid. Attention was given to the duration of enteric priming and the magnitude and time course of mucosal anti-cholera toxin responses in rats of defined age. Cholera toxin given only intraduodenally was a more efficient priming immunogen in young rats than in older rats and caused priming that lasted at least 32 weeks; repeated enteric doses increased local priming and repeatedly evoked vigorous mucosal anti-cholera toxin responses which occurred rapidly and declined slowly. Results differed when a portion of the immunizing regimen was parenteral. Cholera toxoid given intraperitoneally (i.p.) caused mucosal priming that peaked promptly and then rapidly declined; parenteral boosting after enteric priming was much more effective given i.p. than subcutaneously; moreover, the booster response was brief, virtually disappearing within 11 days, and could not be reproduced by a second i.p. immunization. These results accord with evidence that parenteral immunization both stimulates and suppresses mucosal secretory immunoglobulin A responses, whereas local immunization is not known to be suppressive. Evidence for parenterally induced suppression was the rapid decline in mucosal priming after i.p. immunization, the shortened mucosal antibody response after i.p. immunization, and possibly the inability to parenterally evoke a booster response twice. In these studies, the level of priming observed at different intervals after parenteral, enteric, or combined immunization appeared to reflect the sum of priming and suppressive effects evoked by the preceding immunization(s).

Optimal methods for inducing specific mucosal secretory immunoglobulin A (sIgA) responses have not been fully defined. One question at issue concerns the relative efficacy of systemic (parenteral) or local (mucosal) routes of antigen administration. Present knowledge of the design and function of mucosa-associated lymphoid tissue seems to favor local immunization with the object of stimulating precursor IgA B cells in submucosal lymphoid follicles, where they are most numerous (4). However, some studies show that systemic immunization (2, 10, 11, 21), or combined systemic-local immunization (1, 8, 17, 18), can evoke mucosal or glandular sIgA responses, sometimes quite efficiently. On the other hand, other reports show that systemic immunization is ineffective (12, 18, 20) or even suppresses mucosal sIgA responses (5, 19).

Possibly, some of the apparent contradictions in the results of previous studies reflect shortcomings in their design. Thus, potentially important determinants of the immune response, such as age and dosage number and interval, have not usually been considered; carefully defined antigen dose-response curves have not often been obtained; and the time course of the desired sIgA response and the ability to evoke the response repeatedly have not been determined.

Cholera toxin (CT) is a potent mucosal immunogen to which rats are not naturally primed (13). We and others have used CT, or its antigenic derivatives, as a model antigen to explore basic features of the mucosal sIgA system (3, 13, 15, 16, 18). We have shown that intestinal sIgA anti-CT responses are evoked in rats given sequential intraduodenal (i.d.) doses of CT (i.d.-i.d.), primed intraperitoneally (i.p.) and boosted i.d. (i.p.-i.d.), or primed i.d. and boosted i.p. (i.d.-i.p.) (13, 18, 19). We have also shown that primary immunization given i.p., or by other parenteral routes, evokes late suppression of the mucosal sIgA antitoxin response and that this suppression is mediated by suppressor T cells, serum antitoxin, or both (9, 14, 19). The present report describes further studies with this model aimed at more precise, quantitative comparison of the efficacy of mucosal immunization with local, or combined systemic-local, antigen administration, giving particular attention to the duration of enteric priming and to the magnitude and time course of evoked mucosal anti-CT responses.

MATERIALS AND METHODS

Rats. Rats were supplied by Charles River Breeding Laboratories, Inc. (Wilmington, Mass.) and were housed in a conventional rodent colony. All were females of the inbred Lewis strain (Lew/CrlBR) and weighed 125 to 150 g (7 to 8 weeks old) when first immunized, unless stated otherwise.

Immunization. Purified CT, purified cholera toxoid inactivated by formalin (CTd), and crude cholera toxin (CrT) are described elsewhere (13). CT was obtained from Schwarz/Mann (Orangeburg, N.Y.). CTd was a gift of R. O. Thomson, Wellcome Research Laboratories (Beckenham, Kent, England). CrT was lot 001, made by Wyeth Laboratories (Radnor, Pa.) and provided by the National Institute of Allergy and Infectious Diseases.

CT was diluted in 0.01 M phosphate-buffered saline, pH 7.4, with 0.1% gelatin. CTd and CrT were diluted in 0.9% NaCl. For parenteral immunization, only CTd was used. It was given in a volume of 0.2 ml i.p., intravenously (i.v.), or subcutaneously (s.c.) over the flank. In some instances it was emulsified with an equal volume of Freund complete adjuvant (CFA) (Difco Laboratories, Detroit, Mich.) or precipitated by adsorption to 20% aluminum hydroxide. Intracolonic (i.c.) or i.d. immunization was with CT, CTd, or CrT injected directly into the bowel lumen in a volume of 0.5 ml with a small laparotomy as previously described (18).

ACC in intestinal lamina propria. The methods used to

identify and count antitoxin-containing plasma cells (ACC) in the lamina propria of intestinal biopsies are described elsewhere (18). In brief, pieces of proximal jejunum or ascending colon were taken from anesthetized rats and frozen over liquid nitrogen, and 5-μm sections were cut on a cryostat and fixed in methanol. ACC were identified by a fluorescent-antibody technique that involved sequential staining with CTd and an immunopurified, fluorescein-conjugated rabbit antitoxin (18). The frequency of ACC is expressed as the number per cubic millimeter in the crypt region of jejunal lamina propria or the full thickness of colonic mucosa. To determine geometric means, biopsies having no detectable ACC in 50 high-power fields (×800) were assigned a value of 115/mm^3, which was the lower limit of sensitivity of this scoring method.

Statistics. Statistical analysis was by Student's *t* test applied to geometric mean frequencies of ACC in intestinal lamina propria. Geometric means were used because they reflect the logarithmic manner in which the ACC response expands after immunization and because mucosal protection against challenge with CT correlates linearly with the geometric mean frequency of ACC in the lamina propria (17).

RESULTS

Influence of age on the ACC response to CT given i.d. Initial studies examined the influence of age at priming on the mucosal antitoxin response to two doses of CT given i.d. The priming dose was given at 3 to 4, 7, 15, 23, or 39 weeks of age; an identical i.d. challenge dose was given 2 weeks later. The numbers of ACC in jejunal biopsies taken 5 days later are summarized in Fig. 1. Rats were most responsive when CT immunization was begun at age 7 weeks, and responsiveness was nearly as great in weanlings aged 3 to 4 weeks. CT doses 11-fold greater were needed to evoke comparable responses in rats aged 15 weeks, and even larger doses were required for rats aged 23 or 39 weeks at priming. Among the latter, maximum ACC responses were lower than in younger rats and appeared to decline with the highest CT dose (400 μg/kg). The age-related decline in response to CT immunization was not due simply to increasing body size, because the doses in Fig. 1 are expressed as micrograms per kilogram of body weight.

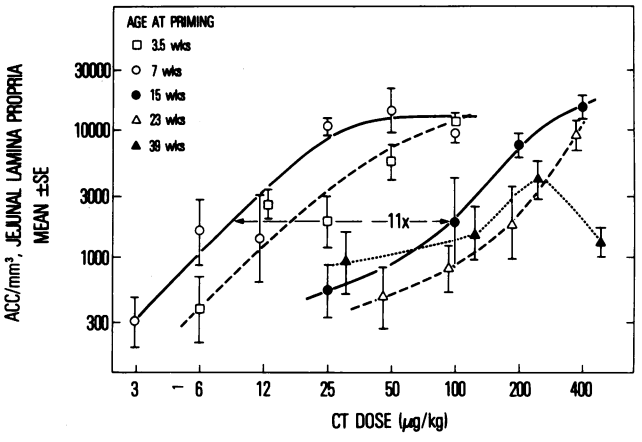

FIG. 1. Effect of age on jejunal ACC response to enteric immunization with CT. Rats were immunized twice i.d. with the indicated dose of CT. Priming was at the age shown, and boosting was on day 14. Jejunal biopsies for ACC content were obtained 5 days later. The arrow indicates the fold difference in the immunizing doses required to evoke mean ACC responses of 2,000/mm^3 in rats aged 7 or 15 weeks at priming. Each point reflects data from at least four rats.

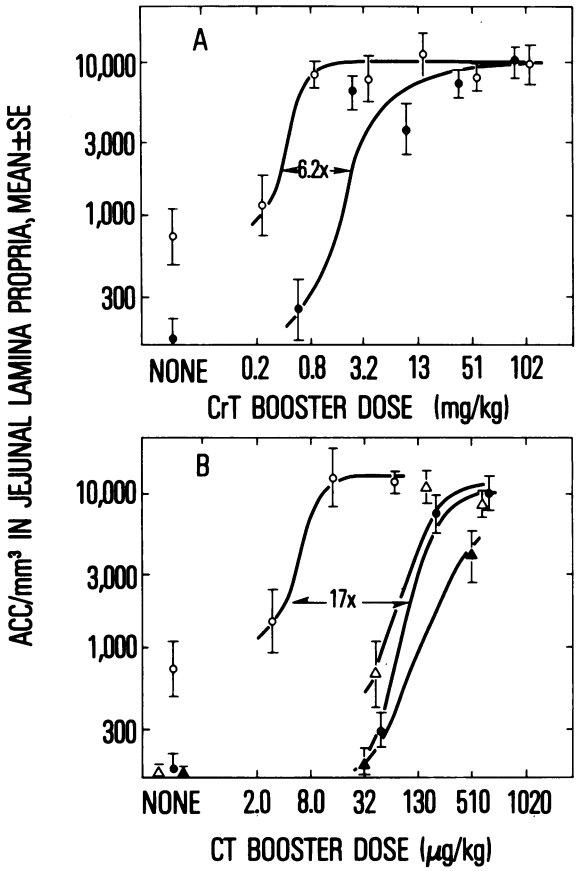

FIG. 2. Duration of enteric priming after i.d. immunization with CT or CrT. Rats aged 7 weeks were primed i.d. with 12.5 μg of CT. Intraduodenal boosting with graded doses of CrT (A) or CT (B) was given after 2 (○), 8 (●), 16 (△), or 32 (▲) weeks. Jejunal biopsies for ACC content were taken 5 days after boosting. Arrows indicate fold differences in the booster doses required to evoke mean ACC responses of 2,000/mm^3 in rats boosted 2 or 8 weeks after priming. Each point reflects data from at least five rats.

Effect of immunization route on duration of enteric priming for an ACC response. (i) Duration of priming after an i.d. dose of CT. The duration of priming for a mucosal antitoxin response was studied in rats given a single 12.5-μg dose of CT i.d. at age 7 weeks (100 μg of CT per kg); this dose is known to prime for a vigorous jejunal ACC response to an identical CT dose given 2 weeks later (13). Intraduodenal challenge with graded doses of CT or CrT was performed 2 to 32 weeks later. ACC responses in jejunal biopsies taken 5 days after i.d. challenge are shown in Fig. 2. With either challenge antigen, greater doses per kilogram were required to provoke comparable responses at 8 to 32 weeks after priming than at 2 weeks after priming. Thus, 6.2- and 17-fold more CrT and CT, respectively, were required at 8 weeks than at 2 weeks after priming to cause a mean response of 2,000 ACC per mm^3. Similar dosage increases were required with intervals of 16 or 32 weeks. Although greater challenge doses were required, the maximum ACC responses seen after challenge intervals of 8 or 16 weeks were similar to those in rats challenged 2 weeks after priming.

(ii) Duration of priming after an i.p. dose of CTd plus CFA. The duration of priming after an i.p. dose of CTd plus CFA

was similarly studied. Rats were given 40 μg of CTd plus CFA i.p. at age 7 weeks; this is known to prime for a vigorous jejunal ACC response to CT or CTd given i.d. 2 weeks later (18, 19). Intraduodenal challenge was with graded doses of CrT 2 or 8 weeks later; CrT was used because the very large booster doses required to evoke responses 8 weeks after priming would have consumed inordinate amounts of CT. ACC responses in jejunal biopsies taken 5 days after challenge are shown in Fig. 3. The maximum ACC responses achieved after either challenge interval were similar. However, much larger doses of CrT were needed after the 8-week interval, the dose required to evoke a mean response of 2,000 ACC per mm^3 being 565-fold greater when challenge was 8 weeks, rather than 2 weeks, after i.p. priming. Thus, although the dose responses to i.d. challenge with CrT were similar 2 weeks after i.d. or i.p. priming, a 90-fold-greater challenge dose of CrT was required 8 weeks after i.p. than after i.d. priming to achieve comparable ACC responses (cf. Fig. 2A and 3).

Compared effects of prior enteric or parenteral immunization on the mucosal ACC response to enteric immunization. Suppression of the mucosal ACC response to enteric doses of CT follows parenteral immunization with CTd (19). The extent of such suppression, however, has not been determined by dose-response studies, nor have age-matched controls been used when parenteral immunization preceded enteric immunization by more than 2 weeks (19). Moreover, the effects of prior parenteral or enteric immunization on the response to subsequent enteric immunization have not been compared. To study these effects, rats were given a single parenteral dose of CTd (40 μg) or an i.d. dose of CT (12.5 μg) at age 7 weeks. Enteric immunization with graded i.d. doses of CT was given 8 and 10 weeks later. ACC responses in jejunal biopsies taken 5 days later are summarized in Fig. 4. The initial parenteral dose of CTd (i.p. or s.c., with or without CFA or aluminum hydroxide) caused reduced responsiveness to subsequent i.d. immunization, whereas an initial i.d. dose of CT had the opposite effect. Thus, i.d. doses of CT required for mean responses of 2,000 ACC per mm^3 were 4- to 14-fold greater in rats previously given parenteral CTd than in those given none. In contrast, they were sixfold lower in rats given an earlier i.d. dose of CT.

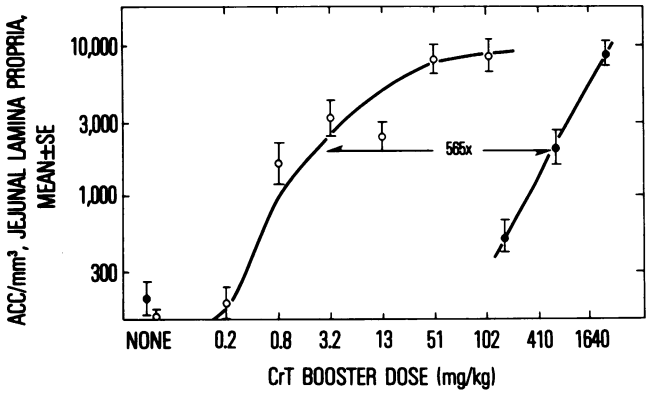

FIG. 3. Duration of enteric priming after i.p. immunization with CTd plus CFA. Rats aged 7 weeks were primed i.p. with 40 μg of CTd plus CFA. Intraduodenal boosting with graded doses of CrT was given after 2 (○) or 8 (●) weeks. Jejunal biopsies for ACC content were taken 5 days after boosting. The arrow indicates the fold difference in the booster doses required to evoke mean ACC responses of 2,000/mm^3 in rats boosted 2 or 8 weeks after priming. Each point reflects data from at least six rats.

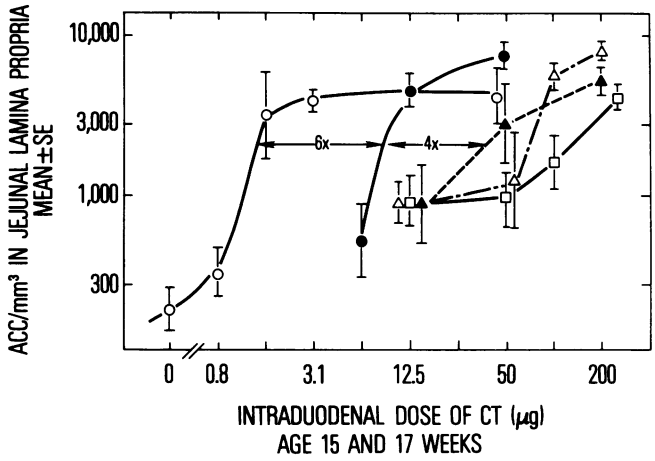

FIG. 4. Compared effects of prior enteric or parenteral immunization on jejunal ACC response to i.d. immunization. Rats aged 7 weeks were given a single parenteral dose of CTd (40 μg), an i.d. dose of CT (12.5 μg) (○), or nothing (●); parenteral CTd was given i.p. in normal saline (△), CFA (▲), or s.c. with aluminum hydroxide (□). At age 15 and 17 weeks, rats were given graded i.d. doses of CT as shown. Jejunal biopsies for ACC content were taken 5 days after the final i.d. immunization. Arrows indicate fold differences in the i.d. doses required to evoke mean ACC responses of 2,000/mm^3 in rats originally immunized i.d. or i.p. with CFA compared with those given no initial immunization. Each point reflects data from at least six rats.

Local priming and parenteral boosting of an enteric ACC response. CTd given i.p. causes a secondary jejunal ACC response in rats primed enterically with a crude toxoid-toxin mixture (18). Further studies aimed to determine (i) the efficacy of parenteral boosting by different parenteral routes and (ii) whether a parenteral booster effect could be repeatedly achieved. In these studies rats were primed i.c. because this primed the entire small bowel without causing a detectable primary jejunal ACC response (15). Thus, all ACC appearing in jejunal lamina propria after i.d. challenge were due to the challenge.

In initial studies, rats were challenged with CTd by various parenteral routes 2 weeks after i.c. priming. Intraperitoneal challenge caused substantial enteric ACC responses that were twofold smaller in the jejunum than those evoked by i.d. challenge (Table 1); challenges given i.v. or s.c. also caused significant, although progressively smaller, jejunal ACC responses. Thus, the jejunal ACC response after s.c. boosting was 90% less than after i.p. boosting ($P < 0.001$) and was still 75% smaller when the s.c. booster dose was increased fivefold ($P < 0.005$). CTd given s.c. was also much less effective than the same dose given i.d., the jejunal ACC response being reduced by 88% ($P < 0.001$).

In a second study, the effectiveness of repeated booster immunizations given i.p. or i.d. was compared. After i.c. priming with CT, rats were boosted once or twice at 2-week intervals with CT given i.d. or CTd given i.p. The first booster immunizations, given i.p. or i.d., evoked similar, vigorous jejunal ACC responses (Table 2). The results differed strikingly, however, after the second booster. The response to a second i.d. boost was almost fourfold larger than the first (not significant), whereas the second i.p. boost caused a response 96% smaller than the first ($P < 0.001$). Thus, the ACC response to a second i.d. booster was 80-fold greater than that after a second i.p. booster. Moreover, the poor response to repeated i.p. boosting was not

TABLE 1. Effects of booster route and dose on ACC in jejunal lamina propria of colon-primed rats

Booster[a]			Jejunal ACC/mm³ response[b]
Route	Antigen	Dose	
	None		115 (–)
i.p.	CTd	40	4,930 (1.4)
i.v.	CTd	40	1,740 (1.5)
s.c.	CTd	40	490 (1.4)
s.c.	CTd	200	1,190 (1.3)
i.d.	CTd	200	9,680 (1.2)

[a] Inbred Fischer male rats (F-344/CrlBR) were primed i.c. with 12.5 μg of CT. Booster immunization was 14 days later. The booster dose is in micrograms.

[b] Measured in jejunal lamina propria 19 days after i.c. priming; geometric mean (× ÷ standard error); each mean represents data from six to eight rats.

significantly improved by a fourfold increase in the size of the second i.p. dose ($P > 0.10$).

Effect of parenteral immunization on time course of jejunal ACC response. A final experiment compared the time courses of jejunal booster responses in rats immunized only enterically (i.c.-i.d. or i.d.-i.d.) or primed enterically and boosted parenterally (i.c.-i.p.). For enteric immunization the CT dose was 12.5 μg; for i.p. immunization the CTd dose was 40 μg. Doses were separated by 14 days. For each regimen, the jejunal ACC response peaked 5 days after boosting (Fig. 5). In enterically immunized rats, the subsequent decline was relatively slow, whereas in those primed i.c. and boosted i.p. the decline was rapid. Thus, by day 11 the jejunal ACC response had declined 30-fold in rats immunized i.c.-i.p. but only 1.4- to 4-fold in those immunized only enterically.

DISCUSSION

This study shows that CT given only i.d. was a more efficient local immunogen in young than in older rats, that a single i.d. dose caused priming which lasted at least 32 weeks, that repeated enteric doses increased local priming and repeatedly evoked vigorous mucosal anti-CT responses, and that the local anti-CT response caused by i.d. boosting after enteric priming occurred rapidly and then declined slowly.

These findings accord with reports that sequential enteric doses of CT, or some other antigens, evoke distinct primary and secondary types of intestinal sIgA responses in several species (3, 7, 13) and that priming, once established, is relatively long lasting (13). Prolonged priming is due to long-lived memory lymphocytes, most of which remain in gut-associated lymphoid tissue, but some of which circulate (3, 6, 15, 16). The greater priming seen after repeated enteric doses of CT than after a single dose (Fig. 4, Table 2) probably reflects increased numbers of such cells; it is known that memory cells are generated after each enteric dose of CT (16). The modest decline in responsiveness observed when rats were boosted enterically 8 weeks or more, rather than 2 weeks, after enteric priming was similar in magnitude to the diminished responsiveness associated with increasing age (cf. Fig. 1 and 2) and could be entirely due to that process. The mechanism of the age-related effect on mucosal immunization is unknown.

When a portion of the immunization regimen was given parenterally, distinctly different results were obtained. It was shown that CTd given i.p. with CFA caused mucosal priming that peaked promptly but then rapidly declined; that the efficacy of parenteral boosting after enteric priming depended upon the parenteral route used, i.p. being most effective and s.c. least; that i.p. boosting was effective only once, a second booster causing almost no jejunal anti-CT response; and that the specific jejunal antibody response evoked by i.p. boosting after enteric priming was brief, virtually disappearing within 11 days.

The causes of these differing effects of parenteral and enteric immunization were not determined. However, they may reflect the fact that parenteral immunization can, by independent mechanisms, both stimulate and suppress a mucosal sIgA response (19); in contrast, enteric immunization is also stimulatory but is not known to have a suppressive effect. The stimulating effects of parenteral antigen included priming of nonimmune animals for a specific mucosal immune response and evoking of such a response in animals previously primed enterically. Because the precursor lymphocytes involved in sIgA responses are largely sequestered in mucosa-associated lymphoid tissue (4), it is likely that parenteral antigen must encounter that tissue, or lymphocytes which migrate to it, for stimulation to occur. The greater efficacy of i.p. than s.c. immunization for mucosal priming, as observed previously (19), or mucosal boosting, as observed in this study, suggests that the i.p. route favors that encounter.

The apparently suppressive effects of parenteral immunization included the rapid decline in mucosal priming 8 weeks after primary i.p. immunization (Fig. 3) and the abbreviated course of the mucosal anti-CT response in rats primed enterically and boosted i.p. rather than i.d. (Fig. 5). Parenterally induced suppression may have been due to T cells that arose systemically and migrated to mucosal lymphoid tissue. Such cells arise slowly (i.e., 4 to 16 weeks) after primary parenteral immunization and efficiently suppress subsequently attempted mucosal priming by locally applied antigen (9). Whether they also interfere with established mucosal priming or can arise with sufficient rapidity after parenteral boosting to shorten the booster response is, however, unknown. The inability to stimulate a mucosal anti-CT response by a second i.p. booster in rats originally primed enterically (Table 2) may also reflect suppression evoked by the first immunization, but other explanations are

TABLE 2. ACC responses in jejunal mucosa after colonic priming and single or repeated duodenal or i.p. booster doses of CT or CTd

Booster				Jejunal booster AAC/mm³ response[b]
Route	Antigen	Dose 1	Dose 2[a]	
	None			115 (–)
i.d.	CT	12.5		4,090 (1.6)
i.p.	CTd	40		4,930 (1.4)
i.d.	CT	12.5	None	970 (1.5)
i.d.	CT	12.5	12.5	15,880 (1.2)
i.p.	CTd	40	None	115 (–)
i.p.	CTd	40	40	200 (1.3)
i.p.	CTd	40	160	630 (1.7)

[a] Inbred Fischer male rats were primed i.c. with 12.5 μg of CT. Booster doses of CT or CTd, shown in micrograms, were given 14 and, if a second dose was given, 28 days later.

[b] Measured in jejunal lamina propria 19 days after i.c. priming; geometric mean (× ÷ standard error); each mean represents data from six to eight rats.

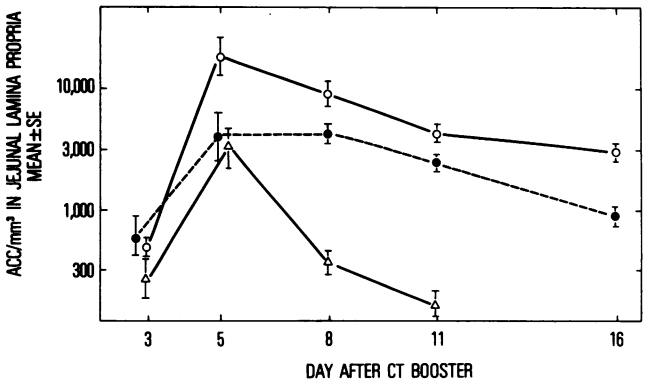

FIG. 5. Effect of parenteral immunization on time course of jejunal ACC response. Rats were given a primary immunization at age 7 weeks and boosted 14 days later. Jejunum was examined for ACC content at the indicated interval after boosting. The immunizing sequences were i.c.-i.d. (●), i.d.-i.d. (○), and i.c.-i.p. (△). All enteric immunizations were with CT (12.5 μg), whereas i.p. immunization was with CTd (40 μg in normal saline). Each point reflects data from at least six rats.

possible. These include reduced delivery of injected antigen to sensitized mucosal lymphocytes due to enhanced antigen trapping by systemic lymphoid tissue, complex formation with serum antibodies, or both.

In this study, the level of priming for a specific mucosal sIgA response was assessed by determining the minimum enteric booster dose that caused a vigorous mucosal antibody response. Such priming was affected by the route(s) and number of previous immunizations and the interval between immunization and challenge. Although other explanations may be possible, two observations suggest that the level of priming observed at different intervals after parenteral, enteric, or combined immunization reflected the sum of priming and suppressive effects evoked by the preceding immunization(s). The first is evidence that i.p. immunization caused priming which, after 2 weeks, equalled that evoked by the i.d. route but after 8 weeks had declined 90-fold, i.e., the i.d. booster dose required for a vigorous mucosal anti-CT response was 90-fold greater than in i.d.-primed rats. Although it is possible that memory cells which mediated priming at 2 weeks had largely disappeared by 8 weeks, this was not the case in rats primed i.d. and thus seems unlikely. More likely, suppressive mechanisms, probably including suppressor T cells, arose slowly after i.p. immunization and imposed their effect upon a persisting population of memory cells. The second is the observation that the suppressive effect of i.p. immunization was largely reversed by a subsequent enteric immunization. This was seen in rats boosted twice i.d., 8 and 10 weeks after i.p. priming. With this regimen, the booster doses required to evoke vigorous mucosal anti-CT responses were only fourfold greater than in rats not given the i.p. immunization (Fig. 4). This marked decline in parenterally induced suppression suggests that the first i.d. booster caused enhanced mucosal priming that nearly overcame the suppression evoked by the earlier i.p. immunization.

These observations may have considerable relevance for efforts to develop effective nonliving vaccines for stimulation of lasting, protective mucosal sIgA responses. They support the view that such responses will probably be evoked best by locally applied antigen and suggest that immunization regimens which include parenteral antigen administration should be carefully studied with regard to the duration of priming or the protective responses which they evoke.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grants AI 14480 and AI 19547 from the National Institute of Allergy and Infectious Diseases. Research facilities were provided by the Gerontology Research Center, National Institute on Aging, under its Guest Scientist Program.

Excellent technical assistance was provided by John B. Sacci, Jr., and Trudy A. Lund.

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