

## Circulating Antitoxin in Rabbits After Ingestion of Diphtheria Toxoid

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Immune responses following antigen ingestion vary from stimulation to suppression depending on animal species, antigen, and experimental protocol. Young adult rabbits were given either 0.02% diphtheria toxoid or 0.1% bovine serum albumin in drinking water for 10-day periods, a protocol previously found to immunize human infants fed bovine serum albumin. Specific serum antibody was detected by radioimmunoassay in 10 of 13 rabbits fed diphtheria toxoid for 10 days and 11 of 13 rabbits fed bovine serum albumin. Response to oral diphtheria toxoid in some animals was equal to that after an injection of alum-precipitated diphtheria and tetanus toxoids. All rabbits fed a second course of either antigen showed an enhanced, rapid rise in antibody concentration. Avidity of the antibody also increased markedly, suggesting that priming and memory had been established during the first feeding. Similar anamnestic responses occurred in animals given a parenteral challenge after ingesting antigen. One month after administration of diphtheria toxoid, no significant difference in serum antibody levels was present between unfed animals challenged with diphtheria-tetanus toxoids and those given a diphtheria-tetanus toxoid challenge after one or two oral courses, although avidity of the antitoxin was higher in fed animals. Antibody activity could be detected only in the immunoglobulin G serum fractions with either antigen. These responses suggest that oral immunization protocols using protein antigens can be adapted for use against toxins causing disease.

Both humoral and secretory antibodies are detectable in normal individuals after the lymphoid tissues associated with mucosal surfaces are exposed to a variety of environmental antigens (1, 2, 8, 16, 21). The mucosal and systemic immune responses to these ingested antigens and the interactions between these two systems appear to vary greatly with the nature of the antigen and differing experimental conditions (1, 5, 11, 20, 23). A better understanding of these variables is important to develop effective ways to stimulate protective immunity by mucosal immunization.

After ingestion of 0.1% bovine serum albumin (BSA) for 52 days, rabbits produce specific anti-BSA of the immunoglobulin G (IgG) isotype which is readily detected in serum (20, 23, 24) and milk (unpublished data), and they respond anamnastically to a subsequent parenteral or oral challenge (20). The migration of lymphocytes of the IgG isotype from the gut-associated lymphoid tissues to secretory lamina propria (15, 25) and the relative lack of plaque-forming cells (24) in the spleen and lymph nodes of rabbits during the first 5 weeks of BSA ingestion suggest that sensitization to antigen probably occurs in Peyer's patches (4) and that most of the circulating antibody detected is produced by cells in

the gut-associated lymphoid tissues and not in the systemic lymphoid tissues.

Antibody responses similar to those of the rabbits were detected in human premature infants fed formula containing BSA for 10 days (22). This systemic immune response of rabbits and infants to ingested 0.1% BSA differs markedly from the suppression of the humoral systemic responses noted after the ingestion of BSA (G. Silverman, B. A. Peri, and R. M. Rothberg, submitted for publication) or other proteins (11) observed in mice and rats.

To compare immune responses to a protein of bacterial origin with those to a mammalian protein (BSA), diphtheria toxin was chosen since protection in humans is determined by the serum antitoxin response, which can be measured by a sensitive, reproducible radioimmunoassay (19), and since diphtheria toxoid has been safely administered orally to humans (9).

Several recent studies have shown that present diphtheria immunization procedures, although effective, have not been administered to a significant number of children in the United States (14, 19). An oral immunization protocol might overcome reluctance to obtain repeated parenteral injections and meet a need in areas where medical personnel are in short supply. To

determine some of the parameters of an optimal but practical method to establish circulating immunity by ingestion of bacterial proteins, the systemic immune response in rabbits to one or two 10-day courses of diphtheria toxoid ingestion was characterized, and the capacity of such orally immunized animals to respond to a subsequent parenteral injection was determined. The 10-day period of toxoid ingestion was used since it has been shown to be sufficient to immunize human infants to a protein added to their formula (22). Since the duration of antigen feeding has been shown to have differing effects on the systemic immune response (5), the rabbit immune response to 10-day periods of BSA ingestion also was characterized and compared with the previously described responses of rabbits to 52 days of BSA ingestion (20, 23).

#### MATERIALS AND METHODS

**Experimental animals.** New Zealand White rabbits weighing 2.5 to 3 kg (Thompson Research Industries Corp., Monee, Ill) were housed individually and fed Purina rabbit chow, performance blend.

**Antigens.** Partially purified diphtheria toxin containing 3,000 Lf/ml (Connaught Laboratories, Willowdale, Ontario, Canada) was further purified with ammonium sulfate precipitation and gel chromatography by the method of Collier and Kandel (3) before radioactive labeling. Two lots of crude toxoid (Commonwealth of Massachusetts Department of Public Health, State Laboratory, Boston) containing 1,100 and 975 Lf/ml, respectively, were diluted in tap water and used for oral immunization. Parenteral immunizations containing 12.5 Lf (approximately 25  $\mu$ g) of diphtheria toxoid were given intramuscularly (i.m.) as alum-adsorbed diphtheria and tetanus toxoid (DT) (Lederle Laboratories, Pearl River, N.Y.).

Crystalline BSA (Reheis Chemical Co., Chicago, Ill.) was used for injection and in the radioimmunoassay, and bovine serum fraction V from the same source was fed to the rabbits.

Purified toxin or crystalline BSA was trace labeled with  $^{125}$ I by a modified chloramine-T method (13) and dialyzed in borate-buffered saline for 48 to 72 h to remove free iodine. The toxin was then passed through a Sephadex G-100 column to remove fragments, and the fractions from the middle of the first radioactive peak were pooled. Total protein nitrogen in each antigen preparation was determined by the Nessler reaction. Labeled toxin or BSA was diluted in 1% normal rabbit serum and frozen in aliquots. Specific antibody or 10% trichloroacetic acid precipitated more than 90% of the radioactivity of these preparations.

**Immunizations.** Drinking water containing 0.1% BSA was given ad libitum for 10 days. The daily average consumption was 300 mg of BSA per day. Eighteen days after the ingestion ceased, BSA was either fed for a second 10-day period or injected i.m. as 5 mg emulsified in incomplete Freund adjuvant. Eighteen days after the second course of antigen ingestion ceased, these animals also received an i.m. injection of 5 mg of BSA in incomplete Freund adjuvant.

Similarly, rabbits were given 250 ml of tap water containing approximately 50 mg of crude toxoid daily for one or two 10-day periods. When this solution was completely consumed, additional drinking water was provided daily. Eighteen days after antigen ingestion ceased, parenteral challenge with 0.5 ml of DT was given i.m. Blood was obtained weekly from the central ear artery, and the serum was separated and stored at  $-20^{\circ}\text{C}$  until used.

**Antibody determinations.** The capacity of the serum to bind BSA was measured using the standard ammonium sulfate method (17). A modification of the ammonium sulfate method previously described (19) was employed to determine the capacity of serum to bind  $^{125}$ I-labeled diphtheria toxin. Specifically, serial dilutions of the rabbit serum to be assayed were prepared with 10% normal rabbit serum in borate-buffered saline. A 0.5-ml portion of each dilution was added to 0.5 ml of  $^{125}$ I-labeled diphtheria toxin, mixed, and incubated for 18 h at  $4^{\circ}\text{C}$ . The antigen-antibody complexes were then precipitated by the addition of an equal volume of 90% saturated ammonium sulfate. After incubation for 30 min at  $4^{\circ}\text{C}$  the precipitates were obtained by centrifugation in the cold at  $2,000 \times g$ . The precipitates were washed with 45% saturated ammonium sulfate and counted in a gamma scintillation counter, and the percent radioactivity precipitated by each dilution was determined. Specific antibody was considered present when a 20% solution of the animals' serum bound more than 10% of the antigen. The minimal amount of toxin binding detectable under these conditions is approximately 2 ng of N. An antigen binding capacity (ABC-33) at concentrations of 0.004 and 0.04  $\mu$ g of N per ml was determined for all serum samples that contained sufficient antibody. The details of the procedures and calculations have been described previously (17).

The avidity of the antibody is expressed as the effect of dilution (ED) on antigen binding in antigen excess:  $\text{ED} = (\text{ABC-33 at } 0.004 \mu\text{g of toxin N} / \text{ABC-33 at } 0.04 \mu\text{g of toxin N}) \times 100$ . The higher the ED, the more avid the antibody (17).

This radioimmunoassay, which measures the primary interaction between the  $^{125}$ I-labeled antigen and antibody, provides a reproducible assessment of the relative antibody concentration (ABC-33) and avidity (ED) of each antiserum which can be quantitatively compared with other antisera to the same or other protein antigens. Although the coefficient of correlation between this assay and the standard passive hemagglutination assay for diphtheria antitoxin is generally good (0.93), the correlation decreases ( $r = 0.70$ ) when the passive hemagglutination titer is less than 1,000 (19). This difference appears to be due to the differing concentrations and/or affinity of antibody detected by the radioimmunoassay and passive hemagglutination. A detailed discussion about possible mechanisms for these discrepancies can be found elsewhere (18, 19). For comparison, 1 IU of standard reference horse antitoxin (lot A-35, Bureau of Biologics, U.S. Food and Drug Administration) bound 730 ng of toxin N per ml with an ED of 83%.

The antibody isotype was determined by radioimmunoassay on microscope slides coated with 1.5% agar (20). The specificity of the reagent antiserum for

rabbit  $\alpha$  chain (kindly supplied by K. Knight, University of Illinois, Chicago) and anti-rabbit  $\gamma$  chain (Miles Laboratories, Elkhart, Ind.) was established by immunodiffusion and immunoelectrophoresis against rabbit serum, rabbit colostrum, and purified IgG and IgA solutions.

**Statistics.** The significance of the differences between the immune responses was determined by Student's *t* test.

## RESULTS

Serum from all rabbits contained no detectable diphtheria antitoxin or anti-BSA before the experiments, suggesting the absence of prior natural infection with toxigenic diphtheria organisms or antigenic exposure. The animals that were to receive only DT injections were immunized with the orally immunized animals 18 days after antigen ingestion ceased. Before immunization, these animals, housed and bled along with the orally immunized animals, did not develop a circulating antitoxin response.

The antigenicity of the crude toxoid used for feeding was compared with that of the purified toxin by a competitive binding assay (Fig. 1). Both the similar slopes of the two curves and the capacity of the toxoid to completely inhibit the binding between toxin and antitoxin indicated that the two preparations contained essentially identical antigenic determinants.

Unlike long-term (52-day) feeding, where 100% of the rabbits eventually responded to the ingestion of BSA (20, 23), 11 of 13 rabbits fed 0.1% BSA for 10 days responded, but only 7 produced sufficient circulating anti-BSA to calculate an ABC-33 (Table 1). The mean antibody concentration, however, was similar during the first 3 weeks in both the 52-day and 10-day fed groups (Fig. 2). Three of the rabbits ingesting BSA were started on a second 10-day feeding period, and five were given i.m. injections of 5 mg of BSA in incomplete Freund adjuvant on day 28 of the experiment. The immune responses

to the second feeding resulted in an abrupt increase in the mean concentration of anti-BSA to levels found in animals ingesting BSA continuously for 52 days. After antigen ingestion ceased, the mean anti-BSA concentration decreased again in contrast to the sustained increase in the mean response of animals continuously ingesting the antigen (Fig. 2). All five rabbits responded to the parenteral challenge with an increase in circulating antibody (Fig. 3), although the animals unresponsive to ingested antigen remained relatively poor responders. No significant difference in initial responses to i.m. or oral challenge was observed (Fig. 3). However, by week 3, the effect of the continuous presence of antigen in adjuvant was evident in the increasing antibody production in the rabbits given parenteral BSA, while the orally challenged animals' antibody concentration decreased. The sustained rise in antibody in the i.m.-challenged animals resembled the response of rabbits fed BSA continuously for 52 days (Fig. 2). In contrast to the suppressed responses of mice fed BSA (Silverman et al., submitted for publication), but consistent with reported responses in humans (12, 22) and in rabbits (20, 23), both oral and parenteral BSA challenge produced a more rapid and greater response in the rabbits fed 0.1% BSA for 10 days than in unfed animals (Fig. 3).

Ten of the 13 animals fed diphtheria toxoid had detectable circulating antitoxin by 14 days after feeding was begun (Table 1). Eleven rabbits were given only a single DT injection, and all were producing detectable circulating antitoxin 2 weeks after the injection. The maximum response of the injected animals (mean ABC-33 =  $0.7 \pm 0.11$   $\mu$ g of toxin N per ml) was significantly higher ( $P < 0.001$ ) than the maximum mean response of the toxoid-fed animals ( $0.2 \pm 0.05$ ). The 13 animals that had been fed toxoid then were divided into two groups 18 days after toxoid ingestion stopped. Group A consisted of six animals fed toxoid for an additional 10 days, and group B consisted of seven rabbits that were given a DT injection. As shown in Figure 4, 7 days after the second toxoid feeding period was begun the amount of antitoxin in group A rabbit sera (mean ABC-33 =  $1.66 \pm 0.22$ ) was significantly greater ( $P < 0.001$ ) than that observed 7 days after unfed rabbits were given DT (mean ABC =  $0.03 \pm 0.01$ ). All seven group B rabbits also responded with increased antitoxin concentration (mean ABC-33 =  $1.81 \pm 0.62$ ) 7 days after DT challenge. Group A and B responses were not significantly different. When group A rabbits were given a DT injection 18 days after the second period of toxoid ingestion, antitoxin production only returned to the same levels seen after the oral toxoid challenge (mean ABC-33

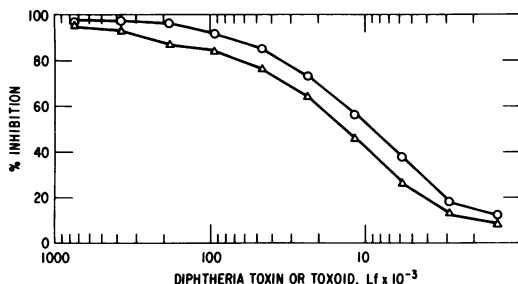


FIG. 1. Comparison of partially purified toxoid used for feeding ( $\Delta$ ) with purified toxin ( $\circ$ ) in the inhibition of  $^{125}$ I-labeled diphtheria toxin binding by rabbit antitoxin.

TABLE 1. Serum antibody concentration in individual rabbits ingesting antigen

Antigen	Serum antibody (ABC-33) concn <sup>a</sup> at day:				
	0	7	14	21	28
0.1% BSA in drinking water	0	0	0	0	0
	0	0	0.4	1.6	0.8
	0	0	0.6	1.2	1.3
	0	0	0.4	0.7	0.6
	0	0	0.2	0.2	0.3
	0	0	0	0	0
	0	0	+	+	+
	0	0	+	+	+
	0	0	0.4	0.2	0.2
	0	0	0.1	0.2	0.1
	0	0	0	+	+
	0	0	+	0.3	0.3
	0	0	+	+	+
	Mean ± SEM <sup>b</sup>	0	0	0.2 ± 0.06	0.4 ± 0.2
0.02% diphtheria toxoid in drinking water	0	0	0	0	0
	0	0	0.6	0.5	0.2
	0	0	0	0	0
	0	0	0.3	0.2	0.1
	0	0	0	0	0
	0	0	0.1	+	+
	0	0	0.2	0.2	0.1
	0	0	0.2	0.1	0.1
	0	0	0.1	0.1	+
	0	0	0.2	0.1	0.1
	0	0	0.1	0.1	0.1
	0	0	+	+	+
	0	0	0.2	0.2	0.1
	Mean ± SEM	0	0	0.2 ± 0.05	0.1 ± 0.04

<sup>a</sup> Antibody concentrations were calculated in a Farr test using [<sup>125</sup>I]BSA or [<sup>125</sup>I]-labeled diphtheria toxin at a concentration of 0.04 µg of N per ml and are expressed as the ABC-33 in micrograms of protein N bound per milliliter of serum. 0, Less than 10% antigen bound; +, binding of between 10 and 33% of the antigen added.

<sup>b</sup> SEM, Standard error of the mean.

= 1.88 ± 0.17) (Fig. 5). In summary, equal antitoxin concentrations were present 1 month after the following: one DT injection, toxoid ingestion followed by a DT injection, two periods of toxoid ingestion, or two periods of toxoid ingestion followed by a DT injection (Fig. 4). A second DT injection given to any group resulted in a major antitoxin increase with no significant difference between rabbits ingesting toxoid and those given only DT (Fig. 5).

The avidity of the antitoxin was lowest in the 13 rabbits that ingested toxoid for 10 days, with a mean ED of only 19 on day 14. The ED then increased to 45 by day 28. Antitoxin avidity in rabbits given a DT injection rose from 37 to 63 during the same period after injection. Orally immunized animals responded to a DT injection (group B) with significantly higher avidity antibody (mean ED = 58 at 7 days) than control rabbits given only DT. The mean avidity of the antitoxin from animals on day 7 of the second

oral exposure (group A) was greater yet (ED = 72), approximately equaling that produced 7 days after two DT injections. During the observation period the avidity of the antitoxin from group A animals remained high. When this group of animals was subsequently challenged with DT, antibody avidity again increased significantly ( $P < 0.01$ ) to a mean ED of 90, although the antitoxin concentrations only returned to those present after the second feeding (Fig. 5). Thus, after a DT injection in animals that had two periods of toxoid feedings, antitoxin response increased over the maximum previous levels qualitatively but not quantitatively. The capacity for further response of group A was estimated by giving a second DT injection to one of these animals. The response to this second parenteral injection (ABC-33 = 13.2 at 7 days) did not differ from those obtained after two DT injections in the rabbits of the other groups.

Specific circulating antitoxin was detected by

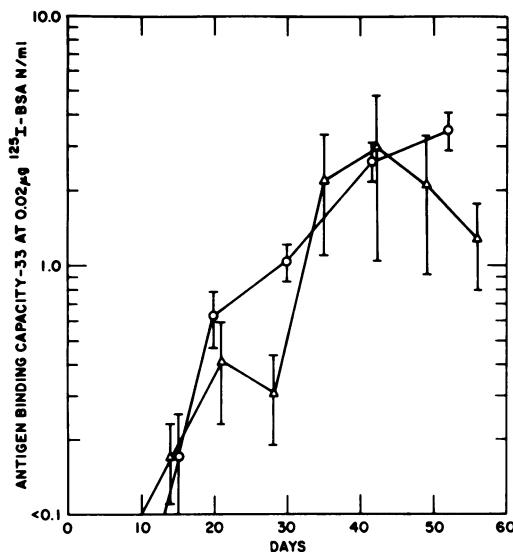


FIG. 2. Serum anti-BSA concentration in rabbits fed 0.1% BSA continuously (O) or fed on days 0 to 10 and 28 to 38 ( $\Delta$ ). Antibody is expressed as the mean micrograms of BSA N bound per milliliter of serum  $\pm$  standard error of the mean.

radioimmunoassay only in the IgG isotype when the ABC-33 was 0.5  $\mu$ g of N per ml or greater. If IgA or IgM antitoxin was present, it was in amounts too low to be detected by this technique.

### DISCUSSION

Considerable evidence indicates that in rabbits continued presentation of BSA to a mucosal surface for more than 50 days immunizes the animal and stimulates the production of significant amounts of circulating anti-BSA (20, 23, 24). In the present studies, the short-term (10-day) BSA ingestion resulted in an initial serum antibody response equivalent to that observed during long-term (52-day) feeding (Fig. 2). The anti-BSA concentrations fell when antigen feeding was discontinued after 10 days, whereas they continued to increase during long-term feeding of BSA. However, intermittent or continuous feeding appeared to result in equivalent antibody production while antigen ingestion was occurring (Fig. 2). A 10-day ingestion of diphtheria toxoid resulted in a serum antibody response similar in magnitude and timing to that observed after the short-term feeding of BSA. The large increase in concentration and avidity and more rapid response observed after a second exposure to either ingested or injected antigen strongly suggests that priming and immunological memory were established during the first oral immunization period by both antigens.

When responses were compared with those of previously unimmunized controls, considerable enhancement of the circulating antibody response was observed. Under these experimental conditions, no evidence of immunological suppression was observed. The poor responsiveness noted in some of the individual animals often has been observed after parenteral immunizations, even among individuals of an established inbred mouse strain (Silverman et al., submitted for publication).

The responses to DT injection of rabbits previously given two periods of oral antigen raise the possibility of immune regulation due to suppressor effects. The second feeding significantly increased both antibody concentration and avidity and was as effective as a DT injection in stimulating antitoxin in a rabbit primed with oral toxoid. The avidity of the antitoxin after the second ingestion period exceeded that pro-

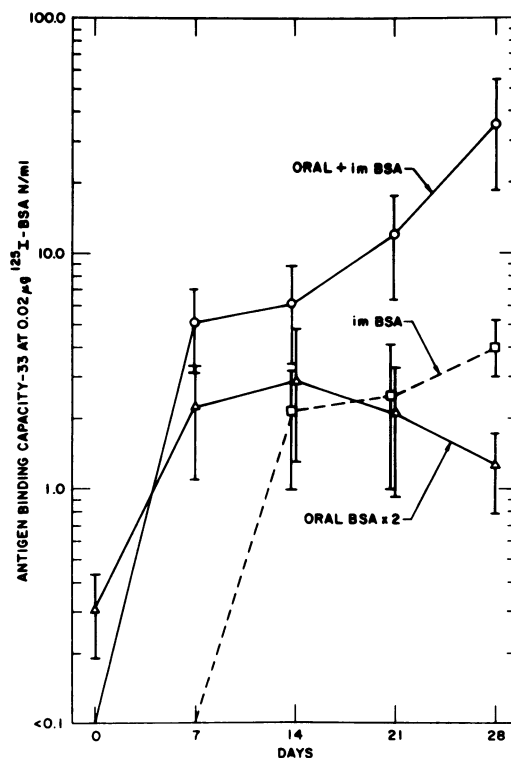


FIG. 3. Effect of BSA ingestion on the circulating anti-BSA response to a second 10-day ingestion period or to i.m. injection of 5 mg of BSA in incomplete Freund adjuvant. Eight rabbits were fed 0.1% BSA for 10 days and then rested for 18 days, and three of these rabbits were given a second 10-day feeding beginning on day 0 ( $\Delta$ ). The remaining five were challenged i.m. with BSA on day 0 (O). Fourteen control rabbits were injected i.m. with BSA on day 0 ( $\square$ ).

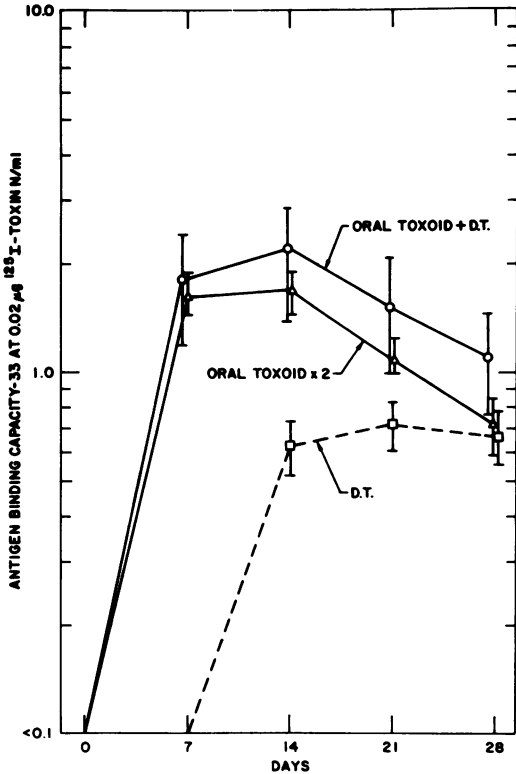


FIG. 4. Effect of prior ingestion of diphtheria toxoid on the circulating antitoxin response to a second exposure to toxoid. Six rabbits were fed diphtheria toxoid for 10 days, rested for 18 days, and given a second 10-day feeding of toxoid on day 0 ( $\Delta$ ). Seven rabbits were similarly fed toxoid for 10 days, rested for 18 days, and injected i.m. with 0.5 ml of pediatric diphtheria-tetanus toxoid (DT) ( $\circ$ ). Eleven control rabbits were given DT on day 0 ( $\square$ ).

duced by a parenteral injection of DT given to orally primed rabbits and increased again after a DT challenge. However, the maximum concentration of antitoxin in orally primed and challenged animals was not increased beyond the previous maximum by the subsequent DT injection, whereas in rabbits fed toxoid only once, a significant "booster" effect was noted after DT injection. These data might be explained by a slight suppression of response to parenteral antigen stimulated by the second oral antigen exposure, or by the stimulation of a different set of antibody-producing cells in the secondary response to oral challenge as compared to the initial exposure to parenteral antigen. The establishment of immunity and immunological memory in orally immunized animals is demonstrated by the increased avidity of the antibody following each antigen exposure. This increased avidity should result in more efficient toxin neutralization.

Evidence that much of the circulating IgG antibody induced by the ingestion of these protein antigens is synthesized in the gut-associated lymphoid tissue includes: (i) few direct or indirect plaque-forming cells (24) or circulating antigen-reactive cells (7) are detected in the mesenteric or peripheral lymph nodes or spleens from orally immunized rabbits despite the presence of large amounts of circulating anti-BSA; (ii) adoptive transfer of murine mesenteric node lymphocytes shows the presence of intracytoplasmic IgG in 25% of labeled B immunoblasts that localize in the small intestine (15); (iii) after adoptive transfer of mesenteric lymphocytes from mice ingesting ferritin, as many as one third of the lymphoid cells synthesizing antiferitin in the recipient mesenteric node and approximately 10% of those in the small intestine contained IgG (25); and (iv) most of the circulating anti-BSA in humans (12, 22) and hamsters (6) after BSA ingestion is IgG. This association of circulating antibody primarily with the IgG or IgA isotypes after antigen ingestion may be related to antigenic structure (C. Theodore, G. Lososky, B. A. Peri, M. Fishaut, R. M. Rothberg, and P. Ogra, unpublished data) or due to species differences in the production of circulating IgA.

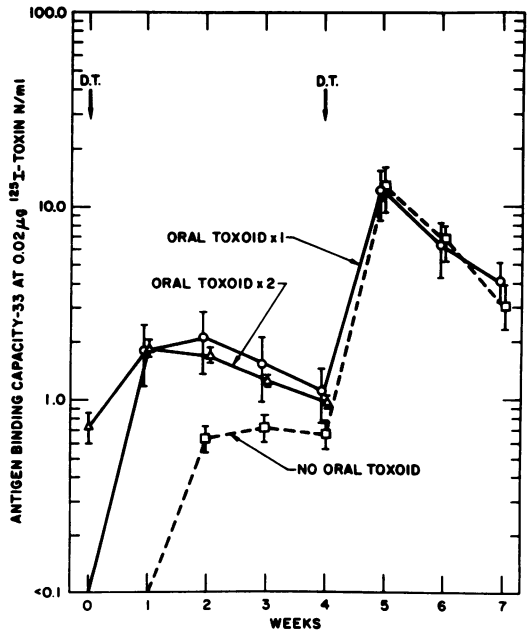


FIG. 5. Effect of multiple exposures to diphtheria toxoid on serum antitoxin levels in rabbits. Six rabbits previously fed toxoid for two 10-day periods were given a DT injection at week 0 ( $\Delta$ ). Seven rabbits previously fed toxoid for 10 days were given DT injections at weeks 0 and 4 ( $\circ$ ). Six rabbits were given DT injections at week 0 and week 4 ( $\square$ ).

Rabbits appear to be more responsive to DT than infants. All rabbits given one DT injection produced detectable antitoxin, whereas none of 18 infants responded to one DT injection and only some had an antitoxin response after a second injection (19). An additional factor that must be considered is the relative inefficiency of oral immunization, requiring long exposure to relatively large amounts of antigen. This relative lack of responsiveness in humans and the inefficiency of oral immunization may in part explain the failure of previous attempts to immunize humans with diphtheria toxoid by the ingestion of small antigen doses over a short period of time (9). These considerations suggest that it would be desirable to define the optimal timing and dose in an animal model and then attempt to increase the antigenicity of the ingested toxoid with orally administered adjuvants. Such an immunization, if practical for humans, could provide protection in areas where medical facilities are limited, provide mucosal surface immunity to toxin and bacterial cell wall products (prevent colonization), and avoid the possible suppression of the mucosal immune response by parenteral immunization (10).

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