Changes in Intestinal Fluid and Mucosal Immune Responses to Cholera Toxin in *Giardia muris* Infection and Binding of Cholera Toxin to *Giardia muris* Trophozoites

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The effect of *Giardia muris* infection on the diarrheal response and gut mucosal antibody response to cholera toxin was examined in mice. The results obtained showed that the fluid accumulation in intestinal loops exposed to cholera toxin was increased in mice infected with a low number (5 × 10⁴) of *G. muris* cysts compared with the response in noninfected mice. This effect was associated with a marked reduction in absorption of oral rehydration fluid from the intestine. In contrast, mice infected with a high dose (2 × 10⁵) of cysts showed a marked decrease in fluid accumulation in response to the toxin. This decrease might be related to the finding that both *G. muris* and *Giardia lamblia* trophozoites can bind significant amounts of cholera toxin. Evidence is presented which suggests that the gut mucosal antibody response, mainly immunoglobulin A but also immunoglobulin G, to an immunization course with perorally administered cholera toxin was depressed in mice infected with *G. muris*. The reduction in antibody levels was particularly evident when the primary immunization was made very early after infection. The serum antitoxin antibodies to the oral immunization with cholera toxin were, however, not affected. Likewise, the delayed-type hypersensitivity response against sheep erythrocytes in animals primed subcutaneously with sheep erythrocytes was not modified during the course of *G. muris* infection.

Although enterotoxin-producing bacteria are responsible for a high proportion of acute diarrheal diseases, little is still known about the host and the environmental factors governing the individual responses to enteric pathogens. A relationship between predisposition to cholera and the ABO blood group antigens has been previously noted (9a). However, of immense importance is the role of the intestinal microecology, an area remaining relatively unexplored. For instance, little is known of the effects of intestinal parasites on the intestinal ecology and intestinal responses to other enteric pathogens.

Ljungström et al. have previously shown (14) that mice infected with the parasitic helminth *Trichinella spiralis* show changes in basal and cholera-toxin-stimulated intestinal fluid transport processes during the intestinal stage of the disease. The intestinal fluid secretion in response to cholera toxin was greatly enhanced, probably related to an observed marked reduction in absorption of fluid from the intestine. There was also a drastic depression of the mucosal immunoglobulin A (IgA) and IgG antibody response to orally administered cholera toxin during the intestinal stage of trichinosis, which seemed to be related primarily to a depression of the development of immunological memory in the gut (14).

Giardiasis is a common intestinal protozoan infection of humans and animals. The interaction between *Giardia* spp. and other enteropathogens is, however, poorly understood. The availability of a mouse model of giardiasis, with infection with *Giardia muris*, has provided an opportunity to study certain aspects of such interactions. In the present study, we have explored the possible effects of *G. muris* on intestinal fluid transport processes and immune responses to cholera toxin in mice.

**MATERIALS AND METHODS**

Mice. Inbred mice (C57BL/6J) of both sexes were used. In each experiment, the animals were closely age and sex matched. The ages varied between 4 and 6 weeks at the onset of infection.

**Parasites.** The *G. muris* strain was kindly provided in 1979 by Western General Hospital, Edinburgh, Scotland. Since then the parasite has been maintained at the National Bacteriological Laboratory, Stockholm, Sweden, by passage in C57BL/6J mice. The *Giardia lamblia* strain was kindly provided in 1978 by the National Institutes of Health, Bethesda, Md. The trophozoites were cultured in Diamond TPS-1 medium (5) by the modification of Visvesvara (29) with sterile filtered medium.

**Reagents.** A highly purified preparation of cholera toxin was purchased from Sigma Chemical Co., St. Louis, Mo. ¹²⁵I-labeled cholera toxin was prepared by the method of Markwell (16). Purified cholera B-subunit was prepared by Institute Merieux as described by Tayot et al. (28).

**G. muris infection.** The mice were infected with 10⁴ *G. muris* cysts when not stated otherwise. The cysts were isolated from stock animals on the same day they were used to infect mice. The cysts were given orally in 0.5 ml of saline with a stomach tube without anesthesia.

**Determination of cyst and trophozoite recovery.** *G. muris* cysts in stools were recovered and isolated as described by Roberts-Thomson et al. (23). In brief, the stools from the individual mice were dispersed in tap water, and the cysts were isolated on a sucrose density gradient. The total cyst output per 2 h of stool collection was calculated. Cysts isolated 1 week after infection were used for passage of the parasite or for experimental infections. For determination of

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trophozoite recovery, mice were killed by cervical dislocation, and the intestine from stomach to cecum was carefully removed. The small intestine was divided into three equal segments. Each segment (the anterior, the medial, and the posterior) was flushed with 0.9% saline, and the number of *G. muris* trophozoites released from each segment was determined. Immediately after the flushing, each segment was cut longitudinally, the mucosa was carefully scraped off, and the number of trophozoites in the mucosal scraping was counted with a hemacytometer. The *G. lamblia* trophozoites were cultured in vitro (29). After 72 h of growth, they were harvested and counted.

**Determination of intestinal secretion and absorption.** Intestinal fluid secretion in response to cholera toxin was studied in ligated small intestinal loops (11). Two 5- to 7-cm-long loops were prepared in each mouse. One loop was inoculated with 0.2 ml of cholera toxin in phosphate-buffered saline (PBS), and the other was inoculated with 0.2 ml of PBS only. After 4 h, the animals were killed, and the length of the loops were determined. Fluid accumulation attributable to the toxin was determined as weight difference (in milligrams per centimeter) between the test and the control loop in each animal. Intestinal fluid absorption was studied in ligated 8- to 10-cm-long jejunal loops which were injected with 0.5 ml of oral rehydration solution composed of (in grams per liter): glucose, 20; NaCl, 3.5; NaHCO₃, 2.5; and KCl, 1.5. After 14 or 21 min, the mice were killed, and the loop fluid content was determined (in milligrams per centimeter) by weighing the loop and an adjacent portion of the intestine.

**Binding studies of cholera toxin to *G. muris* and *G. lamblia* trophozoites.** (i) **Immunological assay.** The trophozoites were washed and incubated in PBS in the presence of 0.1 μg of cholera toxin per ml at 37°C for 1 h. After incubation, the supernatant was collected by centrifugation, and the content of residual cholera toxin was determined by the GM1 ganglioside enzyme-linked immunosorbent assay (24).

(ii) **Immunofluorescence.** Trophozoites were adjusted to a concentration of 10⁶ parasites per ml and used to prepare smears on microscope slides. These were fixed by incubation with 3.5% paraformaldehyde for 5 min at room temperature. Cholera toxin (0.1 μg/ml) was allowed to react with the parasites for 30 min at room temperature. This was followed by incubation with rabbit anti-cholera toxin for 30 min at room temperature and a final incubation (30 min at room temperature) with fluorescein isothiocyanate-labeled anti-rabbit conjugate. Each incubation was followed by a washing step. For the controls, either cholera toxin or rabbit anti-cholera toxin was left out, and PBS was added.

**Binding of radioiodinated cholera toxin.** Trophozoites were adjusted to a concentration of 5 × 10⁶ parasites per ml, and 0.1 ml of the suspension was added to V-formed glass tubes and chilled on ice. To each tube, 0.1 ml of chilled [125I]-labeled cholera toxin was added either in buffer alone (PBS containing 0.2% bovine serum albumin) or in buffer supplemented with 1 μg of cholera toxin B-subunit per ml. The tubes were incubated for 100 min in an ice bath. After incubation, the trophozoites were extensively washed in PBS containing 0.05% Tween 20 by centrifugation at 1,000 × g for 10 min at 4°C between each step and harvested. The uptake of iodine-labeled cholera toxin by the trophozoites was estimated in a gamma-counter. The passive adsorption of radioiodinated cholera toxin to the tube walls was 10% of that of added specific-labeled cholera toxin.

**Immunization with cholera toxin.** Groups of *G. muris*-infected and -noninfected mice were immunized with cholera toxin. Four immunizations, each consisting of 5 μg of cholera toxin in 0.5 ml of 5% NaHCO₃, were given perorally by a stomach catheter at 6-day intervals. Antibody levels were measured 4 days after the last (booster) immunization.

**Intestinal antibody synthesis.** Antibody synthesis by in vitro-cultured intestinal tissue was determined as described previously (25). In brief, the small intestine was quickly excised, and multiple tissue specimens were taken from different parts. Equal amounts of tissue (approximately 500 mg per animal) were taken from experimental animals and controls. The tissue was thoroughly washed, minced into small pieces, and then incubated at 37°C for 24 h in Eagle medium supplemented with 5% heat-inactivated normal rabbit serum and 200 IU each of penicillin and streptomycin per ml (pH 7.2 to 7.4). After incubation, the supernatant was collected by centrifugation at 3,000 × g for 10 min and frozen at −70°C. The titers of specific IgA and IgG antibodies to cholera toxin were determined by the enzyme-linked immunosorbent assay (25).

**Determination of serum antibodies.** Antitoxin of both IgG and IgA classes in serum were determined by the enzyme-linked immunosorbent assay with purified cholera toxin as the solid-phase antigen (25).

**Delayed-type hypersensitivity.** The delayed-type hypersensitivity test was performed essentially as described by Liew (12). Mice were primed subcutaneously with 10⁶ sheep erythrocytes (SRBC) in 50 μl of saline and challenged subcutaneously 5 days later with 10⁴ SRBC in 25 μl of saline in the left hind footpad. As a control, 25 μl of saline was administered subcutaneously in the right hind footpad. The swelling was measured with a caliper 24 h after challenge, and the difference between the left and right footpad was expressed as the percent increase in footpad thickness.

**RESULTS**

**Characterization of *G. muris* infection.** (i) **Kinetics of cyst excretion.** Previous studies have revealed marked strain differences in the resistance of mice to *G. muris* infection. In some mouse strains, the infection is self-limiting and the mice are able to eliminate *G. muris* within 6 to 8 weeks, while in others persistent infection develops (2, 17, 21, 22). To test whether susceptibility to *G. muris* in the inbred C57BL/6J mouse strain used in this study, mice were given 5 × 10⁴ G. muris cysts, and excretion of cysts in stools was followed for 11 weeks (Fig. 1). Cyst excretion was maximal 1 to 2 weeks after initiation of the oral infection. In the next 2 weeks, a progressive reduction in cyst excretion was observed, followed by a gradual decline in cyst numbers throughout the period of study, with mean cyst counts of more than 10⁵/2 h. During the first 2 weeks of infection, doses of 10⁴ and 10⁵ showed almost similar cyst excretion as the 5 × 10⁴ infective dose. The results obtained indicate that C57BL/6J mice, at the age of 4 to 6 weeks, are susceptible to *G. muris* infection and develop a mild but chronic infection.

(ii) **Presence of trophozoites in the small intestine.** The number of trophozoites in the small intestine was determined 1 and 4 weeks after oral infection with 10⁴ or 10⁵ *G. muris* cysts. The total number in each segment was determined by adding the number of trophozoites obtained from the lavage to those obtained from the scraping; 60 to 80% of the parasites were recovered in the scraping. Both 1 and 4 weeks after infection, somewhat higher recoveries of trophozoites were obtained in the segments from mice which had received the higher dose compared with those which had received the
lower dose (Fig. 2). Independent of infectious dose, there was a 10-fold reduction in trophozoite recovery between 1 and 4 weeks. The trophozoites were distributed along the whole intestine, although maximal recovery was consistently observed in the midsection. This part of the small intestine was selected for the studies of fluid transport processes.

**Effect of G. muris infection on intestinal secretion and absorption.** Intestinal fluid secretion in response to cholera toxin was estimated in G. muris-infected mice during the early phase of infection. One group of mice was infected with $5 \times 10^4$ cysts and challenged with 5 μg of cholera toxin. Both 7 and 18 days after infection, these mice responded with an increased fluid accumulation as compared with that in noninfected mice ($P < 0.05$) (Fig. 3). Other groups of mice were given a fourfold higher dose of cysts and challenged with 1.5 or 5 μg of cholera toxin. A significantly decreased fluid accumulation in relation to noninfected controls ($P < 0.01$) in response to both doses of cholera toxin was recorded on both days 7 and 12 after infection (Fig. 3).

To determine the effects on intestinal fluid absorption
during the first 2 weeks after *G. muris* infection, the ligated loops were injected with oral rehydration solution containing glucose. The weight of the loops (in milligrams per centimeter) was determined 14 and 21 min after injection of oral rehydration solution containing glucose and compared with that of an adjacent portion of the intestine. The absorption of oral rehydration solution containing glucose was significantly decreased in the infected mice compared with the noninfected animals (*P* < 0.001) (Fig. 4).

**Uptake of cholera toxin by *G. muris* and *G. lambia* trophozoites.** Incubation of 10^5 *G. muris* or *G. lambia* trophozoites with 0.1 μg of cholera toxin resulted in 41 or 36%, respectively, reduction of cholera toxin from the incubation mixture as measured with the GM1 ganglioside enzyme-linked immunosorbent assay. When the number of trophozoites was decreased (10^3 and 10^5), the reduction in binding was about 10%. Immunofluorescence studies suggested that this reduction was due to binding of cholera toxin to the trophozoites (Fig. 5).

To prove further that *Giardia* spp. trophozoites are able to bind cholera toxin, *G. lambia* trophozoites were incubated with iodine-labeled cholera toxin. The results revealed that there was an uptake of cholera toxin by the trophozoites. It was found that 45% of the added specific-labeled toxin was bound to the trophozoites. This uptake was nearly completely inhibited when 1 μg of B-subunit was added along with the radiolabeled toxin (97.5% inhibition).

**Influence of *G. muris* on immune responses to unrelated antigens.** The ability of the small intestine to respond immunologically to cholera toxin antigen during the parasite infection was tested in mice orally immunized with cholera toxin at various times after infection. An immunization schedule known to induce optimal protection against experimental cholera infection was used (11). The degree of mucosal immune response was assessed by measuring antitoxin IgA and IgG antibodies synthesized in vitro by tissue-cultured intestines. When immunization was started 4 days after *G. muris* infection, there was a marked decrease in the local formation of both antitoxin IgA and IgG as compared with that in noninfected controls (*P* < 0.01 and *P* < 0.02, respectively) (Fig. 6).
respectively) (Fig. 6). A slight but not significant effect ($P > 0.05$) on the local IgA and IgG antitoxin responses was observed when the immunization was started 8 days after infection (Fig. 6).

The serum antibody response against cholera toxin in infected mice given oral immunizations was also determined. No differences in the serum antitoxin responses of either class were observed between $G. \text{muris}$-infected and -noninfected mice (data not shown).

The effect of $G. \text{muris}$ infection on the development of delayed-type hypersensitivity response to SRBC was investigated during the course of the infection. Animals were primed with SRBC 2, 4, 6, 14, and 21 days after infection. No significant effect on the delayed-type hypersensitivity response was observed during this period (data not shown).

**DISCUSSION**

Giardiasis is a common intestinal protozoan infection of humans and animals. The infection can be classified into three categories: self-limiting, recurrent, and persistent. In the present study, we show that C57BL/6J mice infected at the age of 4 to 6 weeks developed a mild but chronic $G. \text{muris}$ infection. Although our finding is not in agreement with an earlier report (2) showing a self-limiting infection in C57BL/6J mice, the difference could be because the authors of the previous study infected the mice at the age of 8 weeks. Indeed, MacDonald et al. (15) have suggested a relationship between the development of chronic infection and the age of mice. Our data showed that trophozoites were distributed throughout the small intestine, an observation similar to findings in Swiss albino (CF-1) mice (18).

The interaction between Giardia spp. and other enteropathogens is poorly understood. We present data showing that infection with $G. \text{muris}$ has a profound effect on intestinal fluid transport processes as well as on the establishment of a gut mucosal immune response to orally administered cholera toxin. When mice were given a high inoculum of parasites, fluid accumulation in response to cholera toxin was greatly decreased during the early phase of infection. To initiate its striking effects on fluid transport processes in the small intestine, cholera toxin has to bind to GM1 ganglioside receptors on the epithelial cells (10). There are several possible means by which the parasites could reduce toxin-induced secretion. They may bind the toxin and thus reduce the concentration of toxin available for binding to the epithelial target cells, they may provide a mechanical barrier which prevents binding to epithelial cells by blocking toxin receptor sites, or they may directly or indirectly, via the immune system, modulate intestinal epithelial functions. Our results do not allow definite conclusions about the relative role of any of these possible mechanisms, although they clearly show that both $G. \text{muris}$ and $G. \text{lambia}$ trophozoites are able to bind cholera toxin, which might explain the decreased fluid accumulation observed in response to cholera toxin. The mechanical barrier hypothesis is less likely, especially since the trophozoites mainly adhere to the microvilli near the bases of the villi (20), and active electrolyte and water secretion is thought to be mediated by the cells in the crypts (7).

In contrast, the net fluid response to cholera toxin was increased when the mice were infected by a lower number of $G. \text{muris}$ cysts. Intestinal fluid accumulation is the net result of absorption and secretion by both passive diffusion and active transport mechanisms. Our results suggest that infection with $G. \text{muris}$ profoundly affects (inhibits) absorption, and this could explain increased fluid accumulation to a secretagogue like cholera toxin in an infection with too few parasites to bind significant amounts of the added cholera toxin. Decreased absorptive capacity in giardiasis is in agreement with earlier findings, which showed that Giardia infection causes reduced brush border enzyme activity (8) and that the small intestine may have decreased capability to absorb sugars (1, 9), vitamins (3, 4, 31), and fat (4, 31) when subjected to Giardia infection. The precise mechanism whereby the absorption is reduced is not clear. One possibility may be damage of microvilli covering the luminal cell surface accompanied by the presence of Giardia spp. adjacent to the epithelial cells (6, 20, 26, 27). The inhibition could also be due to the increased turnover of epithelial cells, which is observed during giardiasis (15), because active absorption is mainly mediated by mature villus cells (7). Proliferation of the intestinal epithelial cells mainly takes place in the crypts, and the new cells are continuously moved upwards during progressive differentiation. Enhanced epithelial cell turnover could therefore result in a greater proportion of functionally immature cells on the villi lacking absorptive capacity.

In addition to the changes in intestinal fluid processes, mice infected with $G. \text{muris}$ demonstrated some clear changes in the local immune response similar to those previously shown for murine trichinosis (13). When oral
immunization with cholera toxin was started 4 days after infection, the intestinal synthesis of both antitoxin IgA and IgG was decreased; this was most pronounced for the IgA antibodies. However, when the immunizations were initiated 8 days after infection, no statistically significant effect on the local antibody response was observed. This effect of giardiasis on mucosal antitoxin immunity seems to be most pronounced during the period when the number of parasites in the intestine is very high. When the immunization was started 4 days after infection, the mice received two antigen stimuli before the rapid diminution in parasite number occurred. When the immunization was started on day 8, only one antigen dose was given before the reduction in parasite number took place.

Earlier studies have shown that the antitoxin response in the intestines of mice is transient and dose dependent. The actual antibody response to the immunization regimen employed reaches its maximum by day 4 to 8 after oral immunization and declines thereafter (25). However, concomitantly, immunological memory of a longer duration develops which is boosted by renewed immunizations and with a lower antigen dose than that needed for priming (11). One interpretation of our results could thus be based on effects on the development of immunological memory for the local antibody response to cholera toxin. This could result from a combination of two events. First, it is known that priming of the gut mucosal immune system is much more dependent on the dose of antigen than the boosting phase of the response. This would make it more sensitive to uptake of cholera toxin by parasites. Second, modification of the response to cholera toxin may be attributable in part to the changes in the mucosal lymphoid-macrophage system that occurs during G. muris infection (9, 19). However, it must be viewed with some speculation that the decreased antibody response to cholera toxin was due to effects on the development of immunological memory since this was not studied. Thus, it could still be speculated that T- and B-cell memory responses were normal but that the parasites evoked specific or nonspecific suppressive mechanisms that prevented the full manifestation of memory.

In this study, we could not demonstrate any influence of G. muris infection on either the systemic IgA and IgG antibody response to orally administered cholera toxin or the delayed-type hypersensitivity response to subcutaneously injected SRBC. The discrepancy observed in this study between the local and the systemic immune responses to orally administered cholera toxin suggests that a noninvasive intestinal parasite only affects the local immunity. In contrast, studies with T. spiralis, a parasite which is invasive during the intestinal stage (30), showed a depression of both local and systemic immune responses during the intestinal stage (13, 13a).

A high frequency of asymptomatic and symptomatic giardiasis occurs in people living in countries in which many other intestinal pathogens are also common. It is clearly difficult to speculate how closely the findings reported here for experimental giardiasis in mice are paralleled in natural giardiasis in humans. Also, the significant effects on intestinal fluid transport processes and mucosal immune response in the murine model were only seen during the first week(s) after infection. Nonetheless, these effects were striking and, as mentioned, similar findings, although perhaps mediated by other mechanisms, were also made in murine trichinosis (14). This warrants studies of this kind to be undertaken in human parasitic infections. If they extend to human infections, effects such as those observed in the experimental system could have considerable implications for the understanding of both aspects of the pathogenesis of enteric infections and disease- or vaccine-induced mucosal immune responses.

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