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Host Defense against Cholera Toxin Is Strongly CD4$^+$ T Cell Dependent

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This study investigates the role of CD4$^+$ T cells in host defense against cholera enterotoxin-induced diarrhea. Antitoxin immunoglobulin A formation and gut protection against cholera toxin (CT) following oral immunizations with CT were evaluated in normal mice and mice that had been depleted of CD4$^+$ T cells by in vivo treatment with specific anti-CD4 monoclonal antibodies. Flow cytometer analysis demonstrated that anti-CD4 monoclonal antibody effectively eliminated CD4$^+$ T cells in the spleen, mesenteric lymph nodes, and Peyer's patches. In contrast, lamina propria lymphocytes demonstrated only some decrease in CD4$^+$ T-cell numbers following antibody treatment. However, CD4 expression of individual lamina propria lymphocytes was strongly down-regulated. Depletion of CD4$^+$ T cells performed prior to oral immunization with CT completely inhibited the ability to respond to CT. No antitoxin production, as detected at the single-cell level by the ELISPOT technique, was found in the spleen, mesenteric lymph nodes, or Peyer's patches, nor did we observe serum antitoxin responses in these mice. Control mice demonstrated strong antitoxin responses in all locations following oral immunization with CT. Anti-CD4 antibody treatment also effectively inhibited the antitoxin immunoglobulin A response in the lamina propria to CT as well as blocked the ability to develop gut protection against CT challenge of ligated intestinal loops after oral CT immunization. Thus, in vivo CD4$^+$ T-cell depletion rendered these mice unable to develop protective immunity in the gut following oral immunization with CT. Moreover, CD4$^+$ T-cell depletion effectively inhibited the antitoxin immune response in the gut lamina propria, mesenteric lymph nodes, Peyer's patches, and spleen when performed prior to both priming and booster immunizations with CT. This study clearly demonstrates the requirement of functional CD4$^+$ T cells in the gut immune system for the development of host defense against CT-induced disease. Our data also reinforce the concept of a strong association between gut protection against CT and local production of neutralizing immunoglobulin A antitoxin.

Our knowledge of regulatory mechanisms controlling local immune responses at mucosal surfaces is poor. Most of our information today stems from studies of gut mucosal humoral immune responses after infections with enteropathogenic microorganisms or immunizations with antigen administered by the oral route (28). A hallmark of antibody responses at mucosal surfaces is that immunoglobulin A (IgA) constitutes approximately 80 to 90% of the total antibody being produced by the mucosa (5). This strongly suggests that antigen priming of resting B cells, e.g., by oral immunization of the gut immune system, involves an immunoglobulin switch of naive IgM$^+$ B cells to differentiate into IgA-producing B cells (28, 34). There is accumulating evidence that regulatory T cells play a central role in the process of isotype switching of B cells (34). For example, in mice interleukin-4, a lymphokine produced by activated CD4$^+$ T cells, is a well-characterized isotype switch factor that will greatly increase B-cell differentiation from IgM to IgG1 or IgE production (2, 13, 33). For IgA differentiation, interleukins 5 and 6 have been shown to act on B cells at the postswitch level, while transforming growth factor beta probably acts as a switch factor inducing IgM B cells to become IgA B cells (1, 11, 20).

Oral vaccination against cholera disease, using a combined toxoid-whole bacterial cell vaccine, is perhaps the best-studied oral immunization protocol that stimulates protective gut immunity associated with local antibacterial and antitoxic antibody responses in the small intestine (28, 35). In a previous study with cholera toxin (CT) as the mucosal immunogen, we addressed the question of whether such local protective immunity and antitoxin production in the gut after oral immunization with CT are thymus-dependent phenomena and thus T cell dependent (24). We used nude mice (lacking the thymus) to demonstrate that both protection against CT challenge of intestinal loops and antitoxin antibody formation in the gut require a functioning thymus and can be restored by grafting of the thymus from syngeneic normal mice to nude mice (24).

Although most earlier studies in various experimental animal models have found a strong association between local antitoxic IgA production and protective immunity in the gut, there is now conflicting information suggesting that protection against enterotoxin-induced disease can be mediated by factors not directly produced by the immune system (18, 22). Furthermore, there are also recent findings to suggest the existence of bidirectional communication between the neuroendocrine and the immune systems (3). In particular, the regulatory T cells of the mucosa might be implicated in neuroendocrine control of the physiology of the gut. Thus, T cells of the mucosa might have regulatory functions other than providing help for IgA antibody produc-

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tion that are important for host defense against diarrheal disease.

This study was undertaken to further investigate the role of $CD4^+$ T cells in the development of antitoxic antibody formation and protection against CT-induced diarrhea in the gut following oral immunizations with CT. We have taken advantage of the knowledge that immunosuppressed, anti-CD4 monoclonal antibody (MAb)-treated mice respond poorly to T-cell-dependent antigens (7, 8). We found that elimination of $CD4^+$ T cells by treatment with anti-CD4 MAb prior to oral CT immunizations rendered the mice unable to respond with local gut antitoxin IgA production or protection against CT challenge of ligated intestinal loops.

MATERIALS AND METHODS

Mice. Inbred female mice of the C57BL/6 strain were obtained from our breeding facilities. All mice were between 7 and 12 weeks old and were age and sex matched at the beginning of the experiments.

Antibodies. The MAbs were generated from cell lines H129.19 (30) and MAR18.5 (19), with specificity for the mouse CD4 antigen and rat immunoglobulin kappa light chain, respectively. The immunoglobulins of the supernatants were precipitated by ammonium sulfate, and MAR18.5 MAb was further purified by affinity chromatography on protein A-Sepharose (Pharmacia, Uppsala, Sweden). The purified antibodies were dialyzed against phosphate-buffered saline (PBS) and sterile filtered, and their relative concentrations were determined by enzyme-linked immunosorbent assay (ELISA), using serial dilutions of rat IgG2a (for H129.19) or mouse IgG2a (for MAR18.5) of known concentrations as standards.

In vivo depletion of $CD4^+$-cells. The mice were injected intraperitoneally (i.p.) with 100 μg of H129.19 MAb followed by 200 μg of MAR18.5 MAb 1 h later, as described previously (10). Control animals received 100 μg of rat serum IgG (Cappel, Malven, Calif.) followed by MAR18.5 MAb 1 h later. Injections were given on days −3 and −1 before analysis of cells by fluorescence-activated cell sorter (FACS; see below) or oral immunizations with CT.

Immunization. The mice were immunized orally with 10 μg of purified CT (a kind gift from Institut Mérieux, Lyon, France) through a baby feeding tube under light ether anesthesia (17). Immunizations with CT were administered in 0.5 ml of PBS. Repeated oral immunizations were administered as follows: 10 days separated the first two and final two doses, and 6 days separated all other immunizations. Analyses of protection and local antitoxin antibody production as well as serum antitoxin antibody levels were performed 4 or 7 days after the final immunization, as indicated.

Preparation of lymphoid cells. Spleen (SP), mesenteric lymph node (MLN), and Peyer's patch (PP) lymphoid cells were prepared by passing the tissues through a nylon net (26). SP cells were subject to lysis of the erythrocytes by ammonium chloride-osmotic shock. The single cell suspensions were then washed three times in Hanks' balanced salt solution (GIBCO, Paisley, Scotland) and diluted in Iscove's medium (GIBCO) containing 5% fetal calf serum (GIBCO). Intestinal lamina propria lymphocytes (LPL) were prepared as described previously (23). Briefly, after thorough washing in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (GIBCO), the tissue pieces were incubated in the same solution containing 5 mM EDTA (Merck, Darmstadt, Germany) to remove epithelial cells and intraepithelial lymphocytes. The intestinal pieces were then incubated in RPMI 1640 (GIBCO) containing collagenase type C-2139 (Sigma Chemical Co., St. Louis, Mo.), 300 U/ml, to extract the LPL enzymatically. Finally, the cells were washed twice in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution and diluted in Iscove's medium with 5% fetal calf serum. The viability of the isolated cells was always higher than 85% as determined by the trypan blue exclusion test.

FACS analysis. The distribution of $CD4^+$ T cells in different tissues after antibody treatment was monitored by flow cytometry, using a FACSTAR analyzer (Becton Dickinson, Mountain View, Calif.). Cells isolated from the SP, MLN, PP, or lamina propria (LP) of two control (untreated) or two anti-CD4-treated (H129.19 plus MAR18.5) animals were pooled together per group, and two groups from each treatment protocol were analyzed and compared on each occasion. Lymphocytes from the various tissues at 200,000 cells per 100 μl of PBS with 0.1% bovine serum albumin (BSA) were incubated with H129.19 antibody at 5 μg/ml for 30 min at 4°C. Thereafter, the cells were washed twice in PBS–0.1% BSA before staining for 30 min at 4°C with goat anti-rat immunoglobulin conjugated to fluorescein isothiocyanate (FITC; Southern Biotechnology, Birmingham, Ala.) at a final dilution of 1/300. To detect the presence of H129.19 antibody bound to the cell surface after i.p. administration of the antibody, we also performed direct staining on a separate set of cells with the anti-rat immunoglobulin FITC-conjugated antibody, i.e., omitting the H129.19 incubation step. In addition, yet other cells from the various tissues were double stained with anti-CD4 conjugated to phycoerythrin (Becton Dickinson) and anti-CD3 (Pharmingen, San Diego, Calif.) or anti-CD8 (Becton Dickinson) conjugated to FITC, used at a 1/100 final dilution. Staining was allowed for 30 min at 4°C, and thereafter the cells were subject to two-color fluorescence analysis with the FACSTAR analyzer (Becton Dickinson). Dead cells were gated out by forward scatter and, when appropriate, also by staining with propidium iodide (Sigma).

Enzyme-linked immunospot (ELISPOT) assay. An antigen-specific spot-forming cell (SFC) assay described previously was used, with minor modifications (9, 27). Briefly, polystyrene petri dishes (Nunc A/S, Roskilde, Denmark) or 96-well nitrocellulose Millititer HA plates (Millipore, Boston, Mass.) were coated with GM1 ganglioside (Sigma), 3 nmol/ml in PBS, at 4°C overnight. After being washed in PBS, the petri dishes and plates were incubated with CT (List Biological Laboratories Inc., Campbell, Calif.), 3 μg/ml in PBS, for 2 h at room temperature. After additional washings in PBS–0.05% Tween 20, coated solid phases were blocked by incubation with Iscove's medium containing 5% fetal calf serum for 30 min at 37°C. Thereafter, cells in appropriate densities (1×10^6 to 5×10^6/ml) in Iscove's medium with 5% fetal calf serum were added to the petri dishes (300 μl) or to 96-well plates (100 μl per well). The cells were analyzed in duplicate or triplicate and incubated for 4 h at 37°C in 8% CO_2 with 90% relative humidity. After thorough washing with PBS–0.05% Tween 20, developing antisera were added to the petri dishes and 96-well nitrocellulose plates.

(i) Nitrocellulose plates. For detection of total immunoglobulin anti-CT antibody-producing cells, anti-CT SFC, rabbit anti-mouse immunoglobulin horseradish peroxidase (HRP)-conjugated antibodies (Dakopatts, Glostrup, Denmark) at a 1/200 dilution in PBS–0.1% BSA were added to 96-well plates overnight, followed, after thorough rinsing of the plate in PBS–0.05% Tween 20, by HRP-conjugated swine anti-rabbit antibodies (Dakopatts) at a 1/200 dilution. When indicated, isotype-specific antitoxin-producing cells were

also detected by using anti-murine IgM-, IgG-, or IgA-specific goat antibodies conjugated to HRP (Southern Biotechnology). After the final wash, the substrate 3-amino-9-ethylcarbazole was added to the wells; 10 mg of 3-amino-9-ethylcarbazole per ml in dimethyl formamide was diluted 1/30 in citrate buffer (adjusted to pH 4.5 and sterile filtered to obtain a clear solution), and 0.05% H_2O_2 was added just before use. The reaction was stopped after 10 to 15 min by rinsing with water.

(ii) Petri dishes. Petri dishes were incubated with goat anti-mouse IgA (Cappel) at a 1/200 dilution overnight, followed by rinsing with PBS–0.05% Tween 20, and then incubated for an additional 2 h with rabbit anti-goat immunoglobulin HRP-conjugated antibodies (Dakopatts) at a 1/200 dilution. The bound antibodies forming the "fingerprints" of single IgA SFC were visualized by adding prewarmed (46°C) paraphenylenediamine (0.5 mg/ml)–0.01% H_2O_2 substrate in a 1% agar-in-PBS solution, which was applied as a thin film of substrate to each petri dish (9).

Spots were counted under low magnification, and SFC numbers were expressed as the means of three to four groups ± standard deviations (i.e., six or eight mice) or, when indicated, for individual mice. In 96-well plates, counting was always attempted in wells containing 10 to 50 SFC.

ELISA. The mice were bled prior to each immunization, i.e., 1 day after treatment with the H129.19 and MAR18.5 antibodies, and at sacrifice. The immune sera were stored at −20°C until assayed. Briefly, polystyrene microtiter plates (Nunc) were coated with GM1 ganglioside (0.5 nmol/ml) followed by CT (0.5 μg/ml) as described before (27). After incubation with PBS–0.1% BSA for 30 min at 37°C, followed by thorough washing, sera were added in 1/20 dilutions and serial threefold dilutions were performed. Incubation was allowed overnight. Bound antibodies were demonstrated with HRP-conjugated rabbit anti-mouse immunoglobulin antibodies (Dakopatts) or, when isotype-specific SFC were determined, with HRP-conjugated anti-mouse IgM, IgG, and IgA at a 1/300 dilution (Southern Biotechnology) and visualized by using *O*-phenylenediamine (1 mg/ml)–0.04% H_2O_2 substrate in citrate buffer (pH 4.5). The reaction was read in a Titertek Multiscan spectrophotometer (Flow) at 450 nm. The antitoxin titer was defined as the interpolated optical density reading giving rise to an absorbance of 0.4 above background. The mice were analyzed individually, and each serum sample was assayed in duplicate.

Ligated loop test. For the ligated loop test, the method described by Lange and Holmgren was used (17). The abdomen was opened under ether anesthesia, and a 6- to 8-cm loop was ligated in the middle part of the small intestine. Purified CT (2.5 μg) in 0.2 ml of PBS was injected into the loop, and the abdomen was closed. After 4 h, the mice were sacrificed; the loop with its fluid content was weighed, and its length was determined. Values for protection were expressed as the weight-per-length ratio (milligrams per centimeter). Mice treated with H129.19 plus MAR18.5 MAbs were also tested for susceptibility to CT challenge prior to oral immunization. Treated mice showed sensitivity to CT injected in ligated intestinal loops equivalent to that shown by nontreated mice.

Statistical analysis. We used the Wilcoxon rank sum test for the analysis of significance.

RESULTS

Effect of in vivo anti-CD4 MAb treatment on T-cell phenotype expression in various lymphoid tissues. T-cell phenotype distribution in various tissues was evaluated in mice following anti-CD4 antibody treatment with H129.19 plus MAR18.5 MAbs. Lymphoid cells were double stained with phycoerythrin-labeled anti-CD4-specific and FITC-labeled anti-CD3- or anti-CD8-specific MAbs. Lymphocytes from the SP, MLN, and PP demonstrated almost complete elimination of $CD4^+$ T cells following anti-CD4 antibody treatment (Fig. 1). This was also seen as a 15 to 20% decrease in the total number of anti-CD3-staining T cells in the various tissues as compared with that observed in untreated mice (Fig. 1). In contrast, LPL showed almost no change in the frequency of $CD3^+$ T cells following the anti-CD4 treatment as compared with cells from control mice (Fig. 2). Although the anti-CD4 staining intensity of the $CD3^+$ population in LPL was markedly decreased after antibody treatment, the frequency of $CD4^+$ T cells remained high. This was also apparent from complementary analysis demonstrating strong LPL staining with an FITC-labeled anti-rat immunoglobulin antibody, indicating that the rat H129.19 MAb had bound to the LPL following i.p. injection of the MAb but had not resulted in effective depletion of $CD4^+$ T cells in the LP (Fig. 2). In contrast, no cells in the other tissues stained with the anti-rat immunoglobulin-FITC antibody following i.p. anti-CD4 MAb treatment (not shown). Even after several weeks of repeated (twice per week) injections with H129.19 plus MAR18.5 antibodies we only saw minor effects on CD4 T-cell frequencies in the LP (not shown). The relative frequency of $CD8^+$ cells in the different tissues increased, from 17% to as much as 27% in MLN, following anti-CD4 MAb treatment. Thus, in vivo treatment with H129.19 plus MAR18.5 MAbs effectively eliminated $CD4^+$ T cells from the SP, MLN, and PP, whereas LPL demonstrated only a minor decrease in these cells.

Effect of $CD4^+$ T-cell depletion on ability to respond to oral immunization with CT. Having established that in vivo treatment with H129.19 plus MAR18.5 MAbs effectively depleted $CD4^+$ T cells in SP, MLN, and PP, we investigated whether T-cell-depleted animals could respond to oral immunization with CT. Mice were given three oral immunizations with CT. Each administration of CT was preceded by two injections with H129.19 plus MAR18.5 or rat serum IgG plus MAR18.5 as a control treatment. Eight days thereafter, animals were sacrificed. Serum was collected, lymphocytes were isolated from the different tissues, and antitoxin immune responses were recorded with the ELISPOT and ELISA techniques. We found that $CD4^+$ T-cell depletion abrogated the capacity to respond to oral immunization with CT (Table 1). Whereas control animals demonstrated strong antitoxin responses in serum (not shown) as well as high numbers of antitoxin SFC in the SP, MLN, and PP, no or very low antitoxin activity was observed in the anti-CD4-treated mice (Table 1). The isotype distribution of the antitoxin SFC response in the various tissues of the untreated mice was dominated by IgG, 88 and 77%, in the SP and MLN, respectively. Antitoxin SFC responses in the PP showed a high IgA frequency of 44%. All tissues demonstrated <5% IgM anti-CT SFC in the untreated mice. In contrast, the poorly responding anti-CD4-treated mice demonstrated relatively high levels of IgM antitoxin SFC, between 40 and 50%, whereas both IgG and IgA relative frequencies were decreased (not shown).

$CD4^+$ T-cell depletion effectively blocks development of antitoxin production and protection against CT in the gut following oral immunization. In the next set of experiments, we addressed the question of whether depletion of $CD4^+$ T cells also affects the ability to respond with intestinal anti-

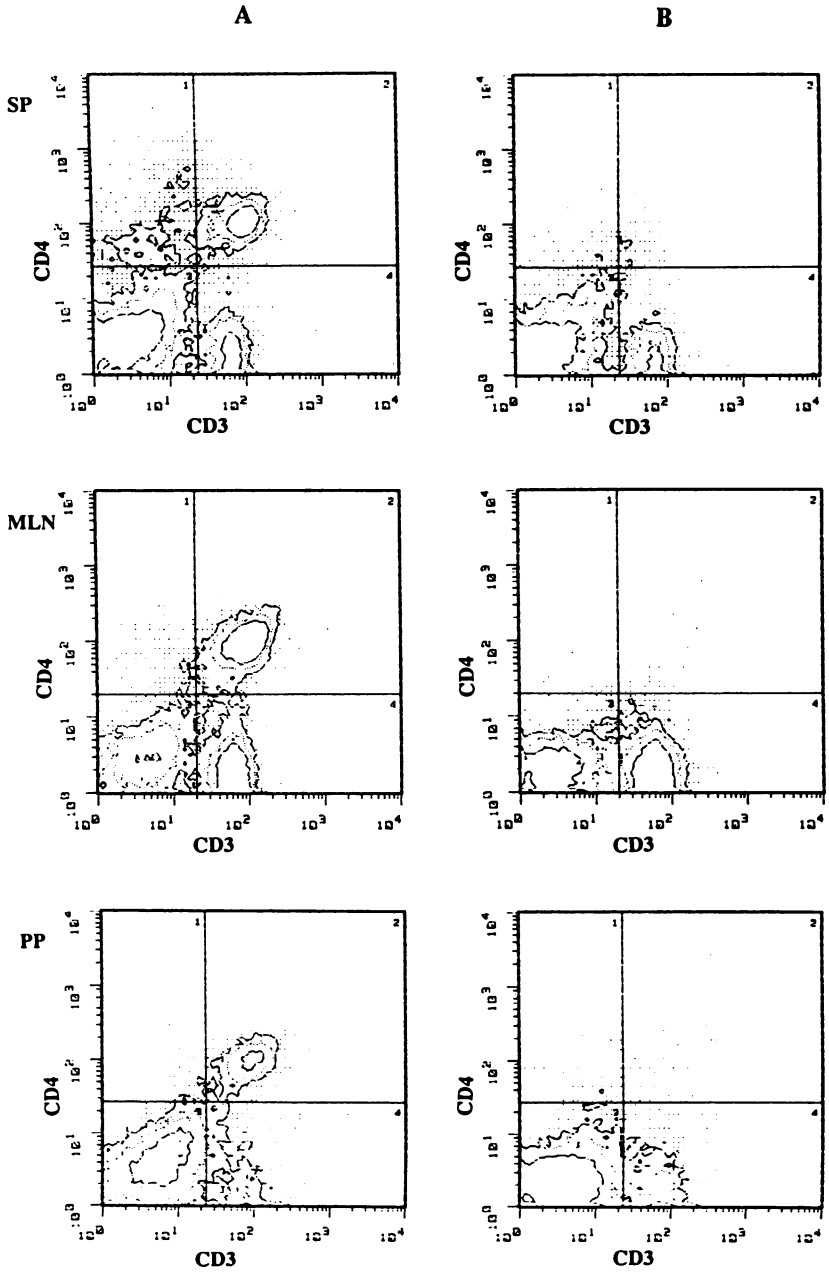

FIG. 1. Fluorescence-activated cell sorter (FACS) analysis of the distribution of CD4+ cells in SP, MLN, or PP after anti-CD4 MAb treatment (H129.19 plus MAR18.5) (B) as compared with cells from untreated mice (A). Cells were double stained with anti-CD4-phycoerythrin and anti-CD3-FITC MAbs, and staining profiles are represented as contour graphs.

toxin IgA production and development of protection against CT-induced fluid secretion following oral immunizations with CT. After five oral immunizations with CT, with each administration preceded by anti-CD4 treatment as described above, we determined the anti-CT SFC activity in LPL and the degree of protection by the ligated loop test. As shown in Fig. 3, anti-CD4 antibody treatment significantly decreased the response to CT in the LP as compared with that detected in control animals. Analysis of serum antitoxin titers of some of the mice in this experiment indicated that anti-CD4 depletion blocked the ability to respond with antitoxin formation (Table 2). Of note, one animal in the anti-CD4-treated group responded with strong serum antitoxin production and also showed strong antitoxin IgA formation (and protection) in the gut (Table 2; Fig. 3 and 4). This individual animal had probably been inadequately depleted of CD4+ cells.

These mice were also subjected to analysis of protection against CT challenge, and the results are shown in Fig. 4. CD4+ T-cell-depleted mice failed to develop protection against CT challenge as compared with rat IgG-treated control animals. The mean fluid accumulation in the loops of anti-CD4-treated mice was almost as high as that of the unimmunized mice (Fig. 4). In contrast, control treated

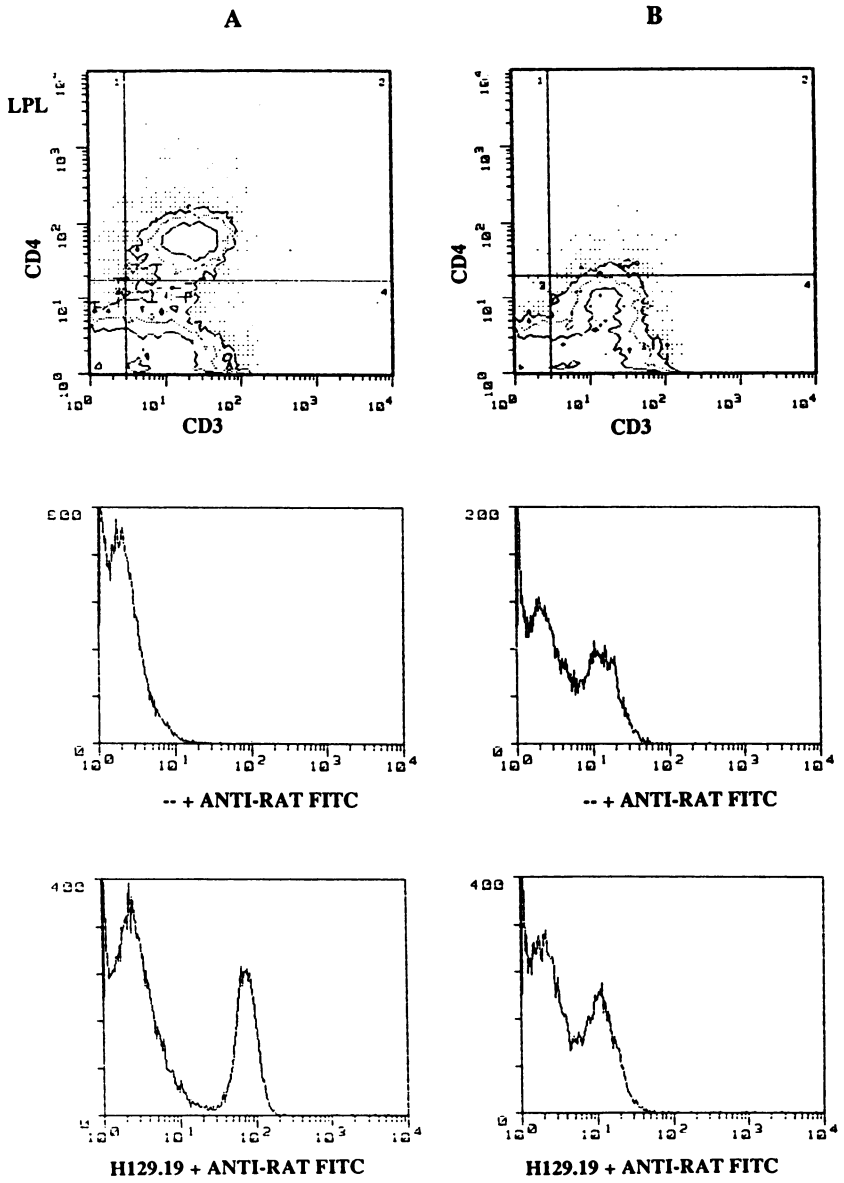

FIG. 2. FACS analysis of the distribution of $CD4^+$ cells in LPL after anti-CD4 MAb treatment (H129.19 plus MAR18.5) (B, top) or no treatment (A, top), using the double-staining protocol described in the legend to Fig. 1. Direct staining of separate sets of cells representing anti-CD4 depleted (B) and control (A) mice was also performed as indicated, using an anti-rat immunoglobulin-FITC antibody with or without prior incubation of these cells with the rat H129.19 MAb.

animals developed strong protection against CT challenge following oral immunization with CT (Fig. 4). Of note, there was no detectable difference between anti-CD4-treated and untreated mice with regard to sensitivity to CT prior to oral immunization, as demonstrated by similar levels of fluid accumulation following injection of CT into the ligated loops in both groups (not shown).

Thus, $CD4^+$ T-cell depletion strongly inhibits the ability to respond with gut IgA antitoxin formation and development of protection against CT challenge of ligated loops after oral immunizations with CT.

Both primary and secondary immune responses to oral CT are abrogated by $CD4^+$ T-cell depletion. $CD4^+$ T-cell depletion was performed prior to oral priming or booster immunizations with CT to investigate whether both types of responses in the local immune system are dependent on the presence of these T cells. Two injections of H129.19 plus MAR18.5 MAbs almost completely blocked the oral priming immunization, whereas it significantly inhibited the booster response in LPL and serum as compared with that observed in control treated mice immunized orally with CT (Table 3). To evaluate whether in these mice we in fact assayed for primary- or secondary-type responses, we analyzed serum antitoxin titers and calculated the ratio between IgG and IgM antitoxin antibody levels. We found the antitoxin IgG/IgM ratio to be highest, >400, for the control treated mice, and ratios were 60 and 2 for the anti-CD4-treated booster and primary immunization groups, respectively. Thus, the re-

TABLE 1. Anti-CT SFC activity in SP, MLN, and PP after oral CT immunization of CD4-depleted mice

Treatment	Animal no.	Total immunoglobulin-antitoxin SFC per 10^7 cells[a]		
		SP	MLN	PP
Control	1	6,750 ± 382	5,880 ± 2,023	1,380 ± 339
	2	10,620 ± 200	1,320 ± 200	1,290 ± 127
	3	730 ± 350	420 ± 176	130 ± 29
	4	230 ± 99	1,200 ± 424	300 ± 85
	5	250 ± 50	730 ± 76	220 ± 29
Mean[b]		4,742 ± 2,121	1,910 ± 1,006	664 ± 276
Anti-CD4	1	40 ± 28	230 ± 71	10 ± 14
	2	180 ± 57	90 ± 42	10 ± 14
	3	50 ± 42	630 ± 127	90 ± 71
	4	0	17 ± 29	0
	5	0	17 ± 29	0
Mean		54 ± 33	197 ± 115	22 ± 17

[a] The mice were given three oral immunizations with CT. Antitoxin SFC in SP, MLN, and PP were determined 7 days after the last immunization. The mice were analyzed individually. SFC values are means ± standard deviations for duplicate or triplicate wells.

[b] Mean values for five mice ± standard errors. There are significant differences between the control treated and the anti-CD4-treated group (SP, $P < 0.01$; MLN, $P < 0.05$; PP, $P < 0.01$).

TABLE 2. Effect of CD4 T-cell depletion on serum antitoxin titers

Treatment[a]	Animal no.	Anti-CT titer[b]
Control	1	7,314
	2	21,540
	3	8,685
	4	4,057
	5	15,010
	6	16,154
Mean		12,126 ± 6,531
Anti-CD4	1	6,544
	2	0
	3	0
	4	13
	5	136
	6	278
Mean		1,162 ± 2,639

[a] The mice were orally immunized five times with CT, with each administration preceded by two injections of H129.19 plus MAR18.5 MAbs (anti-CD4 depletion) or rat IgG plus MAR18.5 MAb (control).

[b] Antitoxin antibody titers were determined in sera from individual animals. Values from individual animals are given together with the mean values ± standard deviations for each group ($P < 0.01$). These mice were also analyzed for protection against CT in ligated loops (Fig. 4) and grouped together in pairs for analysis of antitoxin IgA formation (Fig. 3).

sults suggest that the anti-CD4 MAb treatment effectively blocked primary and booster immunizations, resulting in relatively higher representation of IgM antitoxin antibody levels in sera of CD4-depleted mice as compared with untreated control mice. We conclude that CD4+ T-cell depletion inhibited both the priming and the boosting of an immune response in the local gut immune system following oral immunization.

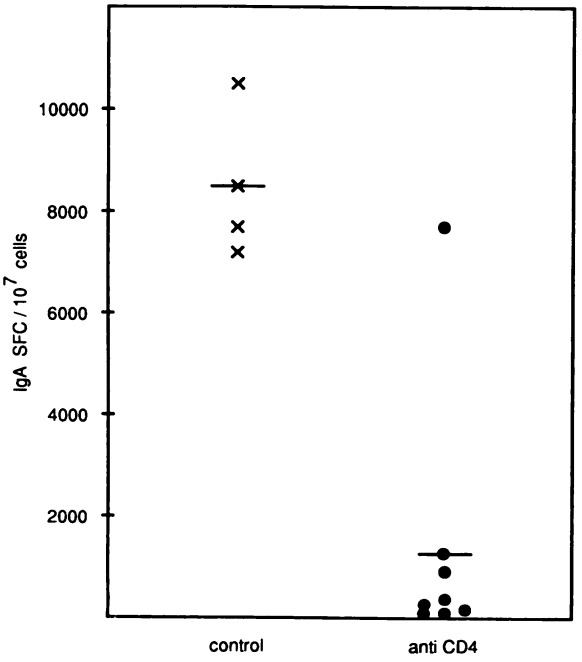

FIG. 3. Comparison of the level of anti-CT IgA SFC in the LP after five oral immunizations with CT in anti-CD4 MAb-treated (●) or control treated (×) mice ($P < 0.05$). Values represent means for two mice, and the mean anti-CT SFC activity in each group is indicated. Serum antitoxin titers were determined in some of these mice (Table 2). The high anti-CT SFC responding mice in the anti-CD4-treated group included the individual animal that showed high serum antitoxin titers in Table 2.

DISCUSSION

This study demonstrates the requirement of functional CD4+ T cells in the gastrointestinal immune system following oral immunization with CT for the development of specific antibody production and protection against CT-induced diarrhea. We have shown previously, using nude thymus-deficient mice (i.e., mice lacking functional T cells) that both antitoxin production and protection against toxin challenge of intestinal loops in mice immunized orally with CT are thymus-dependent, and then probably T-cell-dependent, phenomena (24). In this study we have used normal, instead of nude, mice to extend our analysis of the role of T cells and, more specifically, the role of CD4+ T cells in host defense against CT-induced diarrhea.

A rat MAb, H129.19 (30), specific for the mouse CD4 antigen was used together with a mouse MAb, MAR18.5 (19), directed against rat immunoglobulin in an in vivo treatment protocol that was elaborated to effectively eliminate the CD4+ T-cell population (10). Depletion of the CD4+ population in normal mice has been shown previously to inhibit the ability to respond with antibody production to systemic immunization with various T-cell-dependent antigens (8, 32, 37), to delay allograft rejection (7), and to reduce autoimmune disease (38). This is the first study to show that immune responses to oral immunization are also effectively blocked after depletion of the CD4+ T cells.

Our results from the flow cytometry analysis showing the greatest elimination of CD4+ T cells in the SP, PP, and MLN and much less effective elimination in the LP are interesting from several points. The failure to eliminate CD4+ LP cells suggests that these T cells constitute a subset of cells more

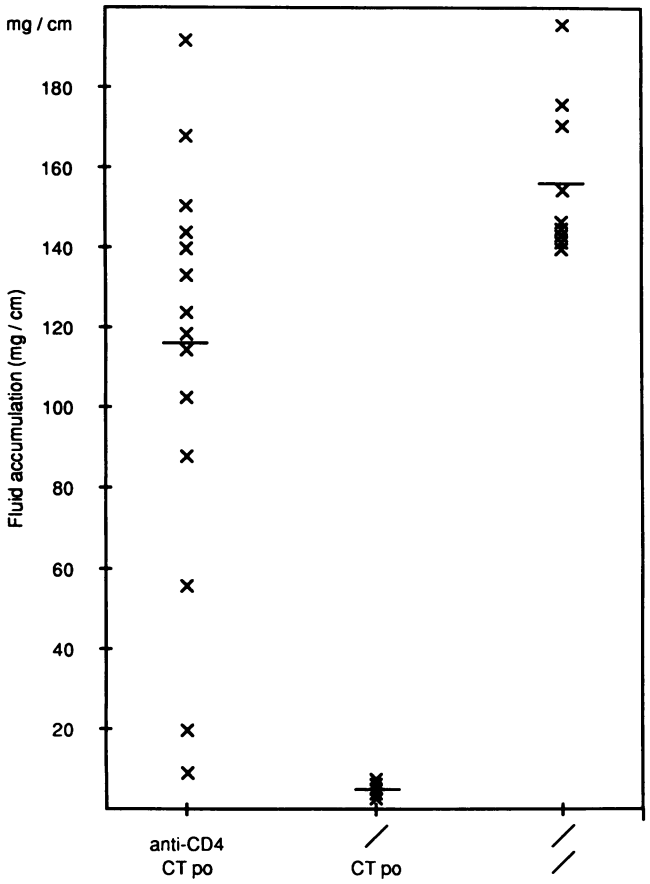

FIG. 4. Protection against CT challenge of intestinal loops of individual mice in anti-CD4-treated (left) or control treated (middle) mice after five oral immunizations with CT ($P < 0.001$). Fluid accumulation in response to CT challenge of the treated and immunized mice was compared with that of untreated mice (right) as indicated. The mean values for each group are indicated. Serum antitoxin titers were determined for some of these mice (Table 2). The individual animal with good protection against CT was also the one that showed high serum antitoxin titers in Table 2. po, Per os.

TABLE 3. Effect of CD4 T-cell depletion on antitoxin responses to oral priming and booster immunizations with CT

Depletion prior to:		Antitoxin response[a]		
		SFC per 10^7 cells		Serum titer[c]
Priming[b]	Boosting[b]	LPL	MLN	
+	−	454 ± 536	667 ± 236	307 ± 328
−	+	2,190 ± 2,626	1,633 ± 939	11,141 ± 8,175
−	−	12,237 ± 2,259	5,183 ± 2,930	36,070 ± 10,974

[a] Antitoxin SFC in LPL and MLN and antitoxin antibody titers in serum were determined 7 days after the booster immunization. The mice were analyzed in pairs for total immunoglobulin anti-CT SFC numbers in the LP and MLN, and the mean values ± standard deviations for three pairs in each group are given. Serum antitoxin titers were determined for individual mice, and the mean values for six mice ± standard deviations for each group are given. This is one representative experiment of two.

[b] The mice were given an oral priming immunization and 10 days later an oral booster immunization with CT. The priming dose (+−) or the booster dose (−+) or neither (−−) was preceded by anti-CD4 MAb treatment.

[c] The differences in serum antitoxin titers among the three groups are statistically highly significant, $P < 0.001$.

refractory to depletion than those in MLN and PP. In studies of systemic immunity after depletion, it has in fact been suggested that secondary $CD4^+$ T cells are less efficiently eliminated by anti-CD4 antibody treatment (32). This might indicate that LP $CD4^+$ T cells are largely secondary or memory types of cells. This notion finds support in recent studies with nonhuman primates, demonstrating that LP T cells carry several characteristics of secondary or activated cells such as high expression of transcripts of the interleukin-2 receptor gene and the interleukin-2 surface receptor (15, 39). In addition, high expression of the CD45RO (recognized by the UCHL1 MAb) isomer, associated with secondary T cells, has been reported in human gut mucosal lymphocytes (4, 16).

However, it might also be that the LP represents a lymphoid tissue to which antibodies injected i.p. have less good access. This is, however, less probable, as the flow cytometry analysis with anti-rat immunoglobulin-FITC revealed that the H129.19 MAbs had indeed bound to the surface of the LPL. It is more likely that good amounts of the anti-CD4 MAb bound to the LPL but still failed to result in lysis of the CD4 cells. A similar phenomenon of poor depletion has been reported for the thymus (37). In that study, it was shown that the anti-CD4 MAb had bound to the cells and down-regulated the expression of the CD4 antigen, but the thymocytes were not substantially depleted. Therefore, in this regard, the LPL $CD4^+$ cells might resemble the T cells in the thymus which have been found to resist both anti-Thy-1 and anti-CD4 antibody treatment (36, 37). The reason for this refractory status of $CD4^+$ T cells in the thymus is unknown, but it has been suggested that it is due to a poor cytolytic environment in the thymus, perhaps insufficient antibody-dependent cellular cytotoxicity (ADCC) (37). Alternatively, antibody-coated cells are not readily killed in situ but are cleared when they circulate through the spleen or liver (37). The latter theory would then indicate that the LPL are relatively stationary cells as compared with lymphocytes from the SP, MLN, or PP. Finally, although the combination of H129.19 and MAR18.5 efficiently eliminates $CD4^+$ cells from the SP, MLN, and PP, they might not be the best MAbs to use for elimination of $CD4^+$ cells from the LP. Further studies are needed to resolve why $CD4^+$ T cells in the LP resist H129.19-plus-MAR18.5 treatment and whether other anti-CD4 MAbs (e.g., GK 1.5) would be more effective in this regard.

Although there were $CD4^+$ T cells present in the LP but absent from the PP, no antitoxin response was observed in either the LP or any other location. These findings are consistent with the theory that the PP is normally the site for priming of a humoral immune response in the gut (5). However, in this study not only primary responses but also secondary responses to oral CT were inhibited by anti-CD4 treatment, suggesting that secondary $CD4^+$ cells in the gut LP were also impaired to respond to challenge with oral antigen. One explanation for this might be that binding of anti-CD4 MAb to the cell surface is sufficient to disrupt normal $CD4^+$ T-cell function to the same extent as that produced by $CD4^+$ T-cell depletion (6). An alternative explanation would advocate that secondary immune responses in the gut LP also must be triggered through cells in the PP, and since these cells are eliminated by the anti-CD4 MAb treatment, there are no cells available to respond to specific antigen. This notion, implying a strong dependence of the gut secondary IgA response on cell traffic from PP to LP, is at variance, however, with our previous studies of gut

immunological memory to CT showing brisk (within hours) antitoxin IgA responses in the LP to oral challenge with CT, as well as with the studies of others providing evidence of local activation of secondary immune responses in the LP without the involvement of cells from the PP (14, 25, 29).

Most previous studies on protection against CT challenge of the intestine and the formation of antitoxin IgA antibody following oral immunization with CT have documented a close association between these two phenomena (17, 24, 28, 29). We have also demonstrated in mice that oral CT immunization stimulates LP cells to produce IgA antitoxin antibodies that effectively neutralize CT as determined by the complete inhibition of the effect of CT in the rabbit skin test (24). However, it is possible that other mechanisms of host defense against enterotoxins exist and might be involved in local gut mucosal protection. Recently, it was reported that gamma interferon produced by activated T cells could protect gut epithelial cells in vitro from fluid loss, i.e., salt loss due to changes in ion transport over the epithelial cell membrane, induced by CT (12). Related to such a protective mechanism by T cells is the recent finding that oral booster immunization with the combined whole cell-cholera B-subunit vaccine gave rise to high numbers of gamma interferon-producing cells in the mucosa (31).

In contrast to these host defense mechanisms dependent on a functioning local immune system, Lönnroth and coworkers have reported that CT, and other agents, stimulates the formation of antisecretory factors which are suggested to function in host defense against cholera disease (21, 22). These factors, found in the highest concentration in the pituitary gland but also in the gut mucosa, were induced by CT as well as hyperosmolar solutions and were not antigen specific, and their formation and protective capacity against CT challenge did not correlate with the production of antitoxin IgA in the gut (18). However, the results of this study clearly associate the presence of $CD4^+$ T cells with the development of host defense against CT, strongly suggesting that antitoxic protection in the gut is a function of the immune system.

Local gut mucosal IgA production might not be the only protective factor in host defense against cholera disease; e.g., CT-specific $CD4^+$ T cells might produce regulatory factors, gamma interferon and others, that interact with the epithelial cells as well as with the nervous system of the gut. Further studies are needed to understand what protective and regulatory factors produced by the local immune system in the gut are necessary for host defense against cholera disease. In particular, it will be important to understand whether T cells in the gut have a regulatory role other than supporting B-cell differentiation in host defense against enterotoxin-induced disease.

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