Morphologic and Functional Alterations of Mucosal T Cells by Cholera Toxin and its B Subunit[1]

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Despite the mucosal immunogenicity and adjuvanticity in vivo of cholera toxin (CT), both CT and CT B subunit are strong inhibitors of T cell activation in vitro. This study asked whether such T cell inhibition is relevant to the mucosal effects of CT in vivo. The activation of T cells pulsed in vitro for only 15 to 120 min with CT or CT B subunit, respectively, was inhibited, consistent with the expected short exposure times in vivo. Although both $CD8^+$ and $CD4^+$ T cells were inhibited in vitro, $CD8^+$ T cells bound more toxin and were inhibited to a greater degree than were $CD4^+$ T cells. Intestinal gavage of mice with 10 μg CT did not alter the overall composition of Peyer's Patch, mesenteric lymph node, or spleen but did cause a marked depletion of intraepithelial lymphocytes, mainly $CD8^+$ T cells, and of lymphocytes in the dome epithelium over Peyer's Patch. To determine whether such inhibition of T cells was functionally relevant in vivo, T cells from mice fed keyhole limpet hemocyanin (KLH) were adoptively transferred into naive recipients, who were then parenterally immunized. T cells from mice fed KLH alone inhibited both the systemic IgG and secretory IgA anti-KLH response, but T cells from mice fed KLH plus CT did not, indicating that mucosally applied CT was able to abrogate the induction of this suppressor T cell. We conclude that one of the mechanisms of CT's mucosal effects in vivo is the inhibition of certain mucosal T cell functions and alteration of the regulatory T cell environment in gut-associated lymphoid tissue. *The Journal of Immunology,* 1995, 154: 1032–1040.

Cholera toxin (CT)[3], an enterotoxin produced by *Vibrio cholerae*, mediates clinical cholera (1). CT is composed of two subunits: an enzymatic A subunit and a binding or B subunit (CT-B) (2). CT-B is a homopentamer that mediates attachment to intestinal epithelial and other cell surfaces via its ligand monosialoganglioside, G_M1 (3). The genes for CT have been cloned (4, 5), and the crystal structure is emerging. By analogy to the crystal structure of the closely related *Escherichia coli* heat-labile enterotoxin, the B subunit pentamer has a central pore through which the helical A2 peptide extends, linking the B subunit to the enzymatically active A1 peptide (6). CT is a strong mucosal immunogen that has been used for some years as a probe of the mucosal immune system (7–9). More recently, CT has been found to act as a mucosal adjuvant, inducing mucosal immune responses to unrelated Ags, when Ag and CT are delivered to the mucosa together (10–12).

The mechanisms involved in the potent mucosal immunogenicity and adjuvanticity of CT remain unexplained. The mucosal administration of most protein Ags fails to stimulate a mucosal Ab response but instead induces a state of unresponsiveness or oral tolerance (13, 14). CT is an exception to this rule in that the feeding of either CT or CT-B does not induce oral tolerance for Ab response; moreover, CT is able to abrogate oral tolerance to unrelated Ags administered with it orally (10). These effects suggest that CT is able to alter the regulatory environment in gut-associated lymphoid tissue (GALT) in such a way as to favor responsiveness over tolerance. Based on these considerations, and because T cells are the major regulators of immune responses within the mucosal immune system (15), we

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[3] Abbreviations used in this paper: CT, cholera toxin; CT-B, cholera toxin B subunit; GALT, gut-associated lymphoid tissue; IEL, intraepithelial lymphocytes; KLH, keyhole limpet hemocyanin; MLN, mesenteric lymph node; PP, Peyer's patch; PE, peritoneal exudate.

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have investigated in more detail the effects of CT and CT-B on T cells.

Despite their stimulatory effects in vivo, CT and CT-B are strong inhibitors of both mitogen- and antigen-induced T cell activation in vitro (16). Such inhibition requires direct binding of CT or CT-B to T cells, and either can be added many hours after the T cells are stimulated with polyclonal activators or with Ag and still mediate their inhibitory effects. The inhibitory signal to the T cell is not mediated either through adenylate cyclase (16) or through the phosphatidylinositol system, but does result in the down-regulation of IL-2 mRNA and protein synthesis (17). In studies reported here, we asked whether these in vitro observations were relevant to what occurs in vivo after mucosal exposure to CT. We report here that T cells are inhibited in vitro after only brief exposure to CT or CT-B, that the $CD8^+$ T cell subset is preferentially inhibited in vitro, and that similar effects occur in vivo in that a profound reduction in $CD8^+$ intraepithelial lymphocytes (IEL) occurs shortly after a mucosal exposure to CT. These in vivo effects have biologic significance in that mucosal CT was shown also to inhibit the generation of suppressor cells that mediate oral tolerance. These results indicate that CT has profound effects on regulatory T cells in the mucosal immune system, and these effects are likely to be involved in its mucosal immunogenicity and adjuvanticity.

Materials and Methods

Animals

C57B1/6 or CB6F1 female mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and were certified virus-free. Mice were used for all experiments at 8 to 12 wk of age.

Materials

Reagents and materials were purchased from the following sources: CT, CT-B, and biotinylated-CT-B were obtained from List Biologicals (Campbell, CA); OVA, Con A (ConA), and PMA were obtained from Sigma Chemical Co. (St. Louis, Mo); ionomycin and keyhole limpet hemocyanin (KLH) were obtained from Calbiochem (La Jolla, CA).

Cell isolation and purification

Freshly isolated murine spleens were teased apart. Red cells were lysed by incubation in 0.017 M Tris-0.14 M NH_4Cl on ice for 10 min. The remaining spleen cells were depleted of B cells with BioMag goat anti-mouse IgG beads and a magnet (BioMag Co., Cambridge, MA). The remaining cells were incubated with anti-CD4 (GK1.5) or anti-CD8 (53.6–72 mAb), washed three times, then incubated with mouse anti-rat IgG (MAR 18.5), washed an additional three times, incubated with the goat anti-mouse IgG-coated magnetic beads, then passed over a magnet. The residual, negatively selected cells, enriched for either $CD8^+$ or $CD4^+$ T cells, were used in the in vitro experiments. Purity of the separations was confirmed by flow cytometry.

Cell culture

All tissue culture components were obtained from Life Technologies, Inc. (Grand Island, NY), unless otherwise stated. Cells were resuspended at a concentration of 2×10^6 cells/ml in complete medium consisting of RPMI 1640, 10% FCS, 25 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 5×10^{-5} M 2-ME (Bio-Rad Labs, Richmond, CA). Except where stated, 200-μl aliquots containing 4×10^5 cells were placed in the individual wells of 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) and incubated at 37°C in a 5% CO_2 atmosphere for the time period described for each experiment.

Measurement of T cell proliferation

Cultured cells were pulsed with 0.5 μCi of [^{3}H] thymidine, sp. act. = 6.7 Ci/mmole, (NEN, Boston, MA) during the last 6 h of incubation. Cells were then harvested onto glass fiber filter paper and washed (Whittaker Bioproducts, Walkerville, MD) using a mini-MASH harvester (Cambridge Technology, Watertown, MA). The filters were air dried, placed into vials containing 3a20 counting mixture (RPI, Mount Prospect, IL), and counted in a beta scintillation counter. Thymidine incorporation into DNA was determined in triplicate cultures and was expressed as mean cpm.

Measurement of Ab

Ab in plasma and secretions was measured by ELISA as described (18). Briefly, Ag was coated on the wells of a 96-well polystyrene Immulon plate (Dynatech, Rockville, MD). After an incubation with sample or standard mouse Ab, the wells were incubated with rabbit anti-mouse IgG or rabbit anti-mouse IgA, followed by an incubation with sheep anti-rabbit globulin coupled to alkaline phosphatase. Finally, 0.1 ml of *p*-nitrophenylphosphate (1 mg/ml; Sigma Chemical Co., St. Louis, MO) in 10% diethanolamine buffer at pH 9.8 was added to each well, and the color development after a 30-min incubation at room temperature was measured at OD 405 in an MR 580 micro-ELISA autoreader (Dynatech Instruments, Santa Monica, CA). A standard curve was constructed for each assay, and the values of the samples were interpolated using a program based on Rodbard's four parameter logistic model (19) on an Apple Macintosh computer (Cupertino, CA). The reference standard for anti-KLH was an affinity-purified Ab obtained from the serum of hyperimmunized mice; the results of anti-KLH are thus expressed as μg/ml. Measurement of total IgA present in intestinal secretions was done by sandwich ELISA as described (16).

Flow cytometric analysis

Cells were incubated with FITC-labeled mAbs to CD3 (145–2C11), CD4 (GK1.5), CD8 (53.6–72), Thy1.2 (30-H12), or B220 (6B2), then washed with PBS containing 0.01% sodium azide and 5% FCS. In some experiments, the cells were first incubated with biotinylated-CT-B, washed, and then stained with FITC anti-CD4, or FITC anti-CD8 plus phycoerythrin-avidin. Single and two-color flow cytometric analysis was performed on a FACStar (Becton Dickinson, Sunnyvale, CA).

Immunohistochemistry

Groups of mice of similar age and housed under the same conditions were gavaged with either saline, CT (10 μg), or CT-B (100 μg) by a gastric feeding needle. Three days later, the mice were killed and a segment of proximal small intestine containing a Peyer's patch (PP) was removed from each mouse, positioned in octamer-binding factor compound (Miles Inc, Elkart, IN) in cryomolds and snap-frozen in liquid nitrogen. Samples were stored at −70°C. Serial 7-μm frozen sections were cut on an IEC cryomicrotome, adhered to superfrost slides, and air dried. All slides were stored at room temperature and 0% humidity for at least 24 h in an electronic desiccation chamber before use. Replicate frozen sections were rehydrated and washed in PBS with 10% agammaglobulinemic horse serum for 30 min and then covered with 200 μl of biotinylated mAbs for an additional 30 min, then washed twice in PBS. Biotinylated-anti-CD3, -anti-CD4, and -anti-CD8 were obtained from Life Technologies, Inc., Gaithersburg, MD; biotin-anti-IgA, -anti-IgM, and -anti-IgD were purchased from Southern Biotechnology Associates, Birmingham, AL. Sections were developed using horseradish peroxidase-biotin-avidin complexes (ABC Kit, Vector Laboratories, Burlingame, CA). The diaminobenzidine reaction products were enhanced by the addition of nickel chloride. The number of IEL/unit length of basement membrane was measured blindly on coded 8 × 10 photographic prints at a final magnification of ×5000 using a map reader. At this scale, 1 cm of basement membrane on the print was equal to 0.05 mm of actual length in the section.

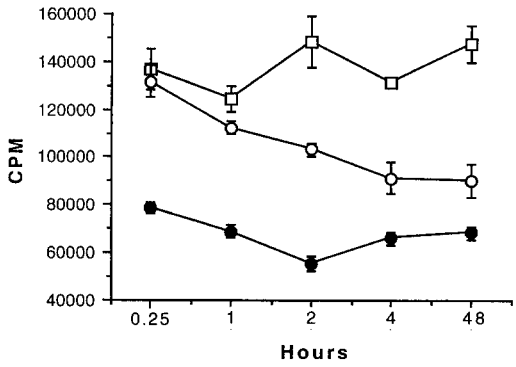

FIGURE 1. Inhibition of T cell activation by short pulses of CT or CT-B. Purified spleen T cells were cultured with ConA in vitro with or without the addition of 4 μg/ml of CT or 4 μg/ml of CT-B. The latter were washed out of individual cultures at 0.25, 1, 2, or 4 h, and the cells put back into complete media with ConA 5 μg for a total duration of culture of 48 h. Hours of exposure to CT or CT-B is given along the ordinate. □, T cells not exposed to CT or CT-B; ○, T cells exposed to CT-B; ●, T cells exposed to CT.

Results

Inhibition of T cell activation in vitro after short pulses of CT or CT-B

In previous reports, CT or CT-B was added to T cell cultures being stimulated for 48 h with ConA or other activators in vitro (16). Cells are likely to be exposed to CT for much shorter periods in vivo. In these experiments, purified T cells were cultured with ConA in vitro with or without the addition of pulsed CT or CT-B for the intervals shown. The latter were washed out of individual cultures, and the cells were put back onto complete media with ConA for a total duration of culture of 48 h. Control cultures were not exposed to CT or CT-B but were otherwise similarly treated. As can be seen in Figure 1, a pulse of 15 min with CT or 2 h with CT-B was sufficient to cause substantial inhibition of T cell activation.

Preferential inhibition of $CD8^+$ T cells in vitro

The effect of CT and CT-B on T cell subsets was assessed in two ways. The first approach was to culture splenic T cells with or without the addition of CT or CT-B and to analyze the cells remaining at the end of culture by flow cytometry for the proportions of $CD3^+$, $CD4^+$, and $CD8^+$ T cells. There was a preferential reduction of $CD8^+$ T cells during culture in the presence of a polyclonal activator, such as ConA (data not shown). Similar inhibition occurred in the absence of a polyclonal activator; representative data using CT-B are shown in (Fig. 2). The same results were obtained with CT. Similar effects on $CD8^+$ T cells were seen with PP T cells activated with ConA from the start of culture, with CT or CT-B added at later time points. As little as 1 h of exposure at the end of the 48-h culture was sufficient to reduce the proportion of remaining $CD8^+$ T cells (Table I).

The second method was to compare the inhibitory effect of CT or CT-B on isolated $CD4^+$ and $CD8^+$ T cell subsets. Thus, whole spleen T cells, CD4-depleted spleen T cells, or CD8-depleted spleen T cells were cultured in the presence of doses of PMA and ionomycin that had previously been found to be optimally mitogenic. Although both subsets were significantly inhibited, there was consistently greater inhibition of $CD8^+$ T cells than $CD4^+$ T cells (Table II).

Greater binding of CT-B to $CD8^+$ T cells than to $CD4^+$ T cells

One explanation for the relatively greater inhibitory effect of CT and CT-B on $CD8^+$ T cells is that more CT-B may bind to $CD8^+$ T cells than to $CD4^+$ T cells. To address this question, cells were analyzed by flow cytometry for the amount of binding by biotinylated-CT-B. T cells were coated with biotinylated-CT-B plus either FITC-anti-CD4 or FITC-anti-CD8. After washing, the cells were incubated with avidin-phycoerythrin. Cells binding both the FITC and PE label were then measured by dual channel flow cytometry. As shown in Figure 3, $CD8^+$ cells bound a greater amount of CT-B than did $CD4^+$ cells.

GALT lymphocyte populations in vivo after gavage with CT

The next question we addressed was whether similar preferential reduction in $CD8^+$ cells in GALT occurred in vivo after CT was administered into the intestine. Mice were given 10 μg CT intragastrically by a feeding needle; controls received only the vehicle. At 6, 12, and 24 h later, PP, mesenteric lymph node (MLN), and spleen were isolated. Single cell isolates were counted, stained with a panel of mAbs to surface markers, including CD3, CD4, CD8, and B220, and analyzed by flow cytometry. As shown in Table III, no significant change in the relative proportions of PP, MLN, or spleen cells was observed. The recoveries of cells from each tissue was equivalent, so there was no change in the absolute number of cells either.

Immunohistochemistry of GALT after gavage with CT or CT-B

Because cells closest to the lumen might be affected more than cells that are not, immunohistochemical analysis of lymphocyte populations in the gut mucosa and PP was performed 72 h after CT 10 μg or CT-B 100 μg were given intragastrically and was compared with the analysis of control mice receiving only vehicle. Although cells in the lamina propria were not visibly affected, there was a marked reduction in IEL number, which consisted mainly of $CD8^+$ T cells (Fig. 4). Lymphocytes in the dome epithelium of the PP, which were mainly CD8 T cells and $sIgD^-$ PNA^+ B cells, were depleted as were cells just

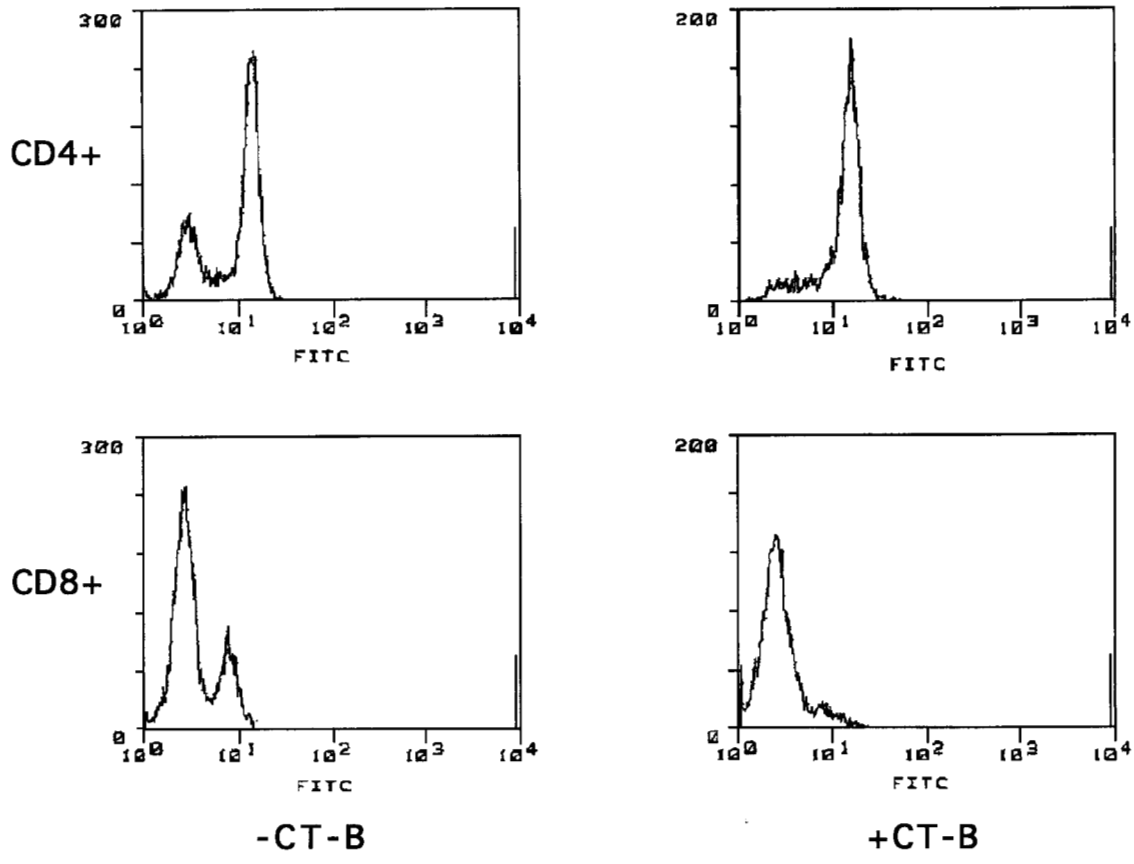

FIGURE 2. Spleen T cells were cultured overnight in complete media. Some cultures received CT-B at 4μg/ml (+ CT-B); control cultures did not (− CT-B). The cells were recovered, incubated with FITC-anti-CD4 or FITC-anti-CD8, and analyzed by flow cytometry.

Table I. *Effect of CT and CT-B on activation of Peyer's patch T cell subsets in vitro*

Addition[a]	Duration of Exposure (h)[b]	% Positive[c] Thy1	CD4	CD8
None		77.7	67.2	7.2
CT	36	79.4	61.2	3.1
CT-B	36	75.6	60.8	3.7
CT	1	73.1	67.9	3.5
CT-B	1	69.3	60.7	4.3

[a] Purified PP T cells were cultured with ConA 5 μg in vitro with or without the addition of CT or CT-B at 4 μg/ml.

[b] The additions of CT or CT-B to culture were made at various times during the 48-h culture period such that the duration of exposure to CT or CT-B was either 36 h or 1 h.

[c] Cells remaining at the end of culture were analyzed by flow cytometry for the percentage of cells expressing Thy1, CD4, or CD8. These are representative data of three experiments.

Table II. *Inhibition of proliferation of isolated $CD4^+$ and $CD8^+$ T cell subsets by CT-B*

Spleen T Cells[a]	CT-B	CPM ± SD Media Alone	Phorbol Myristic Acetate and Ionomycin	% Inhibition
Unfractionated	–	146 ± 10	116,417 ± 12,817	–
	4 μg/ml	77 ± 19	56,262 ± 4,874	52
$CD4^-$ depleted	–	84 ± 6	71,060 ± 8,971	–
	4 μg/ml	42 ± 2	18,460 ± 2,325	75
$CD8^-$ depleted	–	77 ± 14	97,120 ± 1,352	–
	4 μg/ml	59 ± 10	52,948 ± 11,293	45

[a] Spleen T cells were depleted of $CD4^+$ or $CD8^+$ T cells using mAbs and magnetic beads. Depletions resulted in removal of >95% of the relevant cell type. Whole T cells, $CD4^+$ depeleted T cells, or $CD8^+$ depleted T cells were then cultured for 48 h in the absence (media alone) or presence (PMA + I) of phorbol myristic acetate 25 μM ionomycin 100 μM, which had previously been found to be optimally mitogenic. Proliferation was measured as the incorporation of ^{3}H-thymidine during the last 6 h of culture. The percentage inhibition of proliferation caused by the addition of CT-B to the cultures was calculated. These data are representative of the results of three experiments.

below the dome epithelium, where there was a notable reduction of T cells, particularly $CD8^+$ T cells, as well as reduction of $sIgA^+$ and $sIgM^+$ B cells. The IgD^+ corona and regions lying deeper in the PP were unaffected, which is consistent with the flow cytometry data. When the $CD8^+$ IEL/mm length of basement membrane was measured, the number in the group receiving CT (3.6 ± 1.4 cells/mm) was significantly less ($p < 0.02$) at day 3 than in the group receiving saline (16.5 ± 6.5 cells/mm). IEL number in the group receiving CT-B was reduced 50% (8.3 ± 3.8 cells/mm) from control but did not reach statistical significance. There was a concomitant reduction at

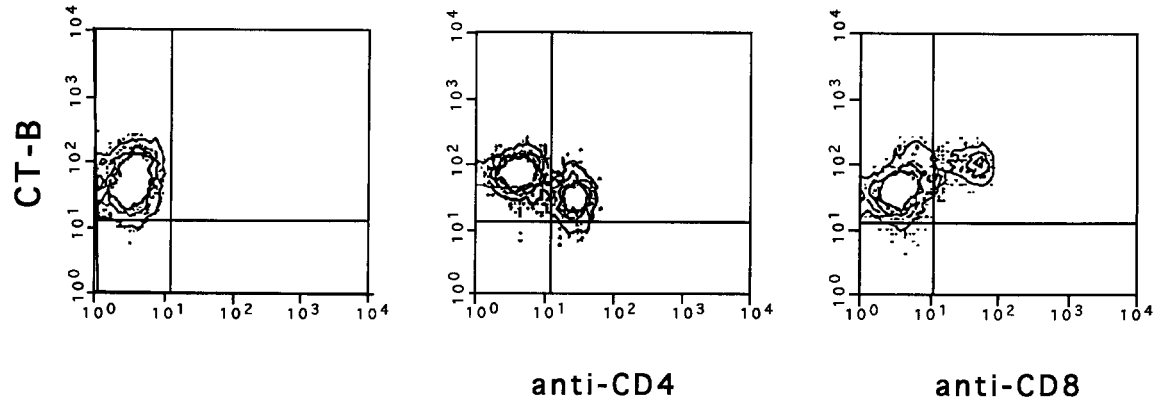

FIGURE 3. Preferential binding of CT-B by $CD8^+$ T cells. Purified spleen T cells were incubated for 30 min with biotin-CT-B plus FITC-anti-CD4 or FITC-anti-CD8. After washing, the cells were incubated with avidin-PE and analyzed by dual channel flow cytometry.

Table III. *Analysis of lymphoid cell populations in mice 24 hrs after CT gavage*

	% Positive							
	Control				CT-Gavage			
Tissue[a]	B	CD3	CD4	CD8	B	CD3	CD4	CD8
PP	75.0	26.0	19.0	5.0	63.0	29.0	22.0	7.0
MLN	15.5	85.5	63.0	22.0	18.0	84.0	60.5	21.0
Spleen	50.0	47.0	31.0	15.0	50.0	45.0	29.0	14.0

[a] Mice were given CT 10 μg intragastrically by feeding needle; controls received only saline. At 6, 12, and 24 h later, PP, MLN, and spleen were obtained, and single cell suspensions were prepared. Cell counts indicated that equivalent numbers of cells were recovered from these organs in control and CT-gavaged mice. Cells were incubated with mAbs to B220 (B), CD3, CD4, and CD8. The percentage of cells expressing these surface markers was measured by flow cytometry. Only data from the 24-h time point are shown, but similar results were obtained at 6 and 12 h after CT gavage.

day 3 of the numbers of IEL recovered from the intestine of mice given CT (0.5×10^6/mouse) as compared with mice given saline (1.7×10^6/mouse).

Abrogation of suppressor T cell function in vivo by mucosal CT

The next experiment asked whether the morphologic alteration in mucosal T cells in vivo was accompanied by any change in immunoregulatory T cell function. Oral tolerance after the feeding of protein Ags has been found to be mediated by a number of mechanisms, one of which is induction of suppressor cells (20–22). Thus, an adoptive transfer system was used to determine whether suppressor cells could be demonstrated after KLH feeding and, if so, whether feeding CT with KLH altered this functional activity. Donor mice were fed either OVA as an irrelevant Ag, KLH 5 mg, or KLH 5 mg plus CT 10 μg on two occasions 1 wk apart. One week after the second feeding, the mice were killed, their spleen cells were isolated, and spleen T cells were purified over nylon columns. After i.v. injection of these T cells into naive recipients, the recipients were immunized with three doses of KLH at biweekly intervals: 100 μg KLH in CFA i.p., 5 mg. KLH intragastrically, and 100 μg KLH in CFA i.p. One week after the last dose, plasma and intestinal secretions were obtained for measurement of anti-KLH by ELISA. The results are shown in Table IV. A strong serum IgG and secretory anti-KLH response was present in the control group receiving T cells from the OVA fed mice. This anti-KLH response was significantly reduced ($p < 0.05$) in the group that received T cells from the mice fed KLH, consistent with the induction of a suppressor cell by the KLH feeding; of note, both the plasma IgG and intestinal sIgA anti-KLH response were significantly inhibited. The group receiving T cells from mice fed CT with the KLH had an anti-KLH response similar to the control, indicating that mucosally applied CT had abrogated this suppressive T cell functional activity.

Discussion

These studies provide new insight into the potential mechanism of immunogenicity and adjuvanticity of CT in the gut. Consistent with a number of studies already reported (16, 17), T cell activation in vitro was shown to be markedly inhibited by CT and CT-B. This occurred even after only short exposures to CT or CT-B. Although both $CD4^+$ and $CD8^+$ T cell activation was inhibited, $CD8^+$ T cells were consistently more sensitive than $CD4^+$ T cells. The

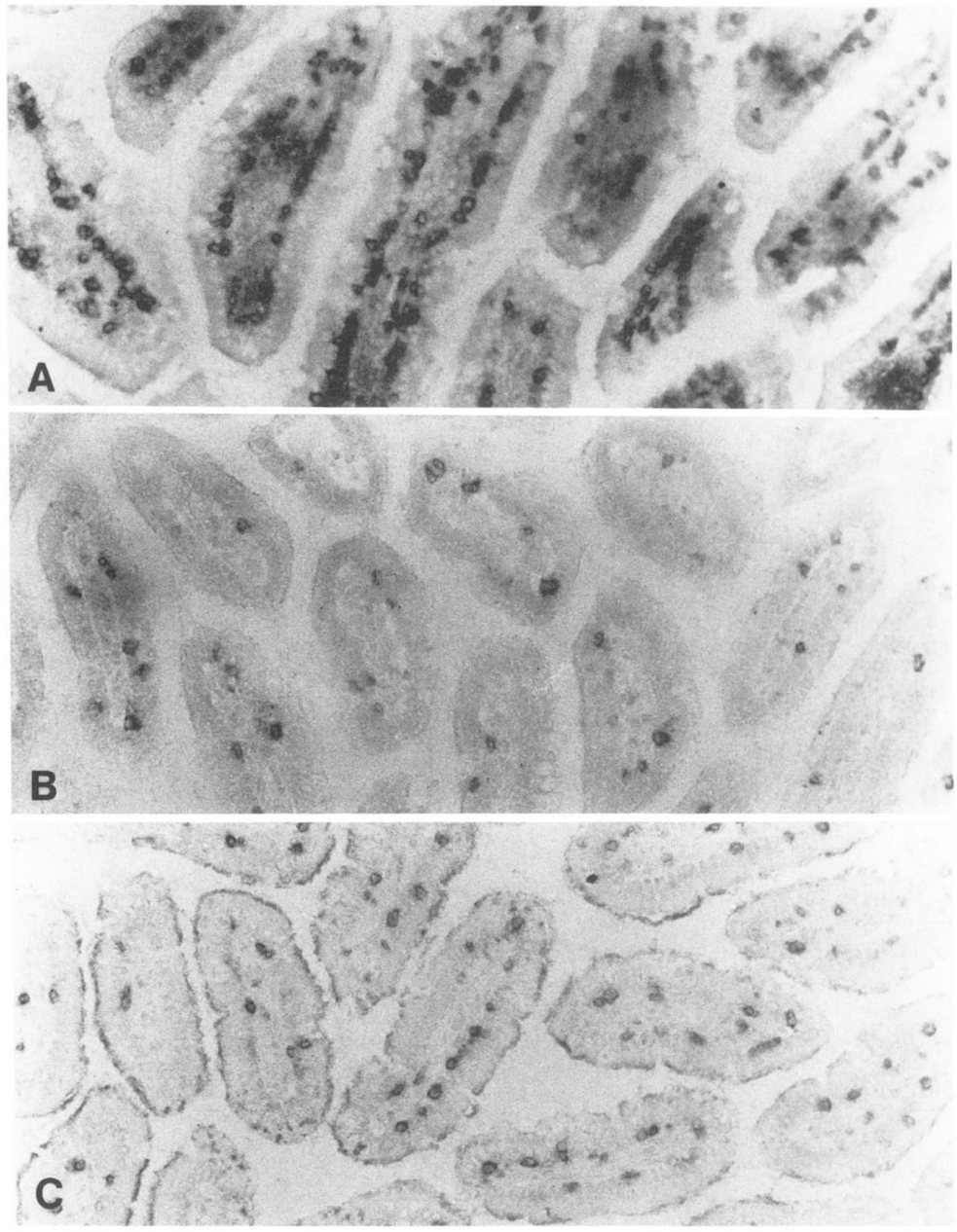

FIGURE 4. Marked depletion of IEL by mucosal CT and CT-B. Segments of jejunum were taken 72 h after saline, CT 10 μg, or CT-B 100 μg were given intragastrically. Frozen sections were stained with anti-CD8. *A*, saline control; *B*, CT; *C*, CT-B.

greater sensitivity of the $CD8^+$ T cell subset to inhibition in vitro is likely due to its membrane having greater amounts of G_M1 ganglioside, as reflected by its increased binding of biotin-CT-B. The mechanism of inhibition of T cell activation by CT and CT-B in vitro is not yet clear, but the inhibitory signal is not mediated by either the adenylate cyclase or the phosphatidylinositol second messenger system (16, 17). We have recently found that CT-B is endocytosed by T cells, which raises the possibility that this might be the signaling pathway (Stransky, G., S. Guy, C. O. Elson, unpublished observations). The binding of larger amounts of CT-B or CT to the $CD8^+$ T cell membrane may result in more of the toxin being endocytosed into the cell and thus lead to greater inhibition. Alternatively, $CD8^+$ T cells may simply be more sensitive to the negative signal provided by the CT or CT-B. The consistent T cell inhibition by CT and CT-B in vitro is in marked contrast to their immunostimulatory properties in vivo, and the challenge has been to demonstrate whether the in vitro effects of CT have biologic relevance in vivo. This was the major aim of this study.

CT has been shown to have marked effects on the murine lymphoid system in vivo after it is injected parenterally; μg quantities given i.v. cause marked atrophy of the

Table IV. *T cell suppression of both secretory IgA and plasma IgG anti-KLH after KLH feeding and its abrogation by CT*

Donors Fed[a]	Cell Transfer	Antibody Response in Recipient: Secretory IgA anti-KLH (ng/μg IgA)[b]	Antibody Response in Recipient: Plasma IgG anti-KLH (ng/ml)[b]
OVA	Spleen T cells	5.72 (2.53)	1648 (1.85)
KLH	Spleen T cells	1.13 (2.26)[c]	644 (2.41)[c]
KLH and CT	Spleen T cells	6.70 (1.34)	1455 (1.35)

[a] Groups of mice were fed OVA 5 μg, KLH 5 mg, or KLH 5 mg plus CT 10 μg on two occasions 1 wk apart. One week after the second feeding, spleen T cells were isolated and were i.v. injected into naive recipients, who were then immunized with three doses of KLH at biweekly intervals: 100 μg KLH in CFA i.p..; 5 mg KLH intragastrically and 100 μg KLH in CFA i.p. One week after the last dose, plasma and intestinal secretions were obtained for assay of anti-KLH by ELISA. Representative data of two experiments.

[b] Geometric mean (standard deviation).

[c] $p < 0.05$ compared to either OVA or KLH + CT groups.

thymus and lymph nodes (23, 24). We have confirmed this observation (data not shown), but the effect is so marked that one is unable to see any preferential effect on T cell subsets. Injection i.v. is clearly not a physiologic experiment, but it does illustrate that CT can have profound effects on lymphoid cells in vivo. We performed a systematic analysis using flow cytometry on bulk populations of PP, MLN, and spleen cells after mucosal administration of CT. There was no measurable effect of CT on their cellular composition. However, flow cytometry averages the data of a large number of cells, many of which are at a distance from the intestinal lumen and thus unlikely to be directly exposed to CT. The lack of effect by flow cytometry does illustrate that the dose of CT that gets through the epithelium and into the general circulation must be small, because effects similar to i.v. administration were not seen.

Because flow cytometry might not reveal effects on the small fraction of cells that are strategically located close to the intestinal lumen, immunohistochemical analysis was done. This analysis showed striking changes in lymphocytes in the epithelium, i.e., those closest to the intestinal lumen. The IEL population, which is predominantly $CD8^+$ T cells, was markedly reduced in number after mucosal exposure to CT. Similarly, the epithelium and subdome region of PP were depleted of lymphoid cells. CT binds to the luminal surface of epithelial cells but then is endocytosed and probably transcytosed (25). Thus, the IEL and the dome of PP are the sites where the highest concentration of CT is likely to occur after mucosal administration, and these are the sites that were most affected. Notably, both CT and CT-B were able to reduce the IEL number, although the effect was more pronounced for CT than for CT-B, which is similar to their relative inhibitory potency in vitro. A 10-fold larger dose of CT-B than CT was used because a previous study with CT-B as a chimeric immunogen found that larger doses were needed to induce immunity with the molecule (26).

Because of the heterogeneity of the IEL compartment (27), we are currently assessing which subtypes of IEL are affected by mucosally administered CT. Although preliminary, the data indicate that the depletion affects all of the major IEL cell types, including $T\alpha\beta$ and $T\gamma\delta$ cells. The mechanism of IEL depletion is not yet clear, but three possibilities are suggested: 1) the migration of IEL out of the epithelial layer, 2) modulation of CD8 cell surface expression, and 3) the induction of apoptosis. Down-modulation of CD8 surface expression on IEL does not explain these data, because there was a concomitant decrease in total recovery of IEL and of the numbers of $CD3^+$ IEL (data not shown). We have no direct data on either of the other two possibilities; however, CT is able to induce apoptosis of activated T cells in vitro (Stransky, G., and C. O. Elson, unpublished data), and IEL have been found to undergo apoptosis rapidly when isolated in vitro (28), so we favor this last possibility. Regardless of the mechanism of depletion of IEL by CT, this effect now makes studies possible on IEL turnover and repopulation. A detailed kinetic study is underway, but the early indications are that the number of IEL is decreased within hours of CT exposure and returns to base line 10 to 12 days later (Anderson, A. O., C. F. Cuff, and M. T. Dertzbaugh, unpublished data).

The above results indicated that T cells, particularly the $CD8^+$ T cell subset, were inhibited both in vitro and in vivo. However, the question remained whether the effect of CT on mucosal T cells was functionally important in vivo. To examine this question, we used an adoptive transfer system that has been used to demonstrate suppressor T cells after Ag feeding (20–22). In these experiments, KLH was fed to mice, after which their spleen T cells were purified and adoptively transferred to naive recipients, which were then immunized. The group fed KLH alone did have a suppressor T cell evident in their spleens, as has been reported for other proteins (20–22). Co-feeding of CT with KLH abrogated the induction of this suppressor cell, consistent with an alteration of the regulatory T cell environment in vivo by mucosal exposure to CT. It is not clear from these experiments where this regulatory T cell is generated. However, in a previous study, $CD8^+$ suppressor T cells generated by feeding were first identifiable in MLN, and were only found several days later in spleen (18). Others have found suppressor T cells in PP after Ag feeding (29). However, we cannot exclude the possibility that the cells are induced in the IEL or lamina propria and travel to the MLN and then the spleen.

A second important feature revealed in the adoptive transfer experiments was that the Ag feeding generated cells that also suppressed intestinal sIgA production. Mucosal IgA responses have seldom been measured directly in oral tolerance studies. Some studies have suggested that systemic (IgG) tolerance after feeding protein Ags is accompanied by mucosal (IgA) immunization (30, 31). When intestinal secretions are directly measured, the feeding of most proteins, even in large doses, does not generate

sIgA responses but does frequently generate oral tolerance (10). The adoptive transfer data shown in Table IV reveal that another potential outcome of protein feeding is the induction of suppressor cells that actively down-regulate not only systemic IgG responses but also mucosal IgA responses. Thus, inhibition of mucosal IgA responses can be a component of oral tolerance. The difficulty most investigators experience in inducing mucosal IgA responses to protein Ags indicates that such mucosal suppression is not infrequent. If so, these results may help explain why it is so difficult to get mucosal immunization by feeding proteins alone.

Although CT inhibits $CD8^+$ T cells preferentially, it also has effects on $CD4^+$ T cells as well. We had previously reported that both CT and CT-B down-regulate IL-2 mRNA and protein expression in vitro (17). CT is known to have differential effects on Th1 vs Th2 clones in vitro, generally inhibiting Th1 but not Th2 clones (32, 33). These observations are consistent with a recent report that mucosally applied CT induces predominantly Th2 responses to itself and enhances Th2-type responses to other proteins given at same time (34). Although CT feeding does not induce oral tolerance for Ab responses, CT feeding has been reported to result in oral tolerance for delayed hypersensitivity (35), a Th1 function. Recent data on tolerance to protein Ags after parenteral injection has implicated IL-4, a Th2 cytokine, as playing a central role in such tolerance, and the same may apply to oral tolerance (36). The present studies have not analyzed in detail the effects of mucosal CT on these two subsets, but our data together with these reports would suggest that the CT preferentially inhibits the $CD4^+$ Th1 subset in vivo, possibly at the same time it is stimulating the $CD4^+$ Th2 subset. $CD8^+$ cells are now being separated into type 1 and type 2 based on patterns of cytokine production and of CD45RA expression (37); it remains to be seen how CT affects these $CD8^+$ subsets.

A number of mechanisms have been proposed to explain the remarkable mucosal immune properties of CT (12). CT has a variety of effects on immune and nonimmune cells, and a single activity is unlikely to explain its special mucosal properties. Although the present studies have focused on inhibition of T cells by CT, we believe this is only one aspect of CT's mucosal effects, albeit an important one. In previous studies, we have shown that inhibition of T cells is not MHC restricted (16), whereas stimulation of immunity to CT is restricted by MHC class II molecules (38). This implies that inhibition of suppressive regulatory T cells is not enough to induce immunity to CT, but that concomitant stimulation of helper T cells is necessary. Because the effects of CT on T cells in vitro is strongly inhibitory, a direct stimulatory effect on T cells seems unlikely in vivo. It seems more likely that T cell stimulation is an indirect effect of cytokines induced by CT in other cell types. For example, CT stimulates the IEC-6 intestinal epithelial cell line to produce IL-6, and such production is markedly enhanced by the simultaneous presence of other cytokines, such as TNF, IL-1, and TGFβ (39). Thus CT-induced cytokine production by epithelial cells in vivo is one potential stimulatory effect. Another cell likely to be important is the macrophage that has been found to produce increased amounts of IL-1 when stimulated by CT (40). We might term this view the "multiple hit theory" of CT's mechanism of action. The present study would add alteration of the regulatory T cell environment in GALT to these mechanisms of action.

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