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Monica Boirivant, Ivan J. Fuss, Lucietta Ferroni, Mariateresa De Pascale and Warren Strober

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Oral Administration of Recombinant Cholera Toxin Subunit B Inhibits IL-12-Mediated Murine Experimental (Trinitrobenzene Sulfonic Acid) Colitis

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Trinitrobenzene sulfonic acid (TNBS)-induced colitis is an IL-12-driven, Th1 T cell-mediated colitis that resembles human Crohn’s disease. In the present study, we showed initially that the oral administration of recombinant subunit B of cholera toxin (rCT-B) at the time of TNBS-induced colitis by intrarectal TNBS instillation inhibits the development of colitis or, at later time when TNBS-induced colitis is well established, brings about resolution of the colitis. Dose-response studies showed that a majority of mice (68%) treated with rCT-B at a dose of 100 μg (times four daily doses) exhibited complete inhibition of the development of colitis, whereas a minority (30%) treated with rCT-B at a dose of 10 μg (times four daily doses) exhibited complete inhibition; in both cases, however, the remaining mice exhibited some reduction in the severity of inflammation. In further studies, we showed that rCT-B administration is accompanied by prevention/reversal of increased IFN-γ secretion (the hallmark of a Th1 response) without at the same time causing an increase in IL-4 secretion. This decreased IFN-γ secretion was not associated with the up-regulation of the secretion of counterregulatory cytokines (IL-10 or TGF-β), but was associated with a marked inhibition of IL-12 secretion, i.e., the secretion of the cytokine driving the Th1 response. Finally, we showed that rCT-B administration results in increased apoptosis of lamina propria cells, an effect previously shown to be indicative of IL-12 deprivation. From these studies, rCT-B emerges as a powerful inhibitor of Th1 T cell-driven inflammation that can conceivably be applied to the treatment of Crohn’s disease. The Journal of Immunology, 2001, 166: 3522–3532.
gene (18), was used as a source for control material to identify culture contaminants. These were kindly supplied by R. Rappuoli (Istituto Ricerche Immunobiologiche Siena, Chiron, Siena, Italy).

rCT-B was produced and purified according to the protocol described by Lebens et al. (19) with minor modifications. In brief, rCT-B was extracted from the medium of V. cholerae cultures by precipitation with sodium hexametaphosphate. The precipitate was spun down and redissolved in a minimal volume of 100 mM sodium phosphate buffer (pH 8) and dialyzed extensively against 10 mM sodium phosphate buffer (pH 7). The dialysate was then centrifuged at 15,000 × g for 20 min to remove undissolved material. As a final purification step, the material was subjected to ion exchange chromatography through a CM-Sepharose column (CL-6B; Pharmacia Biotech, Uppsala, Sweden) and vacuum dialyzed using a collodion membrane (cut off 100,000 MW). CT-B concentration was determined by solid-phase ELISA using immobilized Gm8 ganglioside (Sigma, Milan, Italy) as a primary capture system and goat anti-CT-B Ab (Calbiochem, La Jolla, CA) and mouse antibody-labeled anti-goat Ab (Kirkegaard & Perry Laboratories, Gaithersburg, MD) as a secondary Ab detection system. rCT-B concentrations were obtained with reference to a standard curve obtained using a known amount of purified CT-B (Sigma).

The amount of endotoxin present in the final preparations was determined by the quantitative chromogenic Limulus amebocyte lysate test (QLC-1000; BioWhittaker, Walkersville, MD) according to the manufacturer’s instructions.

Induction of colitis

Specific pathogen-free 4- to 5-week-old male SJL/J mice were obtained from Charles River Breeding Laboratories (Calco, Italy) and the National Cancer Institute (Bethesda, MD) and maintained in a pathogen-free environment provided by the pathogen-free Charles River Breeding Laboratories (Calco, Italy) and the National Cancer Institute (Bethesda, MD) and maintained in a pathogen-free environment. All operations were performed in the animal facility at the Istituto Superiore di Sanita` and the pathogen-free Institute (Bethesda, MD) and maintained in a pathogen-free environment.

In some experiments, 6 mg of the haptenating agent, oxazolone (4-ethylmethylene-2-phenyl-2-oxazolin-5-one) (Sigma) in 150 μl of 0.35% H2O2 for 10 min and then protein blocked with 3% Rad Free 0.05% Tween 20. Before staining, endogenous peroxidase was blocked with infiltration seen in >50% hpf, high vascular density, crypt elongation, with distortion, transmural bowel wall thickening with ulceration.

Isolation of lamina propria mononuclear cells (LPMC)

LPMC were isolated from freshly obtained colonic specimens using a modification of the technique described by Van der Heijden and Stok (21). The colon specimens were initially washed in HBSS-calcium-magnesium free (HyClone Europe, Cramlington, U.K.), cut into 0.5-mm pieces, and incubated in HBSS containing 0.75 mM EDTA and 1 mMol/L DTT (Sigma) at 37°C for 15 min for two cycles. The tissue was then digested further in RPMI 1640 (HyClone) containing 400 U/ml collagenase D and 0.01 mg/ml DNase I (Boehringer Mannheim, Indianapolis, IN) in a shaking incubator at 37°C. The LPMC released from the tissue were then expanded in 100% Percoll, layered under a 40% Percoll gradient (Pharmacia Biotech), and then subjected to centrifugation to obtain the lymphocyte-enriched population at the 40–100% Percoll interface.

Culture of LPMC

Cultures of LPMC for evaluation of IFN-γ, IL-12, and IL-10 secretion were performed in complete medium consisting of RPMI 1640 supplemented with 2 mM l-glutamine, 10 mM HEPEs buffer, 10 μg/ml gentamicin, 100 U/ml each of penicillin and streptomycin and 10% FCS (HyClone). Cultures of LPMC for evaluation of TGF-β production were performed in serum-free medium supplemented with 1% nutridoma-SP (Boehringer Mannheim).

Stimulation and measurement of cytokine production by LPMC

To measure the capacity of isolated LPMC to produce cytokines, the LPMC populations were cultured in complete medium (or serum-free medium in the case of TGF-β) at 105 cells/ml in 24-well plates ( Falcon; Becton Dickinson, Lincoln Park, NJ) coated with or uncoated with anti-CD3e Ab (clone 145-2C11; PharMingen, San Diego, CA). Coating was accomplished by pre-exposure of individual wells to 10 μg/ml anti- CD3e Ab in carbonate buffer (pH 9.6) overnight at 4°C. Culture fluid for cell populations in coated wells also contained 1 μg/ml soluble CD28 Ab (clone 37.51; PharMingen). After 48 h of culture under these conditions (or 72 h for TGF-β), culture supernatants were removed and assayed for the presence of cytokines (IFN-γ, IL-10, IL-4, and TGF-β) by ELISA. To measure IL-12 production, LPMC cultures were preincubated for 18 h with 1000 U/ml recombinant mouse IFN-γ (Genzyme-R&D Systems, Abingdon, Oxon, U.K.) followed by stimulation with 0.03% Staphylococcus aureus Cowan strain I (SAC; Calbiochem). Culture supernatants were harvested after an additional 24 h.

ELISAs

Cytokine concentrations (except for TGF-β) were determined by commercially available specific ELISAs using anti-paired murine cytokines as per the manufacturer’s recommendations (Endogen, Woburn, MA, distributed by Tema Ricerca, Bologna, Italy). TGF-β concentrations were determined using the commercially available TGF-β Quantikine kit (R&D Systems). Optical densities were measured on a Bio-Rad Novaplas ELISA reader (Bio-Rad, Richmond, CA) at a wavelength of 450.

Immunohistochemistry

Colon tissue sections were frozen in embedding medium (OCT compound; Sakura Finetek, Torrance, CA) and cut into 5-μm-thick sections. The latter were then air dried, fixed in acetone, and rehydrated in TBS containing 0.05% Tween 20. Before staining, endogenous peroxidase was blocked with 0.3% H2O2 for 10 min and then protein blocked with 3% Rad Free (Schleicher & Schuell, Keene, NH) for 20 min. In the staining sections were then incubated for 30 min with anti-IL-12p40 (C17.15 1 μg/ml isotype-matched control (IgG2a) Ig and then incubated with biotin-conjugated mouse F(ab’2) anti-rat IgG (Jackson ImmunoResearch, West Grove, PA) for 1 h; subsequently, the sections were incubated with HRP-streptavidin (DuPont, Boston, MA) for 30 min. In a final step, the sections were incubated with a metal-enhanced diaminobenzidine substrate (Pierce, Rockford, IL) to reveal brown/black staining at the site of HRP localization. After staining, sections were washed in distilled water, dried, and mounted in Permount (Fisher Scientific, Fair Lawn, NJ). Stained sections were photographed on an Axioptol microscope (Zeiss, Thornwood, NY).

Confocal immunofluorescence apoptosis studies

For detection of apoptotic cells, colon tissue sections were placed in freezing medium (OCT compound; Sakura Finetek) and frozen on dry ice. The tissue was then cut into 5-μm serial cryosections on silanized glass slides.
Tissue fixation was accomplished with exposure of slides for 20 min at room temperature to freshly prepared paraformaldehyde solution (3% in PBS) followed by a 30-min wash in PBS. For tissue section analysis of TUNEL-positive cells, cell permeabilization was then performed by incubation with 0.1% Triton X-100 and 0.1% sodium citrate solution for 2 min on ice. The labeling of degraded DNA specific to apoptotic cells was performed using an in situ fluorescein detection assay according to the manufacturer’s instructions (Boehringer Mannheim). In brief, after permeabilization, tissue sections were incubated in a humidified chamber with fluorescein-TUNEL reaction mixture for 1 h at 37°C. Sections were then rinsed with PBS and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Sections were analyzed by single immunofluorescence using a Leica TCS-NT/SP confocal microscope (Leica, Deerfield, IL) at ×40 objective and a numerical aperture of 1.2. Fluorochromes were excited using an argon laser at 488 nm for FITC. Images were processed using the Leica TCS-NT/SP software v1.6.551. In some experiments, tissue serial sections were processed for the detection of CD3<sup>+</sup> T cells or apoptotic cells. Staining for apoptotic cells was performed as described above. Staining for CD3<sup>+</sup> cells was performed as follows: acetone-fixed sections (10 min at −20°C) were washed in PBS and then incubated in PBS for 20 min with Fc-blocking mAb (1:100 dilution; BD Pharmingen) to block nonspecific Ab binding. Samples were then incubated with biotin-conjugated anti-CD3e mAb (10 μg/ml; BD Pharmingen) for 30 min followed by three washes in PBS and finally incubated with avidin-Texas Red (1:250; BD Pharmingen) for an additional 30 min. After the final two washes, sections were analyzed by single immunofluorescence using a Leica TCS-NT/SP confocal microscope at ×40 objective and a numerical aperture of 1.2. Fluorochromes were excited using an argon-krypton barrier filter at 595 nm for Texas Red. Images were processed using the Leica TCS-NT/SP software v1.6.551.

**Results**

**Effect of rCT-B administration on the course of TNBS-induced colitis**

In initial studies, we determined the effect of oral administration of rCT-B on the prevention of TNBS-induced colitis. Accordingly, we administered rCT-B (100 μg in 500 μl of 0.35 M NaHCO<sub>3</sub>) on the day of induction of TNBS-induced colitis (see Materials and Methods) and once each day thereafter for 3 days. We found that such administration led to a reproducible inhibition of colitis induction. Thus, as shown in Fig. 1A, rCT-B-treated mice displayed a less sustained weight loss than untreated mice (i.e., mice exposed to intrarectal TNBS with NaHCO<sub>3</sub> given orally). In addition, treated mice suffered a lower mortality rate than untreated mice (at day 7: 30% in 100-μg treated mice as compared with 83% in untreated TNBS-induced colitis mice; also see Fig. 3B below). Control mice (mice administered 50% ethanol alone) administered rCT-B by mouth (per os) in the absence of TNBS administration per rectum exhibited a weight loss pattern and maintained an appearance identical to that of control mice given 50% ethanol per rectum and NaHCO<sub>3</sub> orally; thus, rCT-B administration itself is not grossly toxic.

These effects of rCT-B on the weight of mice given TNBS per rectum were reflected in both the macroscopic and microscopic appearance of mouse colons. Thus, 4–5 days after initiation of colitis, 68% of the colon of rCT-B-treated mice showed no macroscopic evidence of colitis, whereas untreated mice all exhibited colitis (data not shown). Thus, as shown in Fig. 2, whereas low-power microscopic analysis of untreated mice revealed the presence of severe inflammation characterized by a transmural colitis, loss of epithelial cells, and occasional sites of frank epithelial ulceration, (Fig. 2A), such analysis of treated mice revealed minimal inflammation characterized by only mild lymphocyte infiltration and an intact epithelium cell layer, and, this histologic display did not differ markedly from that of ethanol-treated control mice (Fig. 2, B and C). This assessment of low-power microscopic changes was corroborated by semiquantitative grading of the microscopic changes as described in Materials and Methods in which mice are assigned scores ranging from 0 to 4, with 4 indicating the most severe inflammation possible. Colons from mice that had received TNBS intrarectally and NaHCO<sub>3</sub> per os had marked inflammation
and a score of 3.7 ± 0.4, whereas colons from mice that had received TNBS intrarectally and rCT-B/NaHCO₃ per os had little or no inflammation and a score of 0.75 ± 0.8.

In subsequent studies, we determined the effect of rCT-B on previously established TNBS-induced colitis. In these studies, we administered rCT-B using a similar regimen as described above, but in this case beginning such administration on day 5 after induction of TNBS-induced colitis when, as indicated above, the mice manifested severe colitis. As shown in Fig. 1B, the rCT-B-treated mice exhibited a prompt weight gain (and were approaching a precolitis weight at the time of sacrifice), whereas untreated mice exhibited no weight gain. In addition, as also shown in Fig. 2, histologic analysis of mice with untreated TNBS-induced colitis revealed a severe transmural colitis (Fig. 2D), whereas similar mice treated with rCT-B revealed a mild colitis (Fig. 2E) or normal histology (Fig. 2F). These results were also corroborated by semiquantitative microscopic grading in that mice not treated with rCT-B had a score of 3.1 ± 0.6, whereas mice treated with rCT-B had a score of 0.9 ± 0.9.

In yet other studies, we determined whether lower doses of orally administered rCT-B could also exert anti-inflammatory effects on TNBS-induced colitis. Here, we treated mice undergoing induction of TNBS-induced colitis by intrarectal TNBS administration with 10 μg (four times) of rCT-B rather than 100 μg of rCT-B using the same 4-day administration regimen described above. As shown in Fig. 3A, this low-dose rCT-B treatment also led to weight recovery in treated mice, although in this case the recovery was somewhat delayed; in addition, as shown in Fig. 3B, mice treated with 10 μg of rCT-B manifested a lower mortality rate. It should be noted, however, that the overall (average) effect of treatment with 10 μg of rCT-B depicted in Fig. 3 was accompanied by a more variegated response pattern in that while about one-third of the mice so treated exhibited an almost complete resolution of colitis, about two-thirds of the mice so treated exhibited only a partial inhibition of colitis. Thus, we can conclude that although rCT-B still shows anti-inflammatory activity at a dose of 10 μg, this activity is distinctly less than that seen at a dose of 100 μg.

Finally, as an important control to the above studies, we determined whether the anti-inflammatory rCT-B effects were due to adventitious contaminants in the *V. cholerae* cultures from which the rCT-B was extracted. As shown in Fig. 4, we found that supernatants obtained from cultures of *V. cholerae* deficient in genes encoding both the CT-A and -B subunits prepared in an identical fashion as the rCT-B did not have any effect on TNBS-induced colitis. In addition, the endotoxin content of rCT-B preparation and the supernatant preparation obtained from *V. cholera* lacking A and B subunits as determined by the quantitative chromogenic *Limulus* amebocyte lysate test (see Materials Methods), was approximately the same (0.4 and 0.3 EU for a single dose respectively).

**Effect of rCT-B on oxazolone-induced colitis**

Since previous in vitro studies have shown that CT-B can exert suppressive effects on T cell proliferation (22–24), it can be argued that the ability of rCT-B to ameliorate TNBS-induced colitis in the present study is due to general suppression of T cell function rather than a specific effect on the T cell function involved in Th1 T cell-mediated inflammation. To address this possibility, we determined whether oral administration of rCT-B could prevent the induction of oxazolone-induced colitis, an induced colitis mediated by Th2 T cells rather than Th1 T cells and thus associated with the production of IL-4 rather than IL-12/IFN-γ (20). Accordingly, mice were administered oxazolone per rectum (see Materials and Methods) with and without coadministration of 100 μg of rCT-B per os and then monitored for the development of oxazolone-induced colitis. We found that rCT-B administration had no effect on this form of colitis either with respect to induced weight changes, macroscopic and microscopic changes, or to production of IL-4. In particular, lymphocytes isolated from colons of the mice with oxazolone-induced colitis treated with rCT-B and stimulated in vitro with anti-CD3/anti-CD28 mAbs showed the same increased production of IL-4 as observed in the untreated colitis mice (208 and 215 pg/ml, respectively) when compared with IL-4 production by lymphocytes isolated from colons of ethanol-treated control mice (12 pg/ml). In addition, the lymphocyte population from rCT-B-treated and untreated mice showed the same low level of IFN-γ following in vitro stimulation (21 and 19 U/ml, respectively). Finally, we observed no difference in the course of the colitis in the mice treated and untreated with rCT-B as assessed by weight changes. Thus, it appears that oral rCT-B administration under the
experiments (10 μM NaHCO₃ alone per os at the time of TNBS instillation and each day administered 10 μM by rectal instillation to induce colitis or with 50% ethanol alone were rCT-B-treated mice that had undergone induction of TNBS-induced colitis not treated with rCT-B displayed significantly increased IFN-γ secretion, and this level was not different from that of cells from control mice given ethanol alone). As shown in Fig. 5A and B, LPMC from rCT-B-treated and control mice stimulated in the same way produced similar amounts of IL-4.

Thus, it was apparent that the rCT-B treatment had resulted in the prevention of the Th1 T cell response, without at the same time inducing a Th2 T cell response. Similar studies of LPMC obtained from mice with pre-established colitis revealed a similar pattern. Thus, as shown in Fig. 5C, LPMC from mice with established colitis (obtained 9 days after colitis induction) not treated with rCT-B displayed significantly increased IFN-γ secretion as compared with mice treated with rCT-B.

IFN-γ and IL-4 production by lamina propria cells of mice treated with 10 μg of rCT-B was also determined. In this case, the mice that showed complete or almost complete inhibition of colitis yielded cells that produced markedly decreased amounts of IFN-γ and no increase in IL-4 as in mice treated with 100 μg of rCT-B. In contrast, the mice that exhibited moderate or severe colitis despite treatment with 10 μg of rCT-B produced elevated amounts of IFN-γ, albeit less than that produced by cells from untreated mice with TNBS-induced colitis (data not shown).

Effect of CT-B treatment on TGF-β and IL-10 production in mice undergoing induction of TNBS-induced colitis

The ability of rCT-B to affect the Th1 T cell responses noted above could have been due to up-regulation of counterregulatory or suppressor cytokines such as TGF-β or IL-10. To explore this possibility, we initially measured TGF-β and IL-10 production by LPMC stimulated with anti-CD3/anti-CD28 obtained 5 days after induction of TNBS-induced colitis. As shown in Fig. 6A, we found that mononuclear cells from mice with TNBS-induced colitis treated with rCT-B and those from mice with TNBS-induced colitis not treated with rCT-B manifested the same level of TGF-β secretion, and this level was not different from that of cells from

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**FIGURE 3.** Effect of oral administration of 10 μg of rCT-B on TNBS-induced colitis. A, SJL mice treated with 3.75 mg of TNBS in 50% ethanol by rectal instillation to induce colitis or with 50% ethanol alone were administered 10 μg of rCT-B in 500 μl of 0.35 M NaHCO₃ or with 0.35 M NaHCO₃ alone per os at the time of TNBS instillation and each day thereafter for a total of 4 days. Each point represents mean weight data of mice in each group from three different experiments with each group consisting of at least five mice. B, Effect of oral administration of rCT-B on TNBS-induced colitis-associated mortality. Mice were treated as in Fig. 1A and A. Cumulative data from five experiments (100 μg of rCT-B) and three experiments (10 μg of rCT-B) are shown. The effect of oral rCT-B administration on the development of TNBS-induced colitis is not due to adventitious contaminants in the rCT-B derived from the V. cholerae cultures. TNBS-induced colitis was induced in SJL mice as indicated in the legend in Fig. 1A. The mice were treated with rCT-B or an equivalent amount of supernatant from cultures of mutant of V. cholerae lacking both the A and B subunits of CT at the time of rectal instillation of TNBS and each day thereafter for a total of 4 days. Weight changes of SJL mice. Weight data from one representative experiment is shown. Each point represents average weight data pooled from five mice. Bars represent SEs.

**FIGURE 4.** The effect of oral rCT-B administration on the development of TNBS-induced colitis was not different significantly from that of cells from control mice given ethanol alone (with or without concomitant rCT-B, p > 0.05). On the other hand, as shown in Fig. 5B, LPMC from rCT-B-treated and control mice stimulated in the same way produced similar amounts of IL-4. Thus, it was apparent that the rCT-B treatment had resulted in the prevention of the Th1 T cell response, without at the same time inducing a Th2 T cell response. Similar studies of LPMC obtained from mice with pre-established colitis revealed a similar pattern. Thus, as shown in Fig. 5C, LPMC from mice with established colitis (obtained 9 days after colitis induction) not treated with rCT-B displayed significantly increased IFN-γ secretion as compared with mice treated with rCT-B.

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**Effect of rCT-B administration on IFN-γ and IL-4 production by mononuclear cells isolated from colons of mice with TNBS-induced colitis**

TNBS-induced colitis has been shown to be a Th1 T cell-mediated colitis characterized by high IFN-γ production and low IL-4 production by LPMC (17). We therefore assessed production of these cytokines in mice administered rCT-B orally (rCT-B-treated mice) that had undergone induction of TNBS-induced colitis. Accordingly, mononuclear cells from lamina propria of mice sacrificed 5 days after TNBS administration per rectum were stimulated with anti-CD3/anti-CD28 in vitro and then assessed for cytokine secretion by specific ELISA of culture supernatants (see Materials and Methods). As shown in Fig. 5A, LPMC from mice with TNBS-induced colitis not treated with rCT-B displayed significantly increased IFN-γ secretion (p = 0.03), as compared with cells from control mice given ethanol alone per rectum, whereas LPMC from rCT-B-treated mice that had undergone induction of TNBS-induced colitis displayed IFN-γ secretion that did not differ significantly from that of cells from control mice given ethanol alone (with or without concomitant rCT-B, p > 0.05). On the other hand, as shown in Fig. 5B, LPMC from rCT-B-treated and control mice stimulated in the same way produced similar amounts of IL-4. Thus, it was apparent that the rCT-B treatment had resulted in the prevention of the Th1 T cell response, without at the same time inducing a Th2 T cell response. Similar studies of LPMC obtained from mice with pre-established colitis revealed a similar pattern. Thus, as shown in Fig. 5C, LPMC from mice with established colitis (obtained 9 days after colitis induction) not treated with rCT-B displayed significantly increased IFN-γ secretion as compared with mice treated with rCT-B.

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control mice given ethanol alone per rectum. In addition, as shown in Fig. 6B, we found that LPMC obtained from rCT-B-treated mice 5 days after TNBS-induced colitis when stimulated with anti-CD3/anti-CD28 produce lower amounts of IL-10, although not statistically different (p = 0.18), than LPMC from mice not treated with...
rCT-B. This result was corroborated by the fact that, as shown in Fig. 6C, measurement of IL-10 production by pooled LPMC from mice 9 days after TNBS-induced colitis and treated with rCT-B (at 5–8 days) again manifested lower IL-10 production than pooled LPMC from mice not treated with rCT-B. Thus, IL-10 production at both early and late points after colitis induction was, if anything, lower in rCT-B mice than in nontreated mice, probably because IL-10 production subsides as the inflammation is resolved. It can therefore be concluded that neither up-regulation of TGF-β or IL-10 explain the anti-inflammatory effect of rCT-B administration. It should be noted that IL-10 has been shown to be an anti-inflammatory cytokine and therefore it is reasonable to assume that the decreased IL-10 in rCT-B-treated mice is the result rather than the cause of the rCT-B effect.

Effect of CT-B on IL-12 production in mice undergoing induction of TNBS-induced colitis

Another possible mechanism for the activity of rCT-B in TNBS-induced colitis is that it affects the production of IL-12, the APC-derived cytokine that drives the Th1 T cell response. To investigate this possibility, we stimulated LPMC obtained from mice 5 days after TNBS-induced colitis induction with SAC following an 18-h preincubation with IFN-γ (see Materials and Methods) and then evaluated IL-12 (p70) secretion in culture supernatants. As shown in Fig. 7A, although LPMC from mice with TNBS-induced colitis not treated with rCT-B displayed high levels of IL-12 production, those from rCT-B-treated mice displayed almost undetectable levels of such secretion (p < 0.05). In parallel studies, we also determined IL-12 production in LPMC obtained from mice 9 days after TNBS-induced colitis and treated or not treated with rCT-B beginning 5 days after TNBS-induced colitis. As shown in Fig. 7B, here again rCT-B administration was associated with a decrease in IL-12 production. In further studies to corroborate that the administration of oral rCT-B led to a decrease in IL-12 secretion, in situ staining for IL-12 p40 was performed. Thus, as shown in Fig. 8, a marked decrease in IL-12p40 staining is observed in colonic tissue sections obtained from mice with TNBS-induced colitis treated with rCT-B at the time of TNBS-induced colitis as compared with untreated mice. It was therefore evident that treatment of mice with rCT-B at the time of induction of TNBS-induced colitis or later when colitis is established profoundly down-regulates IL-12 secretion.

In further studies, we also determined IL-12 production by cells extracted from mice treated with 10 μg of rCT-B. In this case as well, IL-12 production paralleled the effect of rCT-B: in mice exhibiting little or no inflammation, IL-12 secretion was only slightly elevated, whereas in mice exhibiting some degree of inflammation, IL-12 secretion was considerably increased, but not to the level seen in mice with TNBS-induced colitis not treated with rCT-B (data not shown).

Inhibition of TNBS-induced colitis by rCT-B is associated with increased apoptosis of T cells in the lamina propria

We have recently shown that treatment of TNBS-induced colitis with anti-IL-12 results in the appearance of apoptotic cells in the lamina propria of the treated mice (25). Since rCT-B, like anti-IL-12, inhibits IL-12 secretion, we asked whether rCT-B treatment is also associated with increased apoptosis of lamina propria T cells. We therefore stained tissue sections obtained from mice 5 days after induction of TNBS-induced colitis using the fluorescein-labeled TUNEL technique to detect apoptotic cells in situ. As shown in Fig. 9, A–C, the tissue section obtained from rCT-B-treated mice displayed large numbers of randomly distributed mononuclear cells with a fluorescent green stain indicative of apoptosis, whereas few if any such cells were noted in tissue from TNBS-induced colitis mice not treated with rCT-B. In further studies along these lines, we assessed apoptosis in dispersed mononuclear cells by flow cytometry. We found that cells in a gate identified by forward and side light scatter as small cells, greatly enriched in CD3+ T cells and with <2% CD14+ cells, 19% of cells from mice with rCT-B-treated TNBS-induced colitis were TUNEL+, whereas only 3.7% of cells from mice with untreated TNBS-induced colitis were TUNEL+. Furthermore, as shown in Fig. 9, D and E, in serial sections of lamina propria tissue from a mouse with TNBS-induced colitis treated with rCT-B, TUNEL staining of one section and anti-CD3 staining of an adjacent section revealed cells that were both CD3 positive and TUNEL positive. Thus, the cells undergoing apoptosis after rCT-B treatment were, at least in part, CD3+ T cells.
Discussion

TNBS-induced colitis is a well-characterized transmural inflammation of the colon which is due to an IL-12-driven, Th1 T cell-mediated response to trinitrophenyl-haptenated colonic proteins and/or cross-reactive luminal Ags (17, 26, 27). On this basis, TNBS-induced colitis has been considered an experimental model of Crohn’s disease, which is also a transmural inflammation associated with the production of cytokines (IL-12 and IFN-γ) indicative of the Th1 response (28–32). Thus, it is of great interest that orally administered rCT-B prevents development of TNBS-induced colitis and brings about resolution of previously established colitis, both with respect to the clinical/pathologic manifestations of the disease as well as its immunologic features. These studies therefore suggest that rCT-B can be a useful form of therapy for Crohn’s disease.

Previous studies of CT and related bacterial toxins (such as E. coli heat-labile toxin) have emphasized the fact that the adjuvant and toxic effects of the toxin resides primarily in its A subunit, the part of the molecule that has ADP ribosyltransferase activity and accounts for its ability to activate adenylate cyclase and to generate cAMP (5). This is supported by the observation that recombinant LT-A subunit or CT-A bound to protein A (an alternative binding molecule that targets the B cells) both retain potent adjuvant activity (6, 7). Of interest, it appears that the A subunit can function as an adjuvant even in the absence of ribosyltransferase activity (7). In contrast, the B subunit, the part of the molecule that binds to the cell surface via GM1 (or other gangliosides) and facilitates entry of the holotoxin into the cell, has an as yet an uncertain adjuvant effect. This arises from the fact that the adjuvant activity of the A subunit is largely retained in modified toxins in which the ribosyltransferase activity has been greatly attenuated by site-specific mutagenesis (33, 34). Thus, many earlier studies conducted with purified B subunit are suspect since purified B subunit may contain sufficient amounts of A subunit to reflect A subunit effects rather than B subunit effects (8, 9). Clearly, evaluation of the immunologic effects of the B subunit requires the use of recombinant B subunit, such as the B subunit used in the present studies.

Recent studies of the adjuvant activity of recombinant B subunit have provided evidence that coadministration of CT-B or LT-B with Ag usually (but not always) enhances immune responses, especially when the two are given by an intranasal route (10, 11, 35). However, this adjuvant activity was considerably less than that of the holotoxin or, indeed, detoxified holotoxin in the few studies in which this comparison was made (33, 36). Importantly, although there is good evidence that holotoxin or detoxified holotoxin serves as an adjuvant for both B cell and T cell responses, the B subunit adjuvanticity has been determined mainly, if not exclusively, for B cell (humoral immune) responses, and in the one study in which its capacity to enhance a T cell response was investigated, it was found to have little, if any, adjuvant effect (33, 37). This fact suggests that the B subunit acts as adjuvant for humoral immune responses by causing direct effects on B cells rather than by indirect effects on helper T cells.

The latter possibility reconciles the demonstrable adjuvant activity of CT-B with evidence that rCT-B has a marked inhibitory effect on Th1 T cell responses arising in the present study as well two previous studies. In one previous study, it was shown that either i.v. or i.p. administration of CT-B (in this case, nonrecombinant CT-B) to nonobese diabetic mice reduces the occurrence of diabetes in these mice without at the same time impairing the capacity of the mice to mount a humoral immune response. In addition, it was found that the CT-B-treated mice have spleen cells that inhibit the development of diabetes in an adoptive transfer model, suggesting that CT-B had induced regulatory cells that suppress islet cell inflammation (15). In another previous study, it was shown that i.v. administration of recombinant LT-B prevented development of collagen-induced arthritis in mice given collagen in Freund’s adjuvant by an i.p. route (16). Furthermore, it was demonstrated that administration of rLT-B plus collagen to mice led to increased spleen cell IL-4 production and decreased IFN-γ production as compared with administration of collagen alone, suggesting that the prevention of arthritis by rLT-B was due to its ability to influence the Th1/Th2 profile of the collagen-induced response. This latter result differs somewhat from those obtained in the present study of TNBS-induced colitis, in which it was found that although rCT-B administration led to decreased IFN-γ secretion, it had no effect on IL-4 secretion; this difference, however, does not erode the fact that the effect of rLT-B on collagen-induced arthritis and rCT-B on TNBS-induced colitis is fundamentally similar in that in both cases the B subunits abrogate a Th1 response. Finally, it should be noted that previous in vitro data suggesting
FIGURE 9. Confocal immunofluorescence studies indicate increased apoptosis occurs in mice with TNBS-induced colitis treated with rCT-B as compared with untreated mice. At day 5 after induction of TNBS-induced colitis, cryosections of tissues from the indicated colons were subjected to a fluorescein-labeled TUNEL staining for detection of apoptotic cells (A–C; see Materials and Methods). Representative confocal microscopy fields from three different experiments. In each experiment, five slides per mouse were stained: A, mice treated with 50% ethanol; B, mice with TNBS-induced colitis treated with NaHCO₃ alone; and C, mice with TNBS-induced colitis treated with rCT-B (×40 objective). See text for description of figures. Serial cryosections of tissue from mice with TNBS-induced colitis treated with rCT-B were subjected to fluorescein-labeled TUNEL staining for detection of apoptotic cells (D) and concomitant Texas Red staining for CD3 (E). Arrows mark TUNEL- and CD3-positive cells. See text for description of figures.

that the effects of either rCT-B or rLT-B in these studies are due to nonspecific inhibition of lymphocyte proliferation (22–24) is unlikely in that both B subunits do not suppress in vivo Th2 (IL-4) responses (11, 33). This was particularly evident in the present study in which it was shown that rCT-B did not inhibit the robust IL-4 response characteristic of oxazolone-induced colitis. Overall, the picture that emerges from these various in vivo studies of the immunologic effects of toxin B subunits is that the latter inhibit Th1 T cells responses by one or another mechanism even though they act as adjuvants for B cell responses.

Additional data relating to the immunologic activity of toxin B subunits comes from studies of the effect of rCT-B conjugated to various Ags on the immune response to the bound Ags (12–14). In general, these studies have shown that orally administered CT-B-Ag conjugates enhance the induction of tolerance to the Ag as compared with Ag alone and thus these findings, at least superficially, support the idea that CT-B favors immunosuppression. However, studies in which the mechanism by which CT-B-Ag conjugates enhance oral tolerance is examined are quite equivocal. In some cases, they support the idea that conjugated rCT-B inhibits Th1 responses (38, 39), whereas in other cases they suggest that conjugated rCT-B enhances Th1 responses (13). In addition, in one study in which Ags conjugated to rCT-B were administered intranasally before aerosol sensitization was studied in an in vivo model of asthma, it was found that the effect of rCT-B conjugates depended on the nature of the Ag: such administration suppressed Th2 responses to OVA whereas it enhanced Th2 (and Th1) responses to Betv1 (birch pollen Ag) (40). One explanation of these disparate data sets is that when rCT-B is acting as part of a conjugate, it acts mainly as a Ag-focusing agent that does not otherwise influence the normal response to the focused Ag. It should be noted in this regard that in the process of conjugation, one may alter the capacity of rCT-B to act as a general immunosuppressant.

As indicated above, the probable mechanism of action of rCT-B on TNBS-induced colitis is its ability to inhibit the Th1 T cell response responsible for the colitis. We proposed that such inhibition is primarily due to the effect of rCT-B on IL-12 production, since we have shown that rCT-B has a profound effect on IL-12 production and IL-12 is the key cytokine in the initiation of the Th1 response. However, several other possible mechanisms of the rCT-B effect should also be considered. It has been shown, for instance, that when T cells are exposed in vitro to CT-B, there is inhibition of mitogen- and Ag-induced proliferation (22, 24), particularly with respect to CD8⁺ T cells. In the latter regard, it has been shown that CT-B causes preferential CD8⁺ T cell loss by apoptosis (41) and that CT-B leads to intraepithelial T cell depletion (24). Despite these data, it is unlikely that the ability of rCT-B to ameliorate TNBS-induced colitis is due to a suppressive effect of rCT-B on T cell proliferation in general or CD8⁺ T cell proliferation in particular. This follows from the fact that oral administration of rCT-B does not suppress oxazolone-induced colitis, i.e., a colitis mediated by Th2 T cells which also requires T cell proliferation. In addition, it should be noted that rCT-B ameliorates TNBS-induced colitis at a 10-fold lower dose that is not likely to result in the provision of antiproliferative concentrations of rCT-B in the whole animal, as these doses are routinely used when rCT-B is administered as an adjuvant for humoral immune responses.

Yet another explanation of the effect of rCT-B on TNBS-induced colitis is that rCT-B primarily inhibits IFN-γ production by T cells, and the apparent effect of rCT-B on IL-12 production is due to loss of the positive feedback effect of IFN-γ on IL-12 production. This seems unlikely, however, since it has been shown that anti-IFN-γ administration to mice with TNBS-induced colitis is not an effective treatment of the colitis unless the anti-IFN-γ is given repeatedly and in large amounts (25). In addition, such administration does not lead to the level of cellular apoptosis as observed with rCT-B treatment (see discussion below). Finally, a possible effect on IFN-γ production would not be expected to have the profound effect on IL-12 production that was observed in this study. All this being said, we would not rule out the possibility that rCT-B has an independent effect on IFN-γ production that also...
contributes to the activity of rCT-B in TNBS-induced colitis. A final possible explanation for the effect of rCT-B on TNBS-induced colitis is that rCT-B induces the production of suppressor cytokines such as TGF-β, IL-10, and IL-4. However, as we have shown in the present study, rCT-B administration does not result in the increased production of TGF-β and actually leads to lower production of IL-10. Thus, counterregulation by these cytokines cannot be the cause of the anti-inflammatory rCT-B effect. The above considerations taken together make it likely that the primary mechanism of the rCT-B effect is its activity as an inhibitor of IL-12 production. Whether or not such inhibition is a direct effect of rCT-B on APCs is presently somewhat unclear. In the present study and one previous study, an indirect effect via IL-10 was not observed (42). However, in another prior study, in which the effect of choleran holotoxin on IL-12 was assessed, evidence for indirect inhibition of IL-12 via IL-10 was obtained (43).

Yet another support for the view that CT-B acts through its ability to inhibit IL-12 and one that has significance to the use of rCT-B in clinical situations is that rCT-B administration in the present study was associated with the appearance of a greatly increased number of apoptotic cells in intestinal tissues, as compared with tissues in mice with TNBS-induced colitis not administered rCT-B. Previously, apoptosis has been noted in mice with TNBS-induced colitis whose colitis was successfully prevented by or treated with anti-IL-12 (25). Evidently then, IL-12 inhibits apoptosis of Th1 T cells and its down-regulation by anti-IL-12 or rCT-B (as in these studies) leads to Th1 T cell loss. If indeed rCT-B is affecting IFN-γ production not simply by inhibiting IL-12 synthesis but also by inducing apoptosis of Th1 T cells, then rCT-B may be especially effective in the treatment of ongoing Crohn’s disease where it is necessary not only to inhibit IFN-γ synthesis, but also to eliminate the cells that produce IFN-γ.

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