INFLAMMATORY BOWEL DISEASE

Cholera toxin subunit B inhibits IL-12 and IFN- γ production and signaling in experimental colitis and Crohn's disease

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Gut 2005;54:1558–1564. doi: 10.1136/gut.2004.062174

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Revised 26 May 2005
Accepted 21 June 2005
Published online first
16 August 2005

Background and aims: Cholera toxin B subunit (CT-B) is a powerful modulator of immune responses. The authors have previously demonstrated that oral administration of recombinant CT-B (rCT-B) is able to prevent and cure the Crohn's disease (CD)-like trinitrobenzene sulfonic acid (TNBS) mediated colitis. In this study they extended their observations and examined if rCT-B interferes with the molecular signaling underlying the Th1 type response both in TNBS colitis and in ex vivo human CD explants.

Methods: TNBS treated mice were fed with rCT-B, and IFN- γ and IL-12 production by colonic lamina propria mononuclear cells (LPMC) was examined by ELISA. In vitro culture of mucosal explants from CD patients and non-inflammatory bowel disease controls, pre-incubated with rCT-B, were examined for IFN- γ and IL-12 production by ELISA and semiquantitative reverse transcription polymerase chain reactions. STAT-1, -4, -6 activation and T-bet expression were examined following rCT-B treatment by western blotting both in TNBS treated mice and in human mucosal explants.

Results: rCT-B significantly reduced IL-12 and IFN- γ secretion by LPMC from TNBS treated mice. Consistent with this, rCT-B inhibited both STAT-4 and STAT-1 activation and downregulated T-bet expression. Inhibition of Th1 signaling by CT-B associated with no change in IL-4 synthesis and expression of active STAT-6 indicating that rCT-B does not enhance Th2 cell responses. Moreover, in vitro treatment of CD mucosal explants with rCT-B resulted in reduced secretion of IL-12/IFN- γ and inhibition of STAT-4/STAT-1 activation and T-bet expression.

Conclusions: These studies indicate that CT-B inhibits mucosal Th1 cell signaling and suggest that rCT-B may be a promising candidate for CD therapy.

Inflammatory bowel diseases (IBD) denote chronic inflammatory disorders of gastrointestinal tract that comprises two major groups: Crohn's disease (CD) and ulcerative colitis (UC).¹ The aetiology of IBD remains unknown, but there is evidence that a dysregulated immune reactivity against luminal bacteria plays a major role in the induction and perpetuation of the ongoing inflammation.² In CD, this is associated with aberrant production of Th1 cytokines and activation of Th1 transcription factors. Indeed, increased production of IFN- γ and IL-12^{3–6} is seen in CD tissue, and selective downmodulation of Th1 type inflammation appears to be an optimal way to achieve stable remission in these patients.

Cholera toxin (CT) is composed of a monomeric A subunit (CT-A) associated with a pentameric B subunit (CT-B). While the former is responsible for the secretory effects of the toxin, the latter is necessary for both binding to cell surface monosialogangliosides and facilitation of toxin entry. In this form CT is a potent immunogen and mucosal adjuvant, capable of stimulating antigen specific secretory IgA and systemic IgG antibody responses to unrelated vaccine antigens when given along with them via the oral or nasal route.^{7,8} The CT-B has been shown to be a potent immunomodulator besides its vector function for the toxic subunit A. It was recently shown that parenteral administration of purified CT-B, in the absence of autoantigen, delays the onset of diabetes in non-obese diabetic mice and that the administration of recombinant B subunit of *E coli* heat-labile enterotoxin, a molecule structurally and functionally related to CT-B, prevents collagen induced arthritis.^{9,10} We have

recently explored the immunological properties of recombinant CT-B (rCT-B) in a well characterised model of mucosal inflammation, the IL-12 driven, Th1 cell mediated inflammation occurring in SJL/J mice after rectal administration of trinitrobenzene sulfonic acid (TNBS).¹¹ We showed that oral administration of rCT-B inhibits the onset of TNBS induced colitis and reverses the established colitis.

Recent studies have focused on the molecular mechanisms underlying Th1 and Th2 cell development.^{12–14} Polarisation of naïve T cells into differentiated effector subsets is a tightly controlled process dependent on the activity of specific transcription factors.^{12–15,16} STAT-1 and STAT-4 transcription factors are specifically associated with IFN- γ and IL-12/IL-23 signaling in T lymphocytes and play a key role in regulating Th1 cytokine production at the transcriptional level.^{12–17,18} However, STAT-1 and STAT-4 deficient cells still retain the ability to produce some IFN- γ thus suggesting the role of alternative signaling pathways in development and expansion of Th1 cells. In this context, it was recently shown that full polarisation of Th1 cells also requires the activity of the transcription factor T-bet.¹⁹ In contrast, optimal differentiation of Th2 cells strictly relies on the activity of STAT-6.²⁰ In the present study we examined if rCT-B modulates mucosal Th1 cell signaling. We show that oral administration of rCT-B in mice with TNBS induced colitis inhibits the activation of Th1 associated transcription factors STAT-4, STAT-1, and

Abbreviations: CD, Crohn's disease; CT, cholera toxin; IBD, inflammatory bowel disease; LPMC, lamina propria mononuclear cells; TNBS, trinitrobenzene sulfonic acid; UC, ulcerative colitis.

T-bet without affecting the expression of active STAT-6. Additionally, we provide evidence that rCT-B inhibits secretion of Th1 cytokines and expression of Th1 associated transcription factors in CD mucosal explants.

MATERIALS AND METHODS

Production and purification of rCT-B

The *Vibrio Cholerae* strain 0395-tacCTB, lacking the CT-A gene, was used as source to produce rCT-B (kindly supplied by Dr R Rappuoli, Istituto Ricerche Immunobiologiche, Chiron, Siena, Italy). rCT-B was produced and purified as previously described¹¹ according to the protocol described by Lebens *et al*²¹ with minor modifications. All rCT-B preparations contained <10 pg/ml LPS as determined by Quantitative Chromogenic Limulus Amebocyte Lysate test (QLC-1000, Biowhittaker, Walkersville, MD, USA).

Induction of colitis and feeding of rCT-B

Specific pathogen free 4–5 week old male SJL/J mice were obtained from Charles River (Calco, Italy) and maintained in a pathogen free environment animal facility. Animals were treated in accordance with the European Community guidelines and the local institute ethics committee. To induce TNBS-colitis, 3.75 mg of TNBS (pH 1.5–2.0; Sigma Chemical Co, St Louis, MO, USA) in 150 µl of a 50% ethanol solution was administered via rectum to lightly anaesthetised mice as previously described.¹¹

In some experiments, 6 mg of the haptenating agent, oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one, OXA) (Sigma Chemical Co) in 150 µl of 50% ethanol solution was administered intrarectally.

Mice, which received TNBS per rectum, were fed with 100 µg of rCT-B in 500 µl of 0.35 M NaHCO₃ or 500 µl of 0.35 M NaHCO₃ at the time of TNBS instillations and every day, over a four day period, using an 18 gauge feeding needle. Mice treated with 50% ethanol intrarectally and which had received 500 µl of 0.35 M NaHCO₃ alone were used as controls for TNBS colitis.

Isolation and culture of murine lamina propria mononuclear cells

Lamina propria mononuclear cells (LPMC) were isolated from freshly obtained colonic specimens using a modification of the technique described by Van der Heijden and Stok²² as previously described.¹¹ Cultures of murine LPMC 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 (Hy-Clone). To measure the capacity of isolated LPMC to produce cytokines, the LPMC populations were cultured in complete medium at 1×10⁶ cells/ml in 48-well plates (Costar, Corning Incorporated, NY, USA) coated or uncoated with anti-CD3ε Ab (clone 145-2C11; Pharmingen, San Diego, CA, USA) as previously described.¹¹ Cultures were also added with 1 µg/ml soluble CD28 Ab (clone 37.51; Pharmingen). For STAT-6 activation LPMC were stimulated with 10 ng/ml recombinant murine IL-4 for 30 minutes (Pharmingen). After 48 hour culture, culture supernatants were harvested and assessed for the presence of IFN-γ by ELISA. To measure IL-12 production, murine LPMC cells were preincubated for 18 hours with 1000 U/ml recombinant murine IFN-γ (Genzyme-R&D Systems, Abingdon, Oxon, UK) followed by stimulation with 0.03% *Staphylococcus aureus* Cowan strain I (SAC; Calbiochem, La Jolla, CA, USA) for an additional 24 hours. Cytokine concentrations were determined by commercially available specific ELISA kits as previously described.¹¹

Cell extracts from LPMC

Whole cell extracts were prepared as previously described.²³ Briefly, mouse LPMC were lysed in ice cold whole cell extraction buffer (20 mM Hepes pH 7.9, 50 mM NaCl, 0.5% NP-40, 1 mM DTT, 10 mM EDTA and 2 mM EGTA, 10 µg/ml leupeptin, 100 mM sodium fluoride (NaF), 0.5 mM PMSF, 10 mM sodium orthovanadate and sodium molybdate). The lysate was incubated 30 minutes on a shaker at 4°C and insoluble debris was removed by centrifugation (10 000 g at 4°C, 10 minutes) and the lysate was stored at –80°C.

Patients and samples

Mucosal samples were taken from intestinal resection specimens of inflamed and non-inflamed regions of 11 patients (median age, 31 years; range 29–57) with moderate to severe CD undergoing surgery. In seven patients, the primary site of involvement of the disease was the terminal ileum; in the remaining four patients the disease involved the terminal ileum and the colon. Four patients were receiving corticosteroids at the time of resection. Indication for surgery was fibrostenosis in seven patients and a poorly responsive disease to medical therapy in four patients. Control samples included macroscopically and microscopically unaffected ileal areas from five patients undergoing bowel resection for right colon neoplasia. All the experiments were approved by the local ethics committee. Informed consent was obtained from all patients before collecting the samples.

Human LPMC and tissue explant cultures and tissue extract preparations

Intestinal mucosa was cut in small pieces (2–3 mm). Some of these pieces were immediately analysed, whereas the remaining were placed in 24-well plates in complete medium. Tissue specimens were incubated with or without the addition of rCT-B (10 µg/ml) at 37°C for four hours. Thereafter, mucosal samples were washed in complete medium to remove rCT-B and incubated for an additional 16–18 hours in complete medium. In addition, mucosal specimens from two CD patients were used to isolate LPMC.²⁴ An aliquot of LPMC was incubated with or without rCT-B (10 µg/ml) at 37°C for four hours, then stimulated with IFN-γ (200 U/ml; Peprotech Inc Ltd, London, UK) for additional 14 hours, followed by 0.03% of SAC for a further 24 hours. At the end, culture supernatants were collected and analysed for IL-12 production. The remaining LPMC were incubated with or without rCT-B (10 µg/ml) at 37°C for four hours and then stimulated with plate bound anti-CD3 mAb (OKT3) and soluble anti-CD28 mAb (clone 9.3; 1 µg/ml, kindly provided by Professor Carl June, Cancer Center, University of Pennsylvania, Philadelphia, PA, USA) for a further 48 hours. Cell-free supernatants were then analysed for IFN-γ production. CD3⁺ cells were also isolated from LPMC of one CD patient using anti-CD3 magnetic beads (DynaL Biotech Inc, Oslo, Norway). Purified CD3⁺ cells were then either left untreated or treated with rCT-B for four hours and then directly stimulated according to the manufacturer's recommendation (DynaL Biotech Inc). IL-12 and IFN-γ content was evaluated by specific ELISA kits (R&D Systems).

Total proteins were prepared from mucosal samples using a Dounce tissue grinder (Wheaton Science Products, Millville, NJ, USA) in 0.5 ml of lysis buffer containing 0.01 M Hepes (pH 7.9), 1 mM EDTA, 6 mM KCl, 0.02% NP40, 1 mM DTT, 1 mM NaF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 1 mM PMSF, and 1 mM Na₃VO₄ (all from Sigma Chemical Co). After cell lysis, samples were incubated on ice for 30 minutes and sedimented by centrifuging the lysates at 10 000 g for 10 minutes (4°C).

Determination of phosphorylated STAT-4 level in human mucosal samples

Total proteins (500 µg/sample) were incubated with anti-STAT-4 (C-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing a cocktail of protease and phosphatase inhibitors (20 µg/ml aprotinin, 2 mM PMSF, and 10 mM Na₃VO₄) at 4°C for two hours. Immune complexes were collected by incubation with protein A/G agarose (Santa Cruz Biotechnology), washed three times with lysis buffer, and boiled for five minutes in sample buffer for SDS-PAGE. Immunoprecipitates from extracts containing the same amount of protein were analysed by western blotting with Ab against phosphotyrosine STAT4 (Y693) (Zymed Laboratories Inc, San Francisco, CA, USA) and subsequent incubation with HRP-conjugated goat antirabbit IgG (Calbiochem). The Ab reaction was detected with a chemiluminescence detection kit (Amersham International, Arlington Heights, IL, USA). After STAT-4 p-Tyr analysis, blots were stripped by incubation for 20 minutes at 50°C in stripping buffer (2% SDS, 62.5 mM Tris-HCl, and 100 mM 2-ME) and then incubated with Ab against STAT-4 (Santa Cruz Biotechnology).

Western blot analysis

Whole cell extracts (30 µg) were separated by 7% or 10% SDS-PAGE gel and blotted onto nitrocellulose membranes. Blots were incubated with rabbit polyclonal Abs against STAT-1, STAT-4 and STAT-6 (Santa Cruz Biotechnology, Inc, E-23, C-20 and S-20, respectively) and reacted with antirabbit horseradish peroxidase (HRP) coupled secondary antibody (Calbiochem) using an ECL system (Amersham, Little Chalfont, UK). Tyrosine phosphorylated Abs against STAT-1 (Upstate, Lake Placid, NY, USA), STAT-4, and STAT-6 (Santa Cruz Biotechnology, Inc) were used to detect the phosphorylation status of these transcription factors. For the detection of T-bet in mice, total proteins (30 µg/sample) were separated on a 10% SDS-PAGE gel. A commercially available monoclonal Ab against mouse T-bet (Santa Cruz Biotechnology, Inc), followed by a HRP-peroxidase conjugated goat antimouse IgG antibody (DAKO SpA, Milan, Italy) was used. The analysis of T-bet in human samples (100 µg/sample) was performed using rabbit polyclonal anti T-bet Abs (a generous gift of Dr L H Glimcher, Department of Medicine, Harvard Medical School, Boston, MA, USA). After detection of T-bet, blots were stripped and incubated with a rabbit anti-β-actin antibody (Sigma Chemical Co), followed by a goat antirabbit antibody conjugated to HRP.

RNA and cDNA preparation and semiquantitative reverse transcriptase polymerase chain reactions

RNA was extracted from freshly mucosal samples with RNeasy Protect kit (Qiagen Inc, Valencia, CA, USA) according to the manufacturer's instructions. One µg of RNA was reverse transcribed in a final volume of 50 µl containing 400 U of murine leukemia virus reverse transcriptase (Gibco-BRL Life Technologies Inc, Gaithersburg, MD, USA), 2 U of RNase Block (Roche), 0.5 mM of a mix of dATP, dTTP, dCTP, dGTP (Gibco-BRL Life Technologies Inc), 10 mM DTT, 1 µM of oligo dT and RT buffer according to the manufacturer's instructions. Reverse transcription was carried out at 37°C for 90 minutes. An equivalent amount of cDNA per sample was amplified using specific primers for p40 and p35 IL-12 subunits, IFN-γ (10 µl/reaction) and GaPDH (3 µl/reaction). For the amplification of IL-12/p35 and IL-12/p40 transcripts the PCR reactions were performed by using specific primers from Stratagene (La Jolla, CA, USA) according to the manufacturer's instructions. IFN-γ primers (M-Medical, Milan, Italy) were as follows: 5'-GCA TCG TTT TGG GTT

CTC TTG GCT GTT ACT GC-3' and 5'-GGT CGT CGA TTT TGT CCC TTC GCT TTT TCC TC-3'; GaPDH primers (M-Medical) were: 5'-GTC TTC ACC ATG GAG AAG GTC-3' and 5'-CAT GCC AGT GAG CTT CCC GTT CA-3'. PCR were performed in a total volume of 50 µl as previously described.²⁵ The level of RNA transcripts was measured by Fluor-S Multimager (Bio-Rad Laboratories, Hercules, CA, USA) and expressed as arbitrary units.

RESULTS

Effect of rCT-B administration on IL-12 and IFN-γ production and signaling in mice with Th-1 mediated TNBS colitis

In initial studies we evaluated the effect of rCT-B administration on TNBS colitis course. rCT-B treated mice display a less sustained weight loss than in mice treated with TNBS alone (fig 1A). In addition, a reduction of IL-12 and IFN-γ production by LPMC isolated from colons of mice treated with rCT-B was observed (fig 1B), without affecting IL-4 production (not shown) in LPMC from mice with TNBS colitis.

As IL-12 signals through the STAT-4 pathway to promote IFN-γ gene transcription,^{26, 27} we then investigated whether the rCT-B induced inhibition of IL-12 production was associated with changes in STAT-4 activation. For this purpose, total cell extracts were obtained from LPMC of mice with TNBS colitis treated or not with rCT-B. As shown in figure 2A, tyrosine phosphorylation of STAT-4 was clearly detected in cell extracts obtained from mice with TNBS colitis as compared to ethanol treated mice. However, treatment of mice with rCT-B caused a clear reduction in STAT-4 phosphorylation.

Recent studies have shown that optimal Th1 cell response requires the activation of multiple transcription factors. Among these, T-bet appears to play a key role in driving mucosal Th1 cytokine differentiation in experimental colitis.²⁸ Therefore, we also examined the effect of treatment with rCT-B both on T-bet expression and phosphorylation of STAT-1, one of its major inducer. As shown in figure 2A, STAT-1 phosphorylation and T-bet expression were barely detectable in ethanol treated mice. Induction of colitis associated with enhanced phosphorylation of STAT-1 and T-bet expression. However, the expression of these transcription factors was reduced by rCT-B (fig 2A). As reduction of T-bet expression enhances IL-4 signaling in LPMC,²⁸ we next evaluated if the rCT-B mediated inhibition of T-bet associated with enhanced STAT-6 activation. As a positive control, we used total extracts from LPMC of mice with oxazolone (OXA) colitis, an experimental colitis mediated by IL-4-driven Th2 cells,²⁹ either left untreated or treated in vitro for 30 minutes with IL-4 (OXA+IL-4). As expected, STAT-6 phosphorylation was evident in LPMC isolated from mice with OXA-mediated colitis, and those cells responded to the stimulation with exogenous IL-4 by further enhancing the level of p-STAT-6 (fig 2B). In contrast, no p-STAT-6 was seen in LPMC extracts from mice with TNBS colitis either left untreated or treated with rCT-B (fig 2B), indicating that rCT-B treatment does not stimulate IL-4 production.

Effect of rCT-B treatment on IL-12 expression and STAT-4 phosphorylation in organ culture of CD mucosal explants

As TNBS colitis shares immunological similarities with CD, we then extended our analysis to the effects of rCT-B on the synthesis of Th1 cytokines and expression of Th1 associated transcription factors in mucosal explants from CD patients and non-IBD controls. As expected, transcripts for both IL-12 subunits were expressed at higher level in samples from involved mucosal area of CD patients than non-IBD controls

Figure 1 Oral administration of rCT-B inhibits the induction of TNBS colitis and the mucosal production of IL-12 and IFN- γ . SJL mice were treated by rectal instillation with TNBS to induce colitis or with 50% ethanol alone, as colitis controls. Mice were fed with 100 μ g rCT-B in 0.35 M NaHCO₃ or with 0.35 M NaHCO₃ alone as described in material and methods. (A) Determination of variations in body weight. Each point represents the cumulative mean weight data from five different experiments. In all experiments, each group consisted of at least five mice. Bars represent standard errors. *p<0.01 mice with TNBS induced colitis and concomitant administration of rCT-B/NaHCO₃ versus mice with TNBS induced colitis and concomitant administration of NaHCO₃. (B) The production of IL-12 and IFN- γ was measured in the culture supernatants of LPMC isolated from the colon of mice with TNBS colitis treated or not with oral rCT-B, or from the colon of mice treated with 50% ethanol alone. To enhance IL-12 production, LPMC were treated with SAC and IFN- γ as indicated in materials and methods. For IFN- γ production, LPMC cultures were performed in the absence of any stimulant or in the presence of anti-CD3/CD28. Data indicate mean from two independent experiments. In each experiment, cultures of pooled LPMC extracted from the colon of five mice per group were performed. Bars indicate standard error.

Figure 2 rCT-B administration inhibits IL-12 and IFN- γ signaling in mice with TNBS colitis. Total cell extracts were prepared from LPMC of mice sacrificed four days after TNBS instillation. Mice with TNBS colitis were either left untreated (TNBS) or treated with oral rCT-B (TNBS+rCT-B). As control of TNBS treatment, mice were instilled with 50% ethanol alone (Ethanol). One representative experiment of three independent experiments is shown. (A) Thirty μ g of whole cell lysates were separated on a 7% SDS-PAGE, transferred to a nitrocellulose membrane and blotted sequentially with the indicated antibodies to evaluate both phosphorylation status and total content of STAT molecules. For the analysis of T-bet, 100 μ g of whole cell lysates were subjected to immunoblotting with anti T-bet antibodies. After detection of T-bet, blots were stripped and incubated with a mouse anti β -actin antibody to ascertain equivalent loading of the lanes. (B) LPMC total extracts were obtained from mice with TNBS or OXA colitis either left untreated or treated with oral rCT-B (TNBS+rCT-B). Where indicated, LPMC were treated with IL-4 for 30 minutes. Thirty μ g of whole cell lysates were subjected to immunoblot analysis with the indicated antibodies to evaluate both STAT-6 phosphorylation status and protein content.

(fig 3A, B). Importantly, rCT-B treatment led to a marked inhibition in the expression of both p35 and p40 transcripts (fig 3A, B). These findings were also confirmed by the measurement of IL-12 p70 released into the supernatants of mucosal explant cultures (fig 3C). We also showed that expression of p-STAT-4 was more pronounced in tissue extracts from inflamed areas of CD in comparison to that seen in uninvolved areas from the same patients and non-IBD controls (fig 3D). Consistent with this, rCT-B reduced the level of p-Stat-4 in inflamed mucosal tissues from CD (fig 3D).

Effect of rCT-B treatment on IFN- γ expression and signaling in ex vivo CD mucosal samples

As IL-12 mediated STAT-4 activation promotes the development of IFN- γ producing Th cells, we investigated the effect of rCT-B on expression and signaling of IFN- γ in CD mucosal explants. As shown in figure 4A–C, expression of IFN- γ RNA transcripts as well as the IFN- γ secretion were upregulated in CD mucosal samples, and they were markedly inhibited by rCT-B treatment. In agreement with this, rCT-B also reduced the expression of intracellular transcription factors that are associated with IFN- γ signaling, such as p-STAT-1 and T-bet (fig 4D). P-STAT-6 was barely detectable both in CD and non-IBD mucosal explants regardless of whether tissue was left untreated or treated with rCT-B (not shown).

Effect of rCT-B treatment on IL-12 and IFN- γ production by purified LPMC isolated from CD mucosal explants

To examine whether rCT-B directly affects the production of cytokines by intestinal LPMC, cells were isolated from two CD tissue specimens and incubated with rCT-B before the addition of stimuli that are known to enhance either IL-12 or

IFN- γ production. As shown in table 1, stimulation of CD LPMC with IFN- γ and SAC enhanced the secretion of the biologically active heterodimeric IL-12p70, and this production was reduced by nearly 60% by rCT-B. Similarly, the synthesis of IFN- γ induced in CD LPMC by anti-CD3/CD28 was reduced by rCT-B (table 1). In a separate experiment we also examined whether rCT-B modulated IFN- γ production by purified CD3⁺ lymphocytes. Treatment of CD3⁺ cells with rCT-B reduced IFN- γ secretion (10.1 v 43.2 pg/ml).

DISCUSSION

There is no doubt that polarisation of naïve T cells toward the Th1 type requires the combined action of multiple cytokines and activation of specific signaling pathways. While IL-12 driven STAT-4 signaling appears to be sufficient to induce IFN- γ gene transcription and trigger Th1 cell differentiation, activation of STAT-1 and induction of T-bet play a decisive role in expanding and sustaining Th1 mucosal cell responses. Consistent with this, high expression of active STAT-4 and T-bet has been documented both in CD tissue and TNBS induced colitis.^{28–30–33} In the present study, we demonstrate that oral administration of rCT-B inhibits IL-12 and IFN- γ production and suppresses Th1 associated transcription factors in the gut of mice with TNBS colitis. Therefore, these data confirm and expand results of our previous work which showed the ability of rCT-B to prevent or cure TNBS colitis in mice. In addition in this study, the effects of rCT-B on CD mucosal explants and LPMC isolated from CD tissue specimens were also examined showing that rCT-B reduced IL-12 and IFN- γ production and, in turn, the Th1 cell signaling.

The molecular mechanism by which rCT-B limits the ongoing Th1 mucosal inflammation in mice with TNBS colitis remains unclear. It is unlikely that rCT-B suppresses Th1 cell response by promoting Th2 cell differentiation, given that no

Figure 3 Effect of rCT-B treatment on IL-12 expression and STAT-4 phosphorylation in ex vivo mucosal explants. Mucosal samples from inflamed (I) and uninvolved (U) tissues of a CD patient and from a non-IBD control (Ctr). T0 represents mucosal samples processed immediately as control for the basal ex vivo level and activation of the studied transcription factors. Where indicated, mucosal samples were treated for four hours with rCT-B (10 μ g/ml). Thereafter, mucosal samples were washed in complete medium to remove rCT-B and incubated for additional 16–18 hours in complete medium. One representative experiment of three independent experiments is shown. (A) RNA transcripts for IL-12 p35, p40 and GAPDH were analysed in mucosal tissue homogenates by semiquantitative PCR. (B) The levels of RNA transcripts were measured by the Fluor-S Multimager and the relative values are expressed in arbitrary units. (C) Determination of IL-12 p70 in supernatants of cultured mucosal explants. The results shown are the mean (standard error) of values obtained in three separated experiments. (D) Total proteins (500 μ g/sample) were immunoprecipitated with anti-STAT-4, and immunoblotted with anti-phosphotyrosine STAT-4 (Y693). After STAT-4 p-Tyr analysis, blots were stripped and then incubated with Ab against STAT-4.

Figure 4 Effect of rCT-B treatment on IFN- γ signaling and expression in ex vivo CD mucosal samples. Mucosal samples were taken from inflamed (I) and uninfamed (U) tissues of a CD patient or from a non-IBD control (Ctrl). One representative experiment of three independent experiments is shown. (A) RNA transcripts for IFN- γ and GAPDH were analysed in mucosal tissue omogenates by semiquantitative PCR. (B) The levels of RNA transcripts were measured by Fluor-S Multimager and the relative values are expressed in arbitrary units. (C) Determination of IFN- γ in supernatants of cultured mucosal explants. The results shown are the mean (standard error) of values obtained in three separated experiments. (D) Thirty μ g of whole cell lysates were subjected to immunoblot analysis with the indicated antibodies to evaluate both total and phosphorylated STAT-1. T0 represents mucosal samples processed immediately as control for the basal ex vivo content of the protein. Where indicated, mucosal samples were treated for four hours with rCT-B (10 μ g/ml). Thereafter, mucosal samples were washed in complete medium to remove rCT-B and incubated for additional 16–18 hours in complete medium. Total proteins (100 μ g/sample) were immunoblotted with anti T-bet. After T-bet analysis, the level of β -actin was evaluated as an internal loading control.

change in IL-4 synthesis and p-STAT-6 activation was seen in the colon of mice treated with rCT-B. A possibility is that rCT-B favours the development and/or activity of regulatory T cells which would, in turn, dampen the local Th1 cell response. This would be supported by the demonstration that administration of rCT-B can induce tolerance in the peripheral T cell compartment and protect animals against several immune mediated experimental diseases by inducing suppressive regulatory T cells. In this context, for example, it has been shown that CT-B promotes the induction of both IL-10 and transforming growth factor- β secreting T cells and synergises with low doses of LPS in stimulating IL-10 production by immature murine dendritic cells.^{34–35} However, it is noteworthy that in our experimental model, rCT-B administration did not increase mucosal production of IL-10,¹¹ thus arguing against a primary role of IL-10 in the negative regulation of TNBS colitis by rCT-B.

Pentameric B subunit of CT binds to GM1 gangliosides on the membrane of all nucleated cells. In association with sphingomyelin and cholesterol, GM1 is concentrate in caveolae-like structures of lymphocytes and in caveolae of

other cells that contain a high concentration of signaling molecules.^{36–37} It is possible that binding of CT-B to GM1 ganglioside receptors on IL-12 producing cells and T cells per se can trigger/modify signaling events which, in turn, may affect the function of target cells leading also to a reduced production of Th1 inducing cytokines. This hypothesis is supported by the recent evidence that a mutant CT-B subunit, that does not bind GM1, fails to modulate leucocyte function.³⁸ How the binding of the B subunit to GM1 can trigger/influence signal transduction is not known. One possibility is that the binding of CT-B to GM1 might influence the activity of integral membrane proteins that participate in cell signaling. Whatever the underlying mechanism, we believe that the beneficial effect of oral administration of rCT-B on the Th1 mucosal inflammation is not purely dependent on the ability of rCT-B to enhance tolerance in the gut associated lymphoid tissues. In fact, in the present study we show that the expression of IL-12 and IFN- γ as well as the activation of Th1 transcription factors are dramatically inhibited by in vitro treatment of CD mucosal explants with rCT-B. Importantly, the expression of phosphorylated but not total STAT-1 and -4 was suppressed by rCT-B, clearly indicating that the rCT-B mediated inhibition of Th1 associated transcription factors in CD is not secondary to a toxic effect of rCT-B. Our data also suggest the possibility that the rCT-B-induced changes in the activation of such Th1 transcription factors are secondary to the ability of rCT-B to suppress directly the synthesis of IL-12 and IFN- γ . Indeed, we observed a reduction of IL-12 production by isolated LPMC pre-incubated with rCT-B and then stimulated with SAC, a known IL-12 inducer. Interestingly, we were also able to demonstrate that rCT-B impairs IFN- γ synthesis by purified CD3⁺ cells from CD tissue specimen in the absence of IL-12-producing cells. At the moment we cannot distinguish whether the inhibitory effect on IFN- γ production is due to a direct action of rCT-B on isolated CD3⁺ cells or

Table 1 Production of IL-12 and IFN- γ by Crohn's disease LPMC		
	IL-12	IFN- γ
Unstimulated	<0.5	<8
SAC+IFN- γ	32 (16)	
SAC+IFN- γ +CTB	12.4 (11)	
α CD3+ α CD28		60 (20)
α CD3+ α CD28+CTB		37 (33)

LPMC (200 000 cells in 200 μ l) were cultured as specified in material and methods. Supernatants were collected and analysed by ELISA. The results represent the mean (standard error) of two separate experiments. The values are expressed in pg/ml.

whether is a consequence of an apoptotic process induced by rCT-B as previously demonstrated.^{39,40}

In recent years evidence showing that IL-12/IL-12R signals contribute to the local inflammatory response in CD has been accumulated. Indeed, increased expression of IL-12 and IL-12R correlates with high activation of STAT-4 and IFN- γ production in the inflamed mucosa of CD patients.^{30,41} Evidence has also been provided to suggest that IFN- γ , in turn, contributes to expand the local Th1 differentiation by signaling through the STAT-1 pathway and enhancing the transcription of T-bet.^{19,28,31–33} Consistent with this, it was recently demonstrated that T-bet is expressed in LPMC in CD but not in UC and control patients, and that overexpression of T-bet results in an earlier onset of Th1 mediated colitis.²⁸ Additionally, it was reported that T-bet deficient T cells fail to induce Th1 mediated colitis in an adoptive transfer model of colitis, while IL-4 mediated colitis is enhanced in T-bet deficient mice. Therapeutic interventions aimed at interfering with Th1 cell development and/or perpetuation could therefore be useful in limiting the intestinal inflammation in patients with CD. This is also strongly supported by the recent demonstration that administration of neutralising IL-12 antibodies in CD patients is followed by a significant reduction in the clinical activity.⁴² Therefore, the demonstration that rCT-B dramatically inhibits the activation of Th1 transcription factors and Th1 cytokine production suggests the possibility that oral administration of rCT-B to CD patients represents a useful means to achieve clinical remission or amelioration of the disease.

ACKNOWLEDGEMENTS

The authors are grateful to Dr Rino Rappuoli (IRIS, Chiron SpA, Siena, Italy) for the kind gift of *Vibrio cholerae* 0395-tacCT-B and Dr Laurie Glimcher (Department of Medicine, Harvard Medical School, Boston, MA, USA) for providing antihuman T-bet antibody. A special thank you to Corrado Volpe for the culture of the *Vibrio cholerae*, to N Di Carlo for animal care, and to E Morassi for preparing drawings.

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This work was supported by grants from the Istituto Superiore di Sanità (99F and 2AEI to EC and to MB).

Competing interests: none declared.

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