Contribution of the ADP-ribosylating and receptor-binding properties of cholera-like enterotoxins in modulating cytokine secretion by human intestinal epithelial cells

Marco Soriani, Lorna Bailey and Timothy R. Hirst

Author for correspondence: Timothy R. Hirst. Tel: +44 117 928 7538. Fax: +44 117 930 0543.
e-mail: t.r.hirst@bristol.ac.uk

Department of Pathology
and Microbiology, University
of Bristol, Bristol BS8 1TD,
UK

When epithelial cells first encounter cholera toxin (Ctx) produced by *Vibrio cholerae* they secrete not only chloride ions responsible for causing diarrhoea, but also a number of cytokines that may contribute to the toxin's potent immunomodulatory properties. Much less is known about the ability of the heat-labile enterotoxin of *Escherichia coli* (Etx), a close homologue of Ctx, to elicit cytokine secretion by epithelial cells. This study shows that treatment of human intestinal epithelial T84 cells with Etx induces expression of IL-6, IL-10, IL-1R antagonist, as well as IL-1 α and IL-1 β and low levels of IL-8. Such induction was totally dependent on the intrinsic ADP-ribosylating activity of the toxin A-subunit, and could be mimicked by cAMP-elevating agents, such as forskolin and dibutyryl cAMP. By comparison, neither an enzymically inactive mutant of Etx nor EtxB was able to induce cytokine secretion. The behaviour of Ctx and CtxB was very similar to that of Etx and EtxB, respectively. The spectrum of cytokines released by Etx and Ctx indicates that the toxins may create a local microenvironment that strongly biases the immune response towards an anti-inflammatory and a polarized Th2 response.

Keywords: *E. coli* heat-labile enterotoxin, cholera toxin, cAMP, epithelial cells, cytokine expression

INTRODUCTION

Cholera toxin (Ctx) and *Escherichia coli* heat-labile enterotoxin (Etx) are composed of an A-subunit that possesses ADP-ribosyltransferase activity and five B-subunits that bind with high affinity to GM1-ganglioside receptors (Merritt *et al.*, 1994; Sixma *et al.*, 1992). Whilst these toxins are well recognized as the principal virulence determinants of cholera and traveller's diarrhoeal disease, respectively (Kaper *et al.*, 1995; Nataro & Kaper, 1998), there is a growing appreciation that they possess remarkable immunological properties. Notably, Ctx and Etx can trigger exceptionally potent anti-toxin responses when administered at mucosal or systemic sites and can also act as adjuvants augmenting immune responses to admixed antigens (for reviews see Lycke, 1998; Rappuoli *et al.*, 1999; Simmons *et al.*, 2001;

Williams *et al.*, 1999). More recently, studies have sought to determine the relative contributions of the respective A- and B-subunits in stimulating such effects, and whether or not the two toxins are equally effective. This has led to an increasing consensus that both subunits possess immunomodulatory properties in their own right, and that a combination of both A- and B-subunits engenders the greatest adjuvant effect (Simmons *et al.*, 2001). Studies by Rappuoli, Dougan and coworkers have found that holotoxin mutants of Etx and Ctx with ablated or reduced ADP-ribosyltransferase activity exert a greater adjuvant effect than the B-subunits alone (Douce *et al.*, 1995, 1997). Notwithstanding this, it is clear that if the B-subunit of *E. coli* enterotoxin (EtxB) is administered intranasally it triggers a high-titre immune response to admixed antigens (De Haan *et al.*, 1998; Millar *et al.*, 2001; Richards *et al.*, 2001), and moreover, that this effect is dependent on the receptor-binding property of the molecule (De Haan *et al.*, 1998; Guidry *et al.*, 1997). Such binding triggers signalling events that lead to up-regulation of

Abbreviations: Etx, *E. coli* heat-labile enterotoxin; Ctx, cholera toxin; dbcAMP, dibutyryl cAMP; PKA, cAMP-dependent protein kinase.

activation markers on B-cells, altered CD4+ T-cell differentiation, maturation of dendritic cells, preferential apoptosis of CD8+ T-cells and altered cytokine secretion by macrophages and monocytes (Francis *et al.*, 1992; Gagliardi *et al.*, 2000; Matousek *et al.*, 1996; Nashar *et al.*, 1996a, b, 1997; Truitt *et al.*, 1998). Although cholera toxin B-subunit (CtxB) shares many of the features of EtxB, including a capacity to modulate immune cells *in vitro* and to induce a potent anti-B-subunit antibody response, it does not appear to be as good an adjuvant as EtxB (Millar *et al.*, 2001; Richards *et al.*, 2001). Thus, while most studies to date have taken the view that findings on Ctx would also hold for Etx, it is not evident that this is the case, and at present few studies on the immunomodulatory effects of Etx have been undertaken.

Given that epithelial cells are the initial targets of the toxins, both during infection and following experimental mucosal immunization, it is conceivable that the apparent spectrum of adjuvant activity of the intact holotoxins, attenuated derivatives and B-subunits results from their differential effects on modulating epithelial cell cytokine production. McGee *et al.* (1993) showed that Ctx was able to strongly promote the production of IL-6 by rat IEC-6 epithelial cells, whilst non-recombinant preparations of CtxB had no effect. Similarly, Bromander *et al.* (1993) found that Ctx-treatment of IEC-17 cells stimulated both IL-1 and IL-6 secretion. Here we show that recombinant preparations of fully intact and enzymically active Etx, but not an inactive mutant or EtxB, causes elevated cytokine secretion by human intestinal epithelial T84 cells. Such effects were found to be dependent on activation of adenylate cyclase and protein kinase A. The nature of the cytokines activated, which included IL-10 and IL-1R α , is consistent with the toxins exerting an anti-inflammatory effect (Ryan *et al.*, 2000), which may contribute to the initial survival and growth of toxinogenic pathogens *in vivo* (Triadafilopoulos *et al.*, 1989). These findings provide a further insight into the enhanced immunomodulatory properties of the intact holotoxin as well as explaining how the toxin may alter the mucosal microenvironment in which immune responses occur.

METHODS

Materials. All reagents were purchased from Sigma unless otherwise stated. Recombinant preparations of Etx, Etx(E112D) (an enzymically inactive form of Etx), and Ctx were purified from culture supernatants of *Vibrio cholerae* O395NT harbouring pAM29, pAM220 and pRC9, respectively, as described previously (Rodighiero *et al.*, 1999). Recombinant EtxB and CtxB were purified as described by Richards *et al.* (2001).

T84 cell cultures. The human epithelial cell line T84 was obtained from the European Centre for Applied Microbiology and Research (ECACC, Salisbury, UK) and routinely cultured in Dulbecco's modified minimal essential medium (D-MEM) supplemented with Hams F10, 10% (v/v) fetal bovine calf serum, glutamine, penicillin and streptomycin. Cells were passaged at 5–6 day intervals and were used between passages

3 and 12. T84 cell monolayers were grown by plating (5×10^5 cells per well) in 12-well tissue culture plates and cultured for 5–6 days until they were fully confluent and had the morphological appearance of polarized cells. Experiments were also performed using T84 cells grown for 3 weeks on 6-well polycarbonate plates (Costar) and with addition of fresh medium every 3 days. When cultures were treated with various agents the following concentrations were used unless otherwise stated: Etx 1 nM; Etx(E112D) 1 nM; EtxB 100 nM; *V. cholerae* lipopolysaccharide (LPS) $10 \mu\text{g ml}^{-1}$; forskolin $10 \mu\text{M}$; dibutyryl cAMP (dbcAMP) $100 \mu\text{M}$; H89 $10 \mu\text{M}$.

Immunofluorescence and confocal microscopy. Immunofluorescence microscopy was performed on confluent adherent T84 cells grown for 5–6 days on collagen-coated glass coverslips as described by Casciola-Rosen *et al.* (1996). In order to detect early apoptotic events in Etx-treated cells, we used Annexin-V staining of phosphatidylserine exposed on the plasma membrane. Control or Etx-treated cells were washed three times with ice-cold PBS and subsequently Annexin V-FITC was added for 20 min at 4°C in the dark. Cover slips were then washed by dipping in PBS prior to fixation with 4% (w/v) paraformaldehyde and permeabilization with acetone. Cover slips were mounted on glass slides with Vectashield containing 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories) before viewing on a Leica TCS-SP2 confocal Ar plus UV laser scanning microscope attached to a Leica DM IRBE inverted phase-contrast/epifluorescence microscope (Leica Microsystems). An oil-immersion objective lens $63\times$, NA 1.32, was used, and imaging parameters were selected to optimize resolution.

cAMP detection assay. To measure intracellular cAMP in T84 cells the Biotrak cAMP scintillation proximity assay (SPA) kit from Amersham was used. Confluent T84 cells cultured in a 96-well plate were treated with cAMP-inducing agents for 1 h. Cells were then lysed and the level of cAMP determined. Briefly, the assay is based on the competition between intracellular unlabelled and a fixed quantity of ^{125}I -labelled cAMP for a limited number of binding sites on cAMP-specific antibody. The antibody-bound cAMP is then reacted with the SPA reagent, which contains anti-rabbit secondary antibody bound to fluoromicrospheres. ^{125}I -labelled cAMP that is bound to the primary rabbit antibody is immobilized on the fluoromicrospheres and the radiations emitted measured by a β -scintillation counter.

Analysis of secreted cytokines. Cytokine levels in T84 supernatants were determined by cytokine-specific ELISA. All cytokine standards, capture antibodies and detection antibodies were obtained from Pharmingen. Maxisorp immunoplates were coated overnight with monoclonal anti-human IL-2, IL-4, IL-6, IL-8, IL-10 and TGF- β 1. After blocking, samples and serial twofold dilutions of standards were added to duplicate wells and incubated overnight at 4°C . The plates were then washed and incubated with the respective biotinylated antibody for 1 h at room temperature. After incubation, extravidin peroxidase conjugate was added and the reaction developed with tetramethylbenzidine and hydrogen peroxide.

Analysis of cytokine gene expression by multi-probe RNase protection assay. RNase protection assays were performed according to the RiboQuant manual for human cytokines (hCK-2 template array, Pharmingen). Briefly, total RNA from T84 cells was prepared by the LS TRI-reagent protocol and quantified both spectrometrically and electrophoretically (TAE 1%, w/v, agarose gel). The Pharmingen multi-probe set contained a series of cDNA templates for IL-12, IL-1 α , IL-1 β ,

IL-1R α , IL-6, IFN- γ and the housekeeping genes L32 and GAPDH to allow normalization of the samples. The multi-probe template set was transcribed as antisense RNA probes by T7 RNA polymerase in the presence of [α - 32 P]UTP (ICN). Labelled probes were hybridized overnight with 4–5 μ g total RNA, after which free probe and other single-stranded RNA species were digested with RNase A and T1. The remaining 'RNase-protected' probes were purified and resolved by urea denaturing PAGE. Mouse control RNA and yeast tRNA were also run. The expressed genes were detected by autoradiography and the level of gene expression determined using the Scion Image program (Scion Corporation). To accurately establish the identity of each protected fragment, the migration of each fragment was plotted against a standard curve of the migration distance for undigested probe versus nucleotide length on a logarithmic scale.

Statistical analysis. Differences in cytokine production between treated and untreated T84 cells were analysed for statistical significance using unpaired Student's *t*-test.

RESULTS

Prior to investigating the effect of cholera-like enterotoxins on the modulation of cytokine production, we assessed whether our recombinant preparations of Etx or EtxB exerted any morphological or cytotoxic effects on T84 cells. When T84 cells were treated with 1 nM Etx or 10 μ M forskolin for 6 h, phase-contrast microscopy revealed that the cells had somewhat enlarged nuclei and that there was a reduction in contrast at the cellular junctions (data not shown). Given the recent findings that the B-subunits of cholera-like enterotoxins trigger apoptosis of certain lymphocyte subsets (Nashar *et al.*, 1996b; Truitt *et al.*, 1998), it was decided to determine whether the morphological changes observed in T84 cells were also accompanied by induction of apoptosis. An early apoptotic event is the exposure of phosphatidylserine on the plasma membrane, which can be detected by FITC-Annexin-V. Treatment of T84 cells with 1 nM Etx/Ctx or 100 nM EtxB/CtxB for 6 h and 24 h failed to cause any detectable phosphatidylserine appearance on the cell surface or nuclear condensation (data not shown). Incubation of T84 cells with hydrogen peroxide clearly revealed Annexin V binding to phosphatidylserine (Asada *et al.*, 2001). We therefore conclude that the morphological changes triggered by treatment of T84 cells with Etx are not associated with induction of apoptosis.

Etx induces cytokine gene expression in T84 cells

It has been previously reported that Ctx triggers cytokine activation in epithelial cells (Bromander *et al.*, 1993; McGee *et al.*, 1993), while no information is available on Etx-mediated effects. We assessed the multiple expression of cytokine mRNA transcripts in Etx-treated T84 cells using a highly sensitive and specific human cytokine multi-probe RNase protection assay system, which allows the simultaneous detection of expression of IL-12, IL-1 α , IL-1 β , IL-1R α , IL-6 and IFN- γ . Fig. 1(a) shows that 1 nM Etx was able to induce the

early expression of IL-1 α , IL-1 β , IL-6 and IL-1R α . The data from three independent experiments were normalized against the mRNA levels of the housekeeping gene GAPDH and expressed as fold induction with respect to PBS control, and are shown in Fig. 1(c–j). We noted while the basal level of IL-1 α and β cytokine expression in the absence of treatment (PBS control) appeared to vary, the fold increase in expression of these cytokines following addition of Etx and other modulators of cAMP levels was remarkably consistent. The expression of IL-6, IL-1 α , IL-1 β genes, which peaked at 6 h post-treatment (see Fig. 1c, g and i, respectively), decreased at 24 h (see Fig. 1d, h and j). By contrast, mRNA levels for IL-1R α remained elevated at 24 h post-Etx treatment (see Fig. 1e and f). Protected probes for IL-12 and IFN- γ were barely detectable in samples derived from either PBS- or Etx-treated T84 cells, while their transcripts were present in a control human RNA sample.

Etx-mediated cytokine expression in T84 cells is dependent on an enzymically active A-subunit

In order to assess if the ADP-ribosylating activity of Etx was essential in triggering activation of cytokine expression, identical experiments to those above were carried out on T84 cells treated with Etx(E112D) (an enzymically inactive mutant of Etx), or a 100-fold molar excess of recombinant B subunits (EtxB). Neither Etx(E112D) nor EtxB caused elevation in mRNA levels of any of the cytokines tested (Fig. 1a). Addition of 10 μ M forskolin (an adenylate cyclase activator) or 100 μ M dbcAMP led to similar cytokine patterns to those observed following Etx treatment (Fig. 1b). As shown in Table 1, T84 cells were actively stimulated by Etx and forskolin to produce cAMP, while Etx(E112D) or EtxB failed to elicit a significant cAMP increase. These findings suggest that Etx-mediated ADP-ribosylation of adenylate cyclase and the resultant increase in cAMP levels are responsible for triggering altered cytokine expression. To further corroborate this, H89, a specific inhibitor of the cAMP-dependent protein kinase (PKA), was added 60 min prior to addition of Etx. This led to a complete loss of Etx-mediated elevation in cytokine expression (see Fig. 1a). As a control, 10 μ g *V. cholerae* LPS ml $^{-1}$ was added to T84 cells. This failed to increase mRNA levels of any of the cytokines tested above basal levels (Fig. 1a). We therefore conclude that the ability of Etx to increase cellular cAMP levels leads to transcriptional activation of IL-1 α , IL-1 β , IL-1R α and IL-6. Addition of 1 nM Ctx gave identical results (data not shown).

Effect of cholera-like enterotoxins on cytokine secretion

The multiple activation of cytokine gene expression observed after Etx treatment led us to further investigate whether such effects were associated with an increase in cytokine secretion by T84 epithelial cells. For comparison, experiments were performed with both Ctx and

Fig. 1. For legend see facing page.

Etx. We tested for the presence of TGF- β 1, IL-2, IL-4, IL-6, IL-8 and IL-10 in the supernatant of Etx- and Ctx-treated T84 cells by ELISA. It was found that neither 1 nM Etx, 1 nM Ctx, 100 nM EtxB nor 100 nM CtxB triggered increased secretion of IL-2, IL-4 or TGF- β 1 throughout the 24 h treatment period (data not shown). By contrast, treatment with 1 nM Etx or 1 nM Ctx led to an increase in IL-6 levels, which peaked between 16 h and 24 h post-treatment (Fig. 2a, b) and decreased after 48 h incubation (data not shown). No effect was observed using an identical concentration of Etx(E112D) (Fig. 2b) or 100 nM EtxB/CtxB (Fig. 2a, b). Addition of the cAMP-elevating agents forskolin or dbcAMP led to an increase in IL-6 secretion to levels comparable to those observed using Etx or Ctx (Fig. 2c). Inhibition of

PKA activity by H89 completely blocked the capacity of Etx to trigger IL-6 production, while LPS treatment had no effect on IL-6 levels (Fig. 2c).

cAMP-elevating agents had previously been shown to activate IL-10 expression in macrophages (Procopio *et al.*, 1999) and T-cells (Benbernou *et al.*, 1997). When the levels of IL-10 were tested in the supernatant of T84 cells after 1 nM Etx or 1 nM Ctx treatment, we found a late up-regulation of IL-10 production peaking at 24 h (Fig. 3a, b) and decreasing at 48 h (data not shown). As with IL-6 secretion, neither EtxB and CtxB (Fig. 3a), Etx (E112D) (Fig. 2b) nor LPS (Fig. 3c) triggered elevated IL-10 production. Moreover, in agreement with the concept that Etx-mediated up-regulation in IL-10 se-

Fig. 1. Etx activates IL-1 α , IL-1 β , IL-1R α and IL-6 gene expression in T84 cells by a cAMP-dependent mechanism. (a) Fully confluent T84 cells were incubated for 6 and 24 h in the presence of PBS, 10 μ g LPS ml $^{-1}$, 1 nM Etx, 1 nM Etx(E112D) or 100 nM EtxB, or pre-incubated for 1 h with 10 μ M H89 and then treated with 1 nM Etx (H89). Total RNA was extracted and hybridized with a labelled human cytokine multi-probe template set as described in Methods. Unprotected probes (P), mouse control RNA (C) and yeast tRNA (Y) were also run simultaneously with the samples. Identical results were obtained on three independent occasions. (b) As in (a) except for treatments with 10 μ M forskolin (Fors) and 100 μ M dbcAMP. (c–j) Quantification of cytokine gene expression in T84 cells treated as in (a): IL-6 (c and d), IL-1R α (e and f), IL-1 β (g and h) and IL-1 α (i and j). Quantification was obtained by normalizing cytokine band intensities in comparison with the GAPDH housekeeping gene. Mean values $\pm$ SD of three independent experiments are shown.

cretion depends on cAMP elevation, it was found that both forskolin and dbcAMP also increased IL-10 secretion by T84 cells (Fig. 3c), but that a 60 min pre-incubation with H89 blocked the ability of Etx to elevate

IL-10 levels. We therefore conclude that the ADP-ribosylating activity of both Etx and Ctx is responsible for activating production of IL-6 and IL-10 in epithelial cells.

Table 1. Modulation of cAMP levels in T84 cells

Treatment	cAMP (pmol $\pm$ SD, $n = 3$)
PBS	0.37 $\pm$ 0.05
Forskolin	8.38 $\pm$ 2.31
Etx	2.68 $\pm$ 0.36
H89/Etx	0.27 $\pm$ 0.02
Etx(E112D)	0.33 $\pm$ 0.05
EtxB	0.33 $\pm$ 0.06

Effect of cholera-like toxins on IL-8 production by T84 cells

In addition to an elevation in IL-6 and IL-10 levels, it was found that 1 nM Etx or 100 μ M dbcAMP (Fig. 4) or 1 nM Ctx (data not shown) slightly increased IL-8 levels (approx. 1.5-fold) in the supernatant of T84 cells after 24 h treatment. By contrast, EtxB, CtxB or Etx(E112D) failed to induce significant increase of IL-8 secretion (Fig. 4). Whilst these findings are consistent with cAMP triggering activation of IL-8 production in epithelial cells, we noted that addition of LPS alone also modulated IL-8 expression (Fig. 4).

DISCUSSION

Enzymically active cholera-like holotoxins and their B-subunits are able to trigger cAMP-mediated and GM1-receptor-mediated signalling events, respectively (Peterson & Whipp, 1995; Soriani *et al.*, 2001). However, the relative contributions of these events to creating a local microenvironment leading to immunoresponsiveness, and indeed, which cell types are involved, have remained unclear. Here we provide unequivocal evidence that the holotoxins of both Etx and Ctx can trigger cAMP-dependent up-regulation of a number of cytokines by epithelial cells, and we found no obvious difference in behaviour between the two toxins. During the course of infection released toxins will first encounter epithelial cells and be taken up by a process of endocytosis, which results in delivery of the toxic A-subunits into the cytosolic compartment and to subsequent activation of adenylate cyclase. The B-subunits, by contrast, do not enter the cytosol, and a proportion appear to be transcytosed and released from the basolateral membrane (Lencer *et al.*, 1995). Thus, it is conceivable that the principal activities of the A- and B-subunits are segregated and function on different cell types, with the A-subunits primarily mediating their effect on epithelial cells while transcytosed B-subunits interact with immune cells in the lamina propria and Peyer's patches. Consequently the A-subunit could be responsible for triggering induction of anti-inflammatory cytokines or factors that counter the activity of inflammatory cytokines in epithelial cells, while the B-subunits may exert a similar anti-inflammatory effect on monocytes, macrophages and dendritic cells (Williams *et al.*, 1999). The sum of all of these effects is that the toxins may create a local microenvironment that

Fig. 2. Ctx and Etx induce IL-6 secretion by cultured T84 cells. (a) Cells were treated for 24 h with 1 nM Etx, 1 nM Ctx and 100 nM of their respective recombinant B-subunits. Medium supernatant was then collected and tested for IL-6 levels by ELISA as described in Methods. (b) Time course for IL-6 production by Etx in T84 cells. Cells were cultured as in (a) and treated with Etx, Etx(E112D) or EtxB for 6, 16 and 24 h. After each time-point an aliquot of medium was flash-frozen using liquid nitrogen and stored at -80°C . ELISA was then performed as in (a). (c) Confluent T84 cells were treated for 24 h with 1 nM Etx, 10 μ M forskolin, 100 μ M dbcAMP or 10 μ g LPS ml⁻¹, or pre-incubated for 1 h with 10 μ M H89 prior to Etx treatment (H89). IL-6 levels were assayed in the respective supernatants as in (a). The results are mean values $\pm$ SD of three independent experiments. * $P < 0.05$ vs PBS control.

strongly biases the immune response towards an anti-inflammatory and a polarized Th2 response.

Ctx is known to elicit cytokine secretion by epithelial cells as well as by bone-marrow-derived macrophages (Bromander *et al.*, 1991, 1993; Cong *et al.*, 2001; Elson & Cong, 1995; Klimpel *et al.*, 1995; McGee *et al.*, 1993)

Fig. 3. Ctx and EtX induce IL-10 secretion by T84 epithelial cells. Cells were cultured and treated as in Fig. 2. Supernatants, stored at -80°C after being flash-frozen in liquid nitrogen, were tested for IL-10 levels by ELISA. The results are mean values $\pm$ SD of three independent experiments. * $P < 0.05$ vs PBS control.

and to inhibit the secretion of pro-inflammatory cytokines such as IL-12, TNF- α , RANTES and IL-1 by LPS-stimulated human dendritic cells (Braun *et al.*, 1999; Gagliardi *et al.*, 2000). Because of the similarity in structure and function of Ctx and EtX, it has been generally accepted that results obtained from studies on Ctx will also hold for EtX. Nonetheless, very few studies have been undertaken on EtX, and recent observations have highlighted some striking differences in the immunological properties of their respective B-subunit moieties (Millar & Hirst, 2001). Thus, while CtxB appears to be a poor adjuvant, EtXB can trigger immune responses to co-administered antigens that approach levels seen using the full holotoxins of Ctx or EtX as

Fig. 4. EtX, dbcAMP and LPS slightly up-regulate IL-8 levels in the supernatant of T84 cells. Fully confluent cells were treated for 24 h with 1 nM EtX, 1 nM EtX(E112D), 100 nM EtxB, 100 μM dbcAMP or 10 μg LPS ml⁻¹. Medium was then collected and tested for IL-8 levels by ELISA as described in Methods. The results are mean values $\pm$ SD of three independent experiments. * $P < 0.05$ vs PBS control.

adjuvants. In order to clarify the mechanism of action of such toxins, we investigated the effects of cholera-like enterotoxins on cytokine expression by epithelial cells and whether such modulation depended on (a) the identity of the toxins, (b) their ability to bind to GM1 receptors or (c) their intrinsic ADP-ribosylating activity. Interestingly, Ctx and EtX behaved identically with respect to their effects on cytokine expression by T84 epithelial cells. Moreover, these effects depended on the elevation of intracellular cAMP levels, since the non-toxic B-subunits alone or a mutant form of EtX, lacking enzymic activity, failed to increase any of the cytokines activated by Ctx/EtX or cAMP-elevating agents, such as forskolin and dbcAMP (Fig. 1). By using the highly sensitive RNase protection assay, we were able to screen for transcriptional activation of a series of pro- and anti-inflammatory cytokines. Ctx and EtX, as well as forskolin and dbcAMP, triggered a large increase in IL-6 and IL-1R antagonist mRNA levels, while the expression of Th1-type cytokines like IL-12 was unaffected. The early activation of IL-1 α and IL-1 β observed is in agreement with previous findings showing that Ctx enhances IL-1 production by macrophages (Bromander *et al.*, 1991) and epithelial cells (Bromander *et al.*, 1993). This potentiates antigen presentation by macrophages and modulates T-cell responses. The elevation in cAMP levels by cholera-like enterotoxins will lead to activation of cAMP-dependent protein kinase (PKA) and the translocation of PKA catalytic subunits to the nucleus (Zidek, 1999). The subsequent phosphorylation of cAMP-responsive element binding proteins would be expected to activate transcription of specific genes carrying cAMP-regulatory elements in their promoter region (Seternes *et al.*, 1999), such as IL-6 (Krueger *et al.*, 1991) and IL-1 β genes (Chandra *et al.*, 1995). Our finding that H89, an inhibitor of PKA, abrogates the induction of IL-1 α , IL-1 β , IL-6 and IL-1R α further corroborates the hypothesis of a direct effect of cAMP on Ctx/EtX-induced cytokine expression.

IL-1R α is an important anti-inflammatory molecule that acts by blocking IL-1-induced inflammatory reactions (Dinarello, 1998). Our finding that the expression of IL-1R antagonist is up-regulated in T84 cells by both Ctx and Etx provides evidence that these toxins might create an anti-inflammatory environment in the intestine by blocking IL-1 binding to its own receptor. Recently, Feng *et al.* (2000) reported that Ctx and 8-bromo-cAMP up-regulated expression of IL-1R α , as well as IL-10, in LPS-stimulated macrophages.

IL-6 and IL-10 are believed to play an important role in modulating mucosal immune responses (Klimpel *et al.*, 1995). Both Etx and Ctx up-regulated IL-6 levels in the supernatant of T84 human epithelial cells, while H89 completely blocked such effects (Fig. 2). By contrast, CtxB, EtxB and the non-toxic mutant Etx(E112D) failed to induce the production of IL-6 in T84 cells. This is in agreement with previous findings indicating that Ctx triggers IL-6 production in rat epithelial cells by a mechanism involving cAMP (McGee *et al.*, 1993). While IL-6 is produced in response to certain inflammatory stimuli, it has been shown that this cytokine can exert anti-inflammatory effects in several systems by inhibiting Th1 responses (Barton, 1997). In addition, IL-6 is known to act as a growth factor for activated T-cells, boosting the magnitude of the Th2 response, and promoting immunoglobulin production by B-cells, especially secretory IgA. The high-level production of IL-6 upon treatment of epithelial cells with Etx may contribute to both the polarization of the Th response and the apparent adjuvanticity of the toxin. IL-10 plays a pivotal role in determining the immunosuppressive environment resulting from the action of cholera-like toxins (Wiedermann *et al.*, 1999). This concept is strengthened by the fact that putative cAMP-responsive elements were localized within the promoter/enhancer region of the IL-10 gene (Platzer *et al.*, 1999). IL-10 levels were up-regulated by a cAMP-dependent pathway in the supernatants of Ctx/Etx-treated T84 cells. Whilst IL-10 secretion was clearly dependent on the ADP-ribosylating activity of Ctx/Etx, its delayed onset in production suggests that indirect autocrine mechanisms may be involved. In the intestinal mucosa IL-10 is released primarily by the Th2 subset of CD4⁺ T helper cells in response to common environmental antigens (Panja *et al.*, 1995). However, the fact that IL-10 is also produced by isolated epithelial cells suggests that it may play a role in the regulation of the intestinal cell function. Indeed, it has been shown that IL-10 enhances sodium and chloride absorption in rat small intestine, limits forskolin-induced chloride secretion and prevents disruption of monolayer barrier integrity in T84 epithelial cells (Madsen *et al.*, 1997).

In addition, Etx, as well as dbcAMP, slightly increased IL-8 levels and failed to induce expression of IL-12 in T84 cells. IL-8 is a potent neutrophil, T-cell and basophil chemoattractant involved in acute inflammatory responses (Harada *et al.*, 1994) and it has been postulated that during intestinal invasion by luminal bacteria IL-8 can function as a signal for mucosal barrier penetration

(Eckmann *et al.*, 1993). However, it has been shown that Ctx inhibits *in vitro* IL-8-induced lymphocyte migration (Bacon & Camp, 1990) and that in a rabbit intestine model Ctx did not cause neutrophil infiltration in the lamina propria or necrosis of enterocytes (Triadafilopoulos *et al.*, 1989). Thus, while cAMP elevation by Etx or dbcAMP results in some IL-8 secretion by epithelial cells, the toxins appear to be able to counteract this, possibly by direct effects on leukocytes. Such findings are consistent with the clinical picture of disease in which diarrhoea ensues without any evident inflammatory response.

Taken together, these findings suggest that Etx and Ctx elevate expression of anti-inflammatory molecules such as IL-10 and IL-1R α , and are unable to trigger the potent secretion IL-8 and IL-12. This is consistent with the view that activation of cAMP-mediated pathways in epithelial cells promotes an anti-inflammatory response, as well as a cytokine microenvironment that may favour induction of a polarized Th2 immune response.

The observation that neither an enzymically inactive mutant of Etx nor EtxB was able to induce cytokine secretion may in part explain why these molecules are not such potent mucosal adjuvants as their respective holotoxins. Moreover, the differential adjuvanticity being reported for EtxB, compared to CtxB, does not appear to be due to a differential induction of cytokine expression by epithelial cells, since both B-subunits behaved identically.

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