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Enhancing Effect of Cholera Toxin on Interleukin-6 Secretion by IEC-6 Intestinal Epithelial Cells: Mode of Action and Augmenting Effect of Inflammatory Cytokines

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Oral administration of cholera toxin (CT) induces a strong mucosal immune response to CT as well as having a potent adjuvant effect. Since one of the first cell types to encounter CT during cholera infection or after oral administration is the epithelial cell, we studied the effect of CT on interleukin-6 (IL-6) secretion by the rat intestinal epithelial cell line IEC-6. CT was found to rapidly enhance IL-6 secretion and IL-6 gene expression by these cells. The addition of dibutyryl cyclic AMP (cAMP) to cultures of IEC-6 cells had little effect on IL-6 secretion, yet mRNA levels were elevated, suggesting that the response may have been regulated by cAMP. Purified B subunit of CT did not significantly enhance IL-6 secretion or mRNA expression. CT and transforming growth factor β1 synergistically enhanced IL-6 secretion in IEC-6 cells. The addition of CT with either IL-1β or tumor necrosis factor alpha gave even greater synergistic enhancement of IL-6 secretion, and dibutyryl cAMP could mimic CT's synergy with IL-1β. These results indicate that the intestinal epithelial cell is capable of secreting high levels of IL-6 after encountering CT, especially in the presence of inflammatory cytokines. This high level of IL-6 secretion could be a very important component of the mucosal immune response to CT and may also account for a portion of the adjuvant effect of CT.

Cholera toxin (CT) is a potent enterotoxin produced by the bacterium *Vibrio cholerae*. This toxin is composed of the enzymatic A subunit which is linked noncovalently to five smaller B subunits (CT-B) which bind with high affinity to GM1 gangliosides found on the surface of most cell types (17). The binding of CT to enterocytes allows the A subunit to activate intracellular adenylate cyclase, resulting in increases in cyclic AMP (cAMP) levels that ultimately lead to a massive flow of water and electrolytes from the epithelial cells, giving rise to the diarrhea associated with cholera. Yet, aside from its pathological functions, CT is also an important mucosal antigen and adjuvant. Unlike most soluble proteins, CT can induce an excellent mucosal antibody response when given orally (32). Furthermore, low oral doses of CT have been shown to potentiate the mucosal immune response to a number of orally administered unrelated antigens which by themselves would induce little or no response (12, 22, 24). The impact of this finding on mucosal immunizations could potentially be very important, especially since a recent report has suggested that the use of CT as an oral adjuvant induced long-term immunological memory to an unrelated antigen (35).

There is some debate whether the adjuvant effect can be induced only with CT holotoxin or whether the B subunits alone can induce the effect. Several reports have shown that simply coadministering CT-B with the antigen does not induce the adjuvant effect (22, 24, 25, 29, 35, 36) but conjugating CT-B to the antigen does show an adjuvant effect (29, 36). Still others have found that CT-B can act as an adjuvant when simply coadministered with the antigen (9, 15), yet some suggest that this effect could have been due to small amounts of the holotoxin which may have been present in the CT-B preparation (35).

The precise mechanism by which CT acts as an oral adjuvant has not been elucidated, but several studies have addressed this issue. It is known that the adjuvant effect is only seen when CT is given orally and simultaneously with the antigen (24), suggesting that the critical events responsible for the response occur in a short time interval. However, CT seems to have an inhibitory effect on T cells, since both CT (35) and CT-B (39) have been shown to inhibit concanavalin A-stimulated T cell proliferation and CT can inhibit interleukin-2 (IL-2) production by T cells (23). CT has also been shown to inhibit anti-immunoglobulin M (IgM)-induced B-cell proliferation (39). Recently, CT and CT-B were shown to enhance IgA and IgG secretion in lipopolysaccharide-stimulated spleen B cells (23) by inducing surface IgM-bearing B cells to differentiate to surface IgA- and IgG-bearing cells (26). These studies provided the first possible mechanism by which CT could act as an adjuvant.

In a separate line of study CT was found to enhance antigen presentation by macrophages and this effect was associated with increased production of both soluble and cell-associated forms of IL-1 (6). However, CT had no effect on the expression of class II major histocompatability complex proteins by macrophages (6). Finally, evidence has been provided that CT may increase gut permeability to some antigens, another mechanism by which CT could enhance mucosal immune responses to unrelated antigens (25). Yet one of the first cell types which would encounter CT after oral administration is the intestinal epithelial cell (IEC), and the majority of the CT dose would probably attach to the IEC. Furthermore, it is the effect of CT on the IEC which is so dramatic in an infection with *V. cholerae*. To this end, a recent study has determined that CT can enhance the ability of an IEC line to present alloantigen,

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presumably by inducing enhanced secretion of IL-1 and IL-6 by the IEC (7), suggesting that the binding of CT to IEC may well play a role in the mucosal immune response to CT.

In previous studies, we used the nontransformed crypt-like rat small intestine IEC line IEC-6 (33) as a model to define the mechanisms which regulate IL-6 secretion by IEC. We found that the inflammatory cytokine IL-1β (28) and, to a lesser degree, transforming growth factor β-1 (TGF-β1) (27), can induce IEC-6 cells to secrete significantly elevated levels of IL-6. More important, we found that a combination of these two cytokines can induce an even greater level of IL-6 secretion by these cells (28). These studies have suggested that the IEC may be an important source for IL-6 in mucosal immune responses in the gastrointestinal tract.

IL-6 not only has a role in the inflammatory response (1) but has also been shown to preferentially enhance IgA secretion by Peyer's patch (5) and human appendix (13) B cells and can also act as a costimulator for T-cell proliferation responses (1). Therefore, elevated levels of this cytokine from IEC could result in an enhanced IgA response. Since IEC can produce significant levels of IL-6 (27, 28) and CT has been shown to induce these cells to secrete IL-6 (7), we have begun to study the effect of CT on IL-6 secretion by IEC-6 cells. In this report, we present evidence that CT but not CT-B can induce a rapid increase in IL-6 secretion by IEC-6 cells, which may be mediated through an elevation of the level of the second messenger, cAMP. Moreover, CT plus TGF-β1 synergistically enhanced IL-6 secretion and the combination of CT with IL-1β and tumor necrosis factor alpha (TNF-α) caused an even greater increase, indicating that IEC may have an important role in the immune response to CT as well as a role in the mechanism by which CT acts as an oral adjuvant.

MATERIALS AND METHODS

Reagents. CT was purchased from either Sigma Chemical Co. (St. Louis, Mo.) or List Biological Laboratories (Campbell, Calif.). Purified CT-B, dibutyryl (DB) cAMP, Forskolin, and purified normal rabbit serum IgG fraction were also obtained from Sigma Chemical Co. Recombinant murine IL-6 was a kind gift from Gerald Fuller (University of Alabama at Birmingham), as was an IgG fraction of a rabbit anti-mouse IL-6 antibody described elsewhere (27). Recombinant human IL-1β and TNF-α and porcine TGF-β1 were obtained from R & D Systems (Minneapolis, Minn.).

Cell culture. The IEC-6 cells (ATCC CRL 1592) were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium containing 4.5% glucose, 5% fetal calf serum (FCS; Hyclone Laboratories, Inc., Logan, Utah), 0.1 IU of bovine insulin (Sigma) per ml, 2 mM L-glutamine, 25 IU of penicillin per ml, 25 μg of streptomycin per ml, and 80 μg of gentamicin (Whittaker Bioproducts, Walkersville, Md.) per ml. All experiments were performed on cells at or before the 20th passage.

Experimental cultures of the IEC-6 cells were prepared as reported previously (27). Briefly, 2×10^5 cells were added to triplicate wells in 12-well culture plates. After 2 days of incubation, the culture supernatants were removed, the adherent cells were washed once in serum-free medium, and 1.5 ml of culture medium containing 1% FCS and the appropriate cytokines or reagents was added to each well. At the appropriate time, the culture supernatants were collected, filtered through a 0.45-μm filter, and stored frozen until assayed for IL-6 content. The adherent cells were then enumerated by first treating the cells with trypsin and EDTA (Sigma) for 10 min and then diluting the cells and counting them with a hemacytometer.

Bioassay for IL-6. A proliferative bioassay with the IL-6-dependent 7TD1 mouse hybridoma was used to determine the IL-6 content of the IEC-6 cell culture supernatants (37). The culture supernatants were diluted in duplicate or triplicate with RPMI 1640 containing 10% FCS, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 0.05 mM 2-mercaptoethanol, 25 IU of penicillin per ml, 25 μg of streptomycin per ml, and 80 μg of gentamicin per ml, and 50-μl aliquots were added to 96-well culture plates. To this, 50-μl aliquots of medium containing 2×10^3 washed 7TD1 cells were added, and the cultures were incubated for 4 days. The resulting proliferation was measured by the MTT colorimetric assay (30). One unit of IL-6 was defined as the reciprocal of the dilution giving 50% maximal stimulation of proliferation, and the values for the IEC-6 cell culture supernatants were compared with a standard curve generated with recombinant murine IL-6. As determined previously, this assay was insensitive to IL-1β, IL-2, IL-4, gamma interferon, TNF-α, TGF-β1, and epidermal growth factor and only slightly responsive to IL-5 (27). Cholera toxin at 1 μg/ml was found to have some inhibitory effect on the IL-6-stimulated proliferation of 7TD1 cells, with 1 μg of CT per ml resulting in approximately 30% inhibition of proliferation. This indicates that the IL-6 content of cultures containing CT may be an underestimate of the actual values. Forskolin (1 mM) and DB cAMP (1 mM) were also found to completely inhibit the IL-6-induced proliferation of 7TD1 cells. Although CT had little effect on the proliferation of IEC-6 cells, the IL-6 levels in all culture supernatants were reported as units of IL-6 per 10^5 cells to account for any effect that the cytokines or CT may have had on the growth of the cells.

Analysis of IL-6 mRNA expression by RT-PCR. The IEC-6 cells were cultured at 2.6×10^6 cells per flask for 2 days, and the culture supernatants were replaced with fresh medium containing 1% FCS and the appropriate cytokine. At the appropriate times, the cells were collected by trypsin and EDTA treatment and washed once in medium containing 5% FCS and then twice in cold phosphate-buffered saline. Total cellular RNA was extracted by the acidic guanidinium thiocyanate method (10). The resulting RNA pellets were dried, suspended in diethylpyrocarbonate (DEPC)-treated water, and stored at −70°C.

Reverse transcription (RT) of the RNA samples to cDNA was done with the Perkin-Elmer Cetus Gene Amp RNA polymerase chain reaction (PCR) kit (Norwalk, Conn.). Briefly, the RNA samples were diluted twofold from 0.5 to 0.125 μg of RNA in diethylpyrocarbonate-treated water containing 20 U of RNase inhibitor (Perkin-Elmer Cetus) per ml. The samples were then heated to 65°C for 2 min and chilled on ice before the appropriate volume of master mix containing $MgCl_2$, 10× PCR buffer II, nucleotide triphosphates, RNase inhibitor, oligo$(dT)_{16}$, and reverse transcriptase was added. The samples were incubated at 42°C for 60 min and then for 5 min at 99°C. The resulting cDNA samples were stored at −20°C until used.

PCR primers for rat glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and IL-6 were obtained from Clonetech Laboratories (Palo Alto, Calif.). These primers produced amplified PCR fragments of 983 and 614 bp, respectively. The cDNA samples were PCR amplified with the Gene Amp RNA PCR kit as follows. Aliquots of the samples were

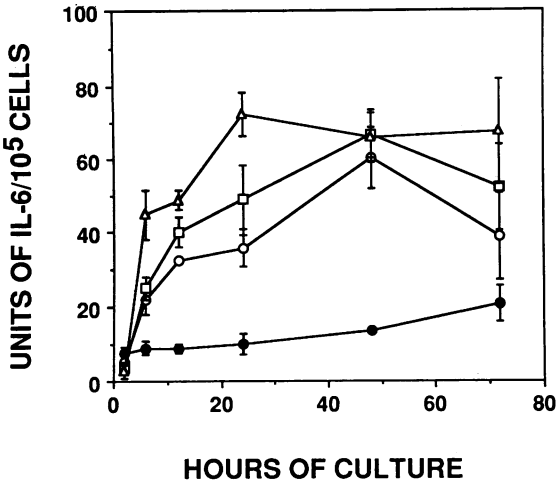

FIG. 1. CT enhances IL-6 secretion by IEC-6 cells. IEC-6 cells were cultured with medium only (●) or medium plus CT at 0.1 (○), 1.0 (□), or 5.0 (△) μg/ml, and supernatants were harvested at the appropriate times for IL-6 determination. This figure is representative of two experiments.

added to a master mix containing $MgCl_2$, 10× PCR buffer II, the appropriate 3′ and 5′ primers, and AmpliTaq DNA polymerase. PCR was performed with a Perkin-Elmer DNA Thermal Cycler model 480 for 30 cycles consisting of 45 s at 94°C, 2 min at 65°C, and 3 min at 72°C. Thirty cycles of PCR amplification were used for subsequent experiments because this method yielded barely detectable cDNA bands from control cultures. Twenty-five PCR cycles yielded virtually no detectable cDNA, and 35 cycles yielded bands which were heavier yet still showed a dilution of the product. Reaction product aliquots (7.5 μl) were separated by electrophoresis at 100 V in a 2% agarose gel and stained with ethidium bromide. As a size marker, a 1-kb DNA ladder (GIBCO BRL, Gaithersburg, Md.) was included. Photographic negatives of the gels were prepared with Polaroid type 665 film.

Statistics. Student's *t* test was used to compare experimental means with control values. An analysis of variance and Fisher's protected-least-significant-differences test were used to determine significant differences between multiple means. The level of significance was set at 0.05% and all values shown are the means ± the standard deviations.

RESULTS

CT enhances IL-6 secretion by IEC-6 cells. Culturing IEC-6 cells with CT induced a rapid increase in IL-6 secretion between 2 and 12 h which began to slow and plateau by 24 to 48 h (Fig. 1). The enhancing effect of CT was also found to be dose dependent for 0.1, 1.0, and 5.0 μg of CT per ml. Next, Northern blotting techniques were used to determine the effect of CT on IL-6 mRNA expression. However, IEC-6 cells were found to express only low levels of IL-6 mRNA in comparison to other species of RNA. Therefore, sensitive semiquantitative RT-PCR analysis was used, and CT was found to enhance IL-6 mRNA expression (Fig. 2). Also shown in Fig. 2 are the PCR products for the expression of G3PDH mRNA obtained with the same RT samples as used for IL-6 analysis. These showed approximately equal amounts of PCR product for each RNA level, which indicates that approximately equal amounts of starting material were used in the various samples. Enhancement of IL-6 mRNA expression was found to begin as early as 4 h and remained elevated at 12 and 24 h (data not shown).

One well-known effect of CT is to enhance intracellular cAMP levels (16), and some of the effects of CT have been attributed to this effect. However, DB cAMP and Forskolin, compounds normally used to raise intracellular cAMP levels, inhibited the proliferation of 7TD1 cells in the IL-6 bioassay, making experiments to determine the effect of these agents on IL-6 secretion by IEC-6 cells impossible to perform. Therefore, RT-PCR analysis was used to determine whether DB cAMP had an effect on the expression of IL-6 mRNA. As shown in Fig. 2, culturing IEC-6 cells for 6 h with 1 mM DB cAMP resulted in elevated levels of IL-6 mRNA, suggesting that the effect of CT on IL-6 production may have been due to alterations in intracellular cAMP levels, at least with respect to mRNA expression.

Next, the possibility existed that CT may have induced the IEC-6 cells to secrete some factor other than IL-6 which may have induced proliferation of the 7TD1 cells. Therefore, 7TD1 cells were cultured with a 2-day CT-stimulated (1 μg/ml) IEC-6 cell culture supernatant and increasing concentrations of either a cross-species-reactive rabbit anti-mouse IL-6 antibody (27) or normal rabbit IgG. As shown in Fig. 3,

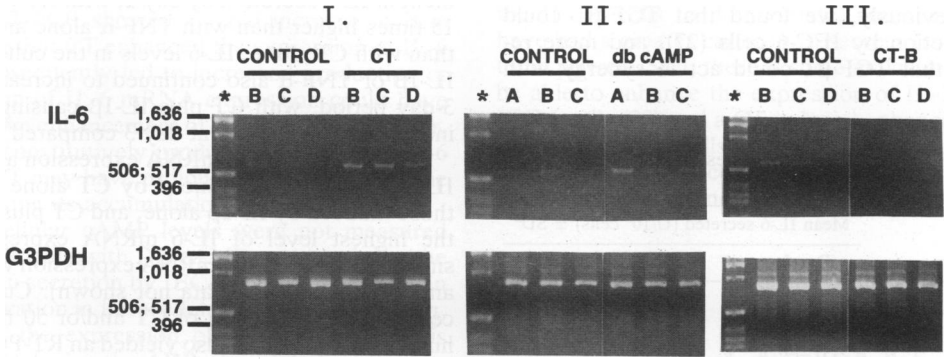

FIG. 2. CT and DB cAMP, but not CT-B, enhance IL-6 mRNA expression by IEC-6 cells. IEC-6 cells were cultured for 1 day before fresh medium containing CT (1 μg/ml), DB cAMP (1,000 μM), or CT-B (1 μg/ml) was added. Shown are the RT-PCR products with primers for IL-6 or G3PDH from either 1.0 μg (A), 0.5 μg (B), 0.25 μg (C), or 0.125 μg (D) of RNA from separate 6-h cultures. Lanes *, 1-kb DNA ladder (GIBCO/BRL). Sizes are shown in base pairs.

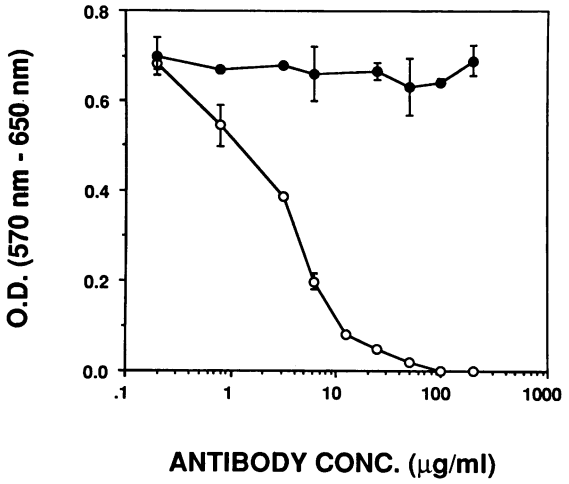

FIG. 3. Rabbit anti-IL-6 antibody completely neutralizes the IL-6 in a CT-stimulated IEC-6 cell culture supernatant. An IEC-6 cell supernatant from a culture stimulated for 2 days with CT (1 μg/ml) was added to 7TD1 cells along with various concentrations of either a rabbit anti-mouse IL-6 antibody (○) or normal rabbit IgG (●). The proliferation of the 7TD1 cells after 4 days is shown as the optical density at 570 nm in a colorimetric proliferation assay (30).

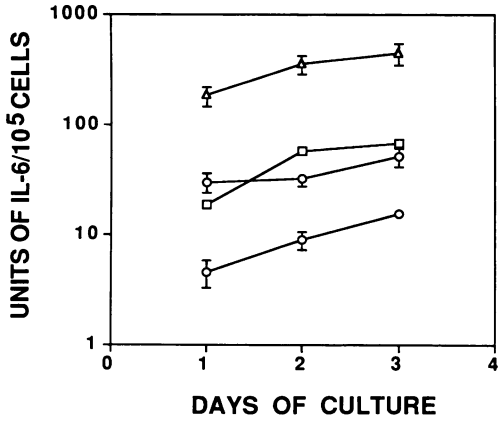

FIG. 4. CT acts in synergy with TGF-β1 to enhance IL-6 secretion by IEC-6 cells. IEC-6 cells were cultured with medium only (●) or medium plus TGF-β1 (2 ng/ml) (□), CT (1 μg/ml) (○), or CT plus TGF-β1 (△). The culture supernatants were collected for determination of IL-6 content. This figure is representative of four experiments.

the presence of 100 μg of the anti-IL-6 antibody per ml completely neutralized the stimulatory capacity of the IEC-6 culture supernatant, confirming that the IEC-6 cells were secreting increased levels of IL-6.

CT-B does not enhance IL-6 secretion. Some of the immunological effects of CT, such as the inhibition of T-cell proliferation (39) and enhancement of IgA and IgG B-cell responses (26), have been attributed to the B subunit of CT. Therefore, the secretion of IL-6 by cultures of IEC-6 cells in the presence of either the holotoxin or purified CT-B was compared. Culturing IEC-6 cells with 1 μg of CT per ml for 1 or 2 days again yielded enhanced levels of IL-6 secretion (Table 1), yet culturing the cells with 1 μg of CT-B per ml yielded IL-6 levels which were not significantly different from those of the unstimulated controls ($P > 0.05$), even though the actual concentration of the CT-B subunit was higher here than in the cultures with CT. Also, an analysis of IL-6 mRNA expression by RT-PCR showed that CT-B did not enhance IL-6 mRNA expression (Fig. 2), confirming that CT-B had no effect on IL-6 secretion by the IEC-6 cells.

CT acts in synergy with inflammatory cytokines to enhance IL-6 secretion. Previously, we found that TGF-β1 could enhance IL-6 secretion by IEC-6 cells (27), and more recently, we found that TGF-β1 could act in synergy with IL-1β to greatly enhance IL-6 secretion (28). Therefore, the effect of adding both CT and TGF-β1 on IL-6 secretion by IEC-6 cells was determined. The addition of both agents to cultures of IEC-6 cells induced a greatly enhanced level of IL-6 secretion which was sixfold higher than in cultures with CT alone at day 1 and as much as eightfold higher at day 3 (Fig. 4). Interestingly, the effect of CT alone plateaued between days 1 and 2, with only slightly elevated levels on day 3. However, the addition of TGF-β1 and TGF-β1 plus CT caused increases in IL-6 secretion, especially between days 1 and 2.

The inflammatory cytokines IL-1 and TNF-α are also known to induce IL-6 secretion by a number of cell types (1, 28). Therefore, we cultured IEC-6 cells with these cytokines in the presence and absence of CT. The doses of IL-1β (28) and TNF-α (unpublished results) were determined previously to yield excellent but suboptimal enhancement of IL-6 secretion by IEC-6 cells. As expected, CT, IL-1β, and TNF-α all induced the IEC-6 cells to secrete enhanced levels of IL-6 ($P < 0.05$; Table 2). However, culturing the IEC-6 cells with both CT and IL-1β resulted in a greatly enhanced level of IL-6 secretion which was more than 100 times higher than the level induced by either IL-1β or CT alone after only 1 day. CT plus TNF-α also caused an impressive enhancement in IL-6 secretion by day 1, with IL-6 levels which were 15 times higher than with TNF-α alone and 49 times higher than with CT alone. IL-6 levels in the cultures with CT plus IL-1β or TNF-α also continued to increase over the entire 3-day period, with CT plus IL-1β causing up to a 200-fold increase in IL-6 levels at day 3 compared with IL-1β alone.

An analysis of IL-6 mRNA expression at 4 h revealed that IL-6 mRNA levels induced by CT alone were higher than those induced by IL-1β alone, and CT plus IL-1β resulted in the highest level of IL-6 mRNA expression (Fig. 5). A similar pattern of IL-6 mRNA expression was also seen at 12 and 24 h of culture (data not shown). Culturing the IEC-6 cells for 6 h with 1 μg of CT and/or 50 ng of recombinant human TNF-α per ml also yielded an RT-PCR pattern similar to that in Fig. 5, suggesting that CT also acted in synergy with TNF-α to enhance IL-6 secretion (data not shown).

Finally, since CT may have acted to increase IL-6 secretion via enhancing intracellular cAMP levels, the role of

TABLE 1. CT but not CT-B enhances IL-6 secretion by IEC-6 cells

Addition to culture (μg/ml)	Mean IL-6 secreted (U/10^5 cells) ± SD[a]	
	Day 1	Day 2
None (control)	7.7 ± 1.2	7.9 ± 0.8
CT (1)	23.7 ± 4.4[b]	32.0 ± 6.1[b]
CT-B (1)	9.4 ± 0.7	12.7 ± 1.6

[a] Values are the mean IL-6 content in the supernatants from three separate cultures for each condition. This experiment is representative of three separate experiments.

[b] Significantly different from control values ($P < 0.05$).

TABLE 2. CT acts in synergy with TNF-α and IL-1β to enhance IL-6 secretion by IEC-6 cells

Addition to culture	Mean IL-6 secreted (U/10^5 cells) ± SD[a]		
	Day 1	Day 2	Day 3
None (control)	3.4 ± 0.5	6.0 ± 1.7	10.3 ± 1.6
CT (1 μg/ml)	20.8 ± 2.5	23.5 ± 7.7	60.8 ± 7.0
TNF-α (50 ng/ml)	68.6 ± 15.1	78.0 ± 4.3	94.1 ± 26.8
CT + TNF-α	1,025.7 ± 58.9	1,817.3 ± 243.7	2,479.0 ± 509.0
IL-1β (1 ng/ml)	55.8 ± 7.2	99.4 ± 12.1	198.3 ± 42.3
CT + IL-1β	6,582.5 ± 802.6	20,079.6 ± 2,553.9	42,093.5 ± 4,672.5

[a] Mean IL-6 content in the supernatants of three separate cultures for each condition. This experiment is representative of three separate experiments.

cAMP in the synergism between CT and IL-1β was investigated. Culturing IEC-6 cells for 3 days in the presence of IL-1β and DB cAMP caused a synergistic enhancement in IL-6 secretion (Fig. 6). With the IL-1β plus DB cAMP cultures, the inhibitory effect of DB cAMP in the supernatants on the proliferation of 7TD1 cells was greatly diminished by the need to dilute the supernatants for the assay. Therefore, we also found that similar cultures with higher doses of DB cAMP (1,000 μM) plus IL-1β yielded even greater levels of IL-6 secretion, 2,000-fold higher than those of cultures containing IL-1β alone (data not shown). These results indicate that the elevated levels of cAMP induced by CT probably played a significant role in the synergistic effect of CT with IL-1β on IL-6 secretion.

DISCUSSION

In this study, we used the IEC-6 cell line as a model to examine the role of IEC in the mucosal immune response to CT. CT was found to rapidly induce IEC-6 cells to secrete significantly elevated levels of IL-6, and this factor was confirmed as IL-6 by complete neutralization of the stimulatory activity in a CT-stimulated IEC-6 cell supernatant by an anti-IL-6 antibody. An analysis of expression levels for IL-6 mRNA by RT-PCR showed a rapid increase in IL-6 mRNA, suggesting that CT enhanced the expression of IL-6 mRNA, which subsequently led to increased production of IL-6. This elevation of IL-6 mRNA expression could have been due to an active enhancement of gene transcription, or, since IEC-6 cells constitutively produce low levels of IL-6 (27) (Fig. 1), the CT may have prolonged the half-life of the IL-6 mRNA, allowing its accumulation.

Although intracellular cAMP levels were not measured directly, the experiments with DB cAMP suggested that the effect of CT on IL-6 secretion by IEC-6 cells may have been the result of an elevation in intracellular cAMP levels which, in turn, enhanced the expression of IL-6 mRNA. CT is known to act by catalyzing the ADP-ribosylation of the $G_{s\alpha}$ protein which then activates adenylate cyclase (8, 14) and the induction of IL-6 gene expression, and IL-6 production has been linked to a cAMP-dependent pathway in fibroblasts (40). In support of this hypothesis, the effect of CT on IL-6 secretion required the intact holotoxin, since CT-B did not enhance IL-6 secretion or IL-6 mRNA expression. This suggests that the enzymatic activity of the A subunit of CT was necessary for the effect and not simply the binding of the B subunits to the cell. Yet a cAMP-mediated pathway may not be the only mechanism by which IL-6 secretion can be enhanced (34).

Finally, the enhancing effect of CT seemed to be short-lived since the rate of IL-6 secretion usually reached a plateau after about 24 to 48 h. To the contrary, IL-6 mRNA levels remained elevated at 24 h, indicating that the IL-6 secretion may have been downregulated by some mechanism other than the downregulation of RNA expression.

Because CT may be acting in an environment where inflammatory or other cytokines may be present, we next looked at the effect of various cytokines in conjunction with CT on IL-6 secretion by IEC-6 cells. First studied was the effect of TGF-β, a multifunctional cytokine produced by many cell types, including B cells (19), T cells (20), and macrophages (3), which is known to have a role in both the inflammatory response and wound healing (38). TGF-β is also produced by IEC (2, 4, 11) and has been suggested to act as an autocrine maturational factor for IEC (21), making it not only present in the vicinity of the IEC but also very important to their function. When IEC-6 cells were cultured with both CT and TGF-β1, these two agents acted synergistically to enhance IL-6 secretion. Since TGF-β has been shown to enhance IL-6 secretion by IEC (27, 28), the TGF-β may have produced a signal to overcome the downregulation of IL-6 secretion noted before at 24 to 48 h in cultures with CT alone, and this plus the elevated IL-6 message levels induced by CT may have resulted in the synergistically enhanced IL-6 secretion seen. Also, since TGF-β may be a maturational factor for IEC, perhaps the TGF-β1 induced the IEC-6 cells to differentiate, and the mature IEC may have had a greater capacity to secrete IL-6, but this has yet to be proven. Still, there is some indication that TGF-β may be able to enhance the expression of IL-1 receptors on the IEC-6 cells (28) and a CT-induced enhancement in secretion of IL-1 by IEC-6 cells, as has been shown with macrophages (6, 23) and IEC (7), could account for an enhanced secretion of IL-6 in this instance.

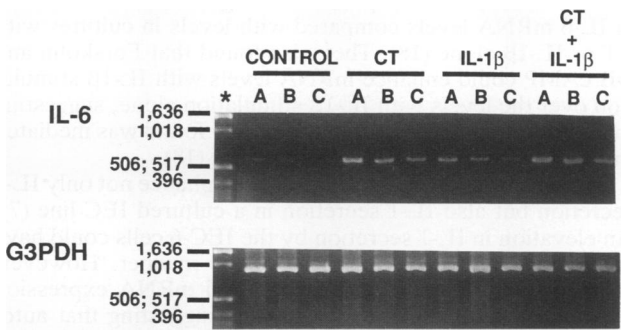

FIG. 5. CT acts in synergy with IL-1β to enhance IL-6 mRNA expression. IEC-6 cells were cultured for 4 h with medium only or with CT (1 μg/ml), IL-1β (1 ng/ml) or both, and samples of 0.5 μg (A), 0.25 μg (B), or 0.125 μg (C) of RNA were analyzed for IL-6 and G3PDH mRNA expression by RT-PCR. Lanes *, 1-kb DNA ladder (GIBCO/BRL). Sizes are shown in base pairs.

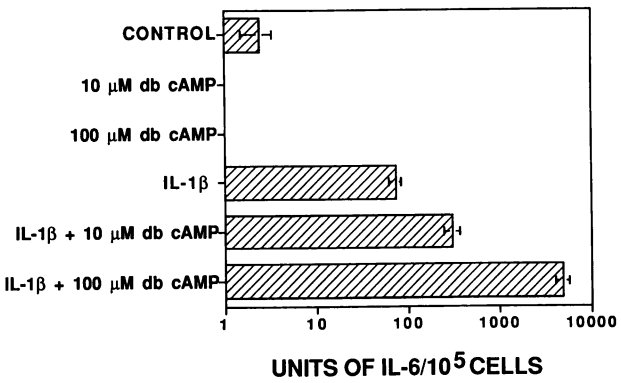

FIG. 6. DB cAMP synergizes with IL-1β to enhance IL-6 secretion by IEC-6 cells. IEC-6 cells were cultured with and without DB cAMP (10 or 100 μM) and IL-1β (1 ng/ml) for 3 days before the IL-6 content in the supernatants was determined.

Culturing IEC-6 cells with CT and IL-1β or TNF-α resulted in an even greater level of synergistic enhancement of IL-6 secretion. Interestingly, when IL-6 mRNA levels were determined by RT-PCR, CT was found to induce higher mRNA levels than IL-1β, yet IL-1β stimulation resulted in greater secretion of IL-6. This suggests that IL-1β enhanced not only IL-6 mRNA expression but also IL-6 protein production, whereas CT greatly enhanced IL-6 mRNA levels without causing a similar increase in protein production. If this is true, then the actions of both agents together could result in enhanced expression of IL-6 mRNA, due mainly to CT but also to IL-1β stimulation, and an enhancement in IL-6 protein production from IL-1β stimulation. Together, these could lead to the massive increase in IL-6 secretion seen. However, further detailed analysis of the regulation and kinetics of IL-6 secretion under these circumstances must be done to confirm this hypothesis. Also, the effect of TNF-α with CT could be due to a similar mechanism.

The experiments with DB cAMP plus IL-1β suggest that the synergism between CT and IL-1β may have been dependent on the ability of CT to elevate intracellular cAMP levels. In a similar study, a synergistic effect of CT plus IL-1β on IL-6 mRNA production has also been shown with a human astrocytoma cell line (18). However, this report presented evidence that CT alone could induce only a small enhancement in mRNA levels for IL-6 and that the combination of IL-1β and CT produced a significant enhancement in IL-6 mRNA levels compared with levels in cultures with CT or IL-1β alone (18). They also found that Forskolin and DB cAMP could enhance mRNA levels with IL-1β stimulation over the levels with IL-1β stimulation alone, suggesting that some of the effect of CT which they found was mediated through its action on adenylate cyclase (18).

In a recent report, CT was shown to enhance not only IL-6 secretion but also IL-1 secretion in a cultured IEC line (7). An elevation in IL-1 secretion by the IEC-6 cells could have affected IL-6 secretion in an autocrine manner. However, the effect of CT on IL-6 secretion and mRNA expression was rapid, occurring within 4 to 6 h, suggesting that autocrine IL-1 production would have played only a very small role in the initial events of the CT effect. Likewise, CT may have affected the production of other cytokines such as TGF-β, by the IEC-6 cells. However, RT-PCR analysis of TGF-β1 mRNA levels after CT stimulation failed to show any effect of CT after 4 and 6 h, and only slightly enhanced levels were seen at 24 h (data not shown). This suggests that CT may not have altered the production of TGF-β early in the response (prior to 6 h) such that it could have affected IL-6 secretion. However, we cannot eliminate the possibility that CT may have enhanced TGF-β production later in the response, either directly or indirectly through the induction of other cytokines, and this enhanced secretion of TGF-β could have affected IL-6 secretion in CT plus IL-1β-stimulated cultures by acting in synergy with IL-1β.

The effect of CT plus IL-1β becomes very relevant when one considers that CT has been shown to induce macrophages to produce IL-1 (6, 23). Therefore, IL-1 could be present in the mucosal tissue during a response to CT and greatly enhanced levels of IL-6 may be produced in vivo by IEC. A large localized infusion of IL-6 into mucosal tissues adjacent to the CT-stimulated IEC could have an enhancing effect on immune responses in that area. Indeed, IL-6 is known to enhance T-cell proliferative responses (1) and immunoglobulin production by B cells and plasma cells (1), especially those of the IgA isotype (5, 13). Therefore, the stimulatory effect of CT on IL-6 secretion by IEC may be an important constituent of the mucosal immune response to CT. It would be interesting to determine whether CT can also enhance IL-6 secretion by the specialized epithelial cells, or follicle-associated epithelium, which cover the Peyer's patches, since an enhancement of IL-6 secretion by these cells could contribute greatly to the immune response generated in the Peyer's patch. Also, the increased levels of IL-6 induced by CT during an infection may have other unknown functions on the epithelial cell itself which could contribute to the defense against cholera.

Aside from its ability to generate a robust mucosal immune response to itself, CT can also enhance the mucosal immune response to an unrelated antigen (12, 22, 24). An enhanced secretion of IL-6 by CT-stimulated IEC could contribute to the adjuvant effect of CT, especially in the presence of the inflammatory cytokines. IL-6 secreted from the IEC would help in the initiation of an immune response not only to CT but also to the unrelated antigen. An increase in IL-6 secretion would even help to enhance secretion of antibodies to both antigens after a boosting immunization. Indeed, one report has shown that the number of anti-CT-producing cells in the lamina propria of the small intestine after enteric priming and challenge was highest at the site of challenge (31). This enhanced number of anti-CT-producing cells was attributed to an antigen-driven proliferation of memory cells, yet it could also have been partially due to increased IL-6 levels driving an increased number of cells to produce antibody. A similar effect could also nonspecifically enhance antibody secretion by B cells specific for an unrelated antigen.

Finally, the enhancing effect of CT on IEC-6 cell IL-6 secretion was limited to the holotoxin since CT-B was ineffective. If the adjuvant effect of CT is due, at least in part, to its ability to induce IEC to secrete enhanced levels of IL-6, then the finding that CT-B alone could not enhance IL-6 secretion would fit well with previous studies which have found that coadministration of CT-B with the antigen did not induce the adjuvant effect (22, 24, 29, 35, 36). However, this does not rule out the possibility that direct conjugation of CT-B to the antigen may work well as an adjuvant (29, 36) since this method may utilize a completely different mechanism of action such as enhancing the presentation of the antigen to the appropriate antigen presenting cell through direct binding mediated by CT-B.

In conclusion, we have presented evidence that IEC may

play an important role in the immune response to CT by producing potentially high levels of IL-6, especially in the presence of the inflammatory cytokines. We have also suggested that this effect of CT on IL-6 secretion by IEC may contribute to the ability of CT to act as an oral adjuvant. We are now initiating studies to determine other effects of CT on IEC-6 cells which may enhance the ability of CT to act as an oral adjuvant.

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