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Anthrax Edema Toxin Differentially Regulates Lipopolysaccharide-Induced Monocyte Production of Tumor Necrosis Factor Alpha and Interleukin-6 by Increasing Intracellular Cyclic AMP

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***Bacillus anthracis* exotoxins mediate most of the symptomatology of severe anthrax. In addition to a clinical syndrome reminiscent of septic shock, which may be mediated by cytokines produced by macrophages stimulated with lethal toxin, infected patients show profound edema at sites of infection. Edema is mediated by edema toxin (ET), which comprises of a binding molecule, protective antigen, and an active moiety, edema factor, which possesses intrinsic adenylyl cyclase activity. Intracellular cyclic AMP (cAMP) regulates the production of several cytokines that modulate edema formation and play important roles in host defense against invading bacteria. To determine whether ET enhanced the accumulation of cAMP in monocytes and thereby influenced cytokine production, we cultured human monocytes with endotoxin (lipopolysaccharide [LPS]) and dilutions of ET and determined the levels of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) in culture supernatant fluids. We further estimated cytokine-specific mRNA accumulation in monocytes by reverse transcription PCR and examined intracellular cAMP concentrations following treatment with ET. ET and LPS each induced monocytes to secrete comparable amounts of IL-6. ET did not inhibit and in most experiments modestly enhanced LPS-induced IL-6 production. In contrast to this stimulatory effect on IL-6 production, ET induced little or no TNF-α production. Moreover, ET profoundly inhibited LPS-induced TNF-α synthesis. These regulatory phenomena were also observed at the mRNA level in association with dose-related enhancement of intracellular cAMP in ET-treated monocytes. Monocytes treated with dibutyryl cAMP, an active analog of cAMP, produced cytokines in a pattern identical to that of cells treated with ET. The disruption of cytokine networks as a consequence of unregulated, ET-induced cAMP accumulation in human monocytes may impair cellular antimicrobial responses and contribute to clinical signs and symptoms.**

Following infection with *Bacillus anthracis*, severe tissue edema occurs at the site of bacterial challenge and in draining lymph nodes. Subcutaneous inoculation of anthrax spores leads to prominent swelling of the skin lesion and surrounding tissues. Inhalation anthrax is associated with pulmonary edema and profound mediastinal widening due in part to edema of the lymph nodes. In addition to these local abnormalities, systemic disease is frequently manifested by fever, hypotension, and diffuse capillary leak reminiscent of endotoxic shock. These pathologic events can largely be recapitulated in animal models by the injection of exotoxins manufactured by anthrax bacilli. The clinical similarities between endotoxic shock and anthrax suggest that cytokines such as interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α), IL-6, and IL-8, characteristically produced by mononuclear phagocytes, may mediate many effects of *B. anthracis* infection. Indeed, a recent report (14) has demonstrated that macrophages play an important role in toxin-mediated lethality.

The anthrax toxin complex is composed of two separate protein toxins, edema toxin (ET) and lethal toxin (LT). These toxins possess a cell receptor-binding B component and an enzymatically active A component responsible for toxicity. Both toxins share the same cell receptor-binding protein, called protective antigen (PA). Thus, ET consists of PA together with edema factor (EF) and LT consists of PA plus lethal factor (LF). PA binds to a high-affinity cell receptor (10, 12) and is then proteolytically cleaved (22), releasing a 20-kDa fragment from the cell surface and exposing a site on the remaining cell-bound 63-kDa protein to which EF or LF can bind. After the binding of EF or LF, the complete toxin is internalized by receptor-mediated endocytosis (11, 13). Individual toxin components are biologically inactive. Earlier studies showed that rodents inoculated intravenously with anthrax toxins died within several hours (3, 9) and primates died within 2 days (42). Necropsied animals showed pulmonary edema, congestion, and pleural effusions, with death primarily attributable to the action of LT (3). In contrast, the administration of ET does not lead to death but induces edema at the site of subcutaneous inoculation (40). Coadministration of ET and LT enhances the lethality of LT (38). The mechanism of this cooperative interaction is not known. Indeed, the physiologic mechanisms by which ET and LT induce their respective symptomatologies have not been clearly delineated. Recently, however, macrophages were implicated as important mediators of anthrax toxicity and were shown to produce IL-1 and TNF-α in response to LT (14). Moreover, the administration of anti-IL-1 or IL-1 receptor antagonist protected mice from lethality after toxin challenge (14).

At the biochemical level, EF has been functionally characterized as an adenylyl cyclase that is activated by cellular

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calmodulin (25, 26, 41). The internalization of ET by target cells leads to rapid increases in cyclic AMP (cAMP) levels in a number of rodent cells and cell lines (25) and in human neutrophils (29). The mode of action of LT is less well characterized. LT kills macrophages and some, but not all, macrophage-like cell lines but does not kill a variety of non-macrophage cells (36). No enzymatic activity has been associated with LF, but a recent report suggests that it may be a metalloprotease (23).

The recent demonstration (14) that LT induces mononuclear phagocytes to produce IL-1 and TNF-α documented a role for cytokine dysregulation in mediating the systemic effects of anthrax infection. The characterization of ET as an adenylyl cyclase further suggests that the local symptoms of anthrax and the enhanced lethality of coadministered LT and ET may also reflect cytokine dysregulation. The capacity of ET to cause edema may be related to its ability to induce cAMP, since the calcitonin gene-related peptide, another agent that augments cAMP, also induces edema (5). Agents that increase intracellular cAMP may increase or decrease production of IL-1, TNF-α, and IL-6 by mononuclear phagocytes treated with lipopolysaccharide (LPS) or may directly induce cytokine synthesis in cells not exposed to LPS (2, 15, 20, 24, 30, 33, 35). A role for cAMP in monokine regulation has been unequivocally determined for TNF-α and IL-6. Augmented cAMP levels induce the production of IL-6 by numerous cell types (19, 27, 37, 45) and inhibit TNF-α production by LPS-treated mononuclear phagocytes (15, 20, 33, 35). The effect of cAMP on these processes is mediated at the level of gene transcription.

To determine whether ET augmented monocyte intracellular cAMP levels and by this mechanism modulated the production of TNF-α and IL-6, we treated monocytes with ET with or without LPS and analyzed cell lysates for levels of cAMP and culture supernatant fluids for cytokine content. We also examined cells for cytokine-specific mRNA. Treatment with ET augmented monocyte intracellular cAMP levels, induced the production of IL-6, and inhibited LPS-induced production of TNF-α. These regulatory effects were associated with corresponding alterations in the accumulation of mRNA. The treatment of monocytes with dibutyryl cAMP (dBcAMP) reproduced the effects of ET. These findings suggest that ET-induced, cAMP-mediated dysregulation of cytokine synthesis may play a role in the promotion of edema formation. Moreover, induction of IL-6 with concomitant impairment in TNF-α response may impair the host's ability to control the invading organism.

MATERIALS AND METHODS

Cells. Mononuclear cells were prepared from leukopacks from healthy donors by centrifugation over lymphocyte separation medium (Organon Teknika, Durham, N.C.). Monocytes were then further purified by counterflow centrifugation-elutriation (43). This procedure resulted in monocyte preparations of >95% viability by trypan blue exclusion and <10% lymphocyte contamination. Monocytes were used immediately or after storage for 16 to 20 h at 4°C. Preliminary experiments indicated that storage at 4°C did not affect responses to LPS.

Culture methods and reagents. EF and PA were prepared as previously described (26). LPS, from *Escherichia coli* O111:B4 (Sigma Chemical Co., St. Louis, Mo.), was used at final concentrations of 1 ng to 1 μg/ml. dBcAMP (Sigma) was used at 100 μM. Monocytes were cultured at 10^6 cells per ml in 10% heat-inactivated (56°C for 30 min) human AB serum (Sigma) in RPMI 1640 medium plus 50 μg of gentamicin (Sigma) per ml at 37°C and 5% CO_2. For the induction of cytokine production, 1 ml of cells was cultured in 24-well tissue culture plates (Costar, Cambridge, Mass.). After incubation at 37°C for 1 h to permit adherence, cells were treated with dilutions of LPS, toxins, or dBcAMP in medium. Controls received medium only. Culture supernatant fluids were harvested 18 to 20 h after the addition of reagents and frozen at −70°C until assayed for cytokine content.

Cytokine assays. IL-6 activity in culture supernatant fluids was determined by the induction of proliferation of B9 cells (a gift of Lucien Aarden) as previously described (17). Dilutions of supernatant fluids or standard recombinant human IL-6 (Boehringer Mannheim, Indianapolis, Ind.) were added to 0.2 ml of B9 cells cultured in 96-well plates at 2×10^3 cells per ml in 10% fetal bovine serum (GIBCO, Grand Island, N.Y.) in RPMI 1640 medium. After 72 h, 0.5 μCi of [^{3}H]thymidine per well was added. Sixteen hours later, cells were harvested onto glass fiber filters and the incorporation of [^{3}H]thymidine was quantitated by liquid scintillation counting. The IL-6 content of supernatant fluids was determined from the standard curve and expressed as laboratory units per ml, where 1 laboratory unit equals half-maximal proliferation of B9 cells. The TNF-α and, where noted, IL-6 contents of supernatant fluids were measured by enzyme-linked immunosorbent assay (ELISA) kits according to the respective manufacturer's instructions (Biokine TNF-α [T cell Sciences, Inc., Cambridge, Mass.] and Quantikine IL-6 [R & D Systems, Minneapolis, Minn.]).

Assay for cAMP. Monocyte cultures were treated with dilutions of toxins for 1 h. Monolayers were then washed twice in Ca- and Mg-free phosphate-buffered saline (PBS) (Sigma), and cAMP was extracted by incubation with 0.1 N HCl for 1 h. Extracts were frozen at −70°C until analyzed by radioimmunoassay (Amersham) performed according to the manufacturer's instructions and expressed as picomoles of cAMP per 10^6 cells.

Analysis of mRNA accumulation. For the induction of mRNA, 30×10^6 cells were cultured at 2×10^6 cells per ml in 75-cm^2 flasks (Costar) for 14 h prior to the addition of stimuli. Four hours after the addition of toxin and/or LPS, flasks were washed with PBS. Cells remaining in the flasks and cells pelleted from the PBS wash were pooled and lysed with 4 M guanidine isothiocyanate (Sigma). Total cellular RNA was extracted by a modification of the method of Chromoczynski and Sacchi (7) with acidic (pH 4.0) phenol-chloroform-isoamyl alcohol (Amresco, Solon, Ohio). RNA in the aqueous phase was precipitated twice with isopropanol, washed in 70% ethanol, dried under vacuum, resuspended in water, and frozen at −70°C until assayed. Cytokine-specific mRNA accumulation was estimated by reverse transcription PCR (Gene-AMP; Perkin-Elmer Cetus, Norwalk, Conn.) with primers for human β-actin, IL-6, and TNF-α (Stratagene, La Jolla, Calif.). A 1/10 dilution of cDNA prepared with oligo(dT) primers was incubated with 1.25 μl of each sense and antisense β-actin primer plus 1.25 μl (each) of either IL-6 primers or TNF-α primers in a total reaction volume of 50 μl. PCR was performed for 30 cycles of 1 min at 94°C, 2 min at 60°C, and 3 min at 70°C followed by 1 cycle of 7 min at 70°C. The reaction mixture was then electrophoresed on a 1.2% agarose (Sigma) gel and stained with ethidium bromide. Reaction products appeared at the expected locations in comparison with a 1-kb DNA ladder standard (GIBCO-BRL). In addition, the DNA sequences of PCR products were determined by a modification of dideoxynucleotide chain termination reaction sequencing (34). PCR products from two to four 50-μl reactions were pooled and concentrated by ultrafiltration spin columns (Ultrafree-MC 30,000; Millipore, Bedford, Mass.). This concentrated

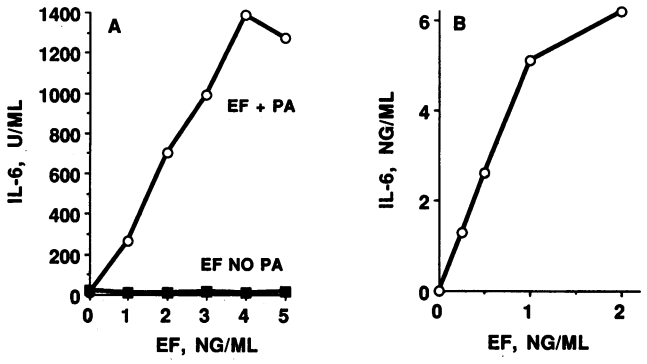

FIG. 1. Induction of IL-6 by ET. Monocytes were treated with dilutions of EF without or with μg of PA per ml. Culture supernatant fluids were collected 20 h after treatment with toxin and assayed for IL-6 content by bioassay (A) or ELISA (B). Data are from two separate experiments.

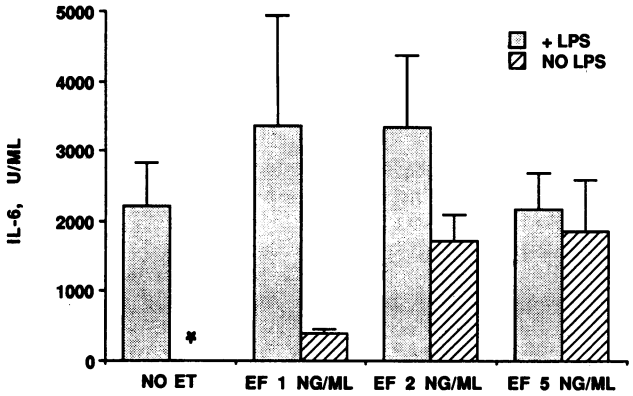

FIG. 2. Production of IL-6 by monocytes treated with ET or with LPS and ET. Monocytes were treated with ET (0 to 5 ng of EF per ml with 1 μg of PA per ml) with or without simultaneous LPS (1 μg/ml). Culture supernatant fluids were collected 18 h after treatment with toxin and bioassayed for IL-6 content. Error bars denote the standard errors of the means of triplicates in bioassay. *, the absence of IL-6 in fluid from cell cultures treated with medium only.

DNA was purified by gel electrophoresis through 1% low-melting-point agarose in 1× TAE (Tris and EDTA) buffer, and DNA was recovered from agarose (Magic PCR; Promega, Madison, Wis.). Fluorescence-based dideoxy sequencing reactions were carried out with Prism cycle sequencing kits (Applied Biosystems, Inc., Foster, Calif.). Cycle sequencing reactions were done with 4 μl of 25-pmol/μl primer–6.5 μl of purified target DNA–9.5 μl of Prism reaction mix. This mixture was thermocyled 25 times at 96°C for 1 min, 50°C for 30 sec, and 60°C for 4 min. Free dye-dideoxynucleotides were removed by centrifugation with Centri-Sep columns (Princeton Separation, Inc., Adelphia, N.J.). Sequence data were collected with a Model 373A DNA sequencer (Applied Biosystems), and data were analyzed with Sequencer 2.1 software (Gene Code Corp., Ann Arbor, Mich.). Direct DNA sequencing confirmed that the PCR products generated by reverse transcription PCR of mRNA from monocytes were human IL-6, TNF-α, and β-actin. The DNA sequences generated were homologous to the regions of IL-6, TNF-α, and β-actin amplified by the PCR primers. Sequencing reactions performed with inappropriate primers did not generate sequence data. To quantitate the intensities of PCR products, gel photographs were scanned into a Macintosh IIci computer, and bands were densitometrically analyzed with NIH Image v1.45 software. The accumulation of cytokine mRNA was expressed as the ratio of the intensity of cytokine bands to that of β-actin bands for the same cDNA sample.

RESULTS

To determine whether ET altered monocyte production of IL-6, adherent cells were cultured for 20 h with dilutions of EF in the presence or absence of 1 μg of PA per ml. Supernatant fluids were then analyzed by bioassay for IL-6 content. Monocytes cultured with EF in combination with PA released substantial quantities of IL-6 (Fig. 1; representative of nine dose-response experiments). IL-6 production was related to EF concentration, with a steep dose-response curve. EF at 60 pg/ml induced little or no IL-6 production; concentrations higher than 10 ng/ml had no additional effect. In other experiments (data not shown), PA concentrations above 250 ng/ml were sufficient to allow maximal effectiveness of EF. The induction of IL-6 production required the simultaneous presence of both toxin components in the cell culture. Monocytes cultured with EF but without PA did not release IL-6 (lower line in Fig. 1A); conversely, PA, in the absence of EF, did not induce IL-6 secretion. These results are consistent with previous studies showing that EF and PA are biologically inactive by themselves and that EF requires PA to bind to and enter cells. The failure of either component alone to induce IL-6 production strongly suggested that potential contaminants (e.g., LPS) in the EF and PA preparations did not explain our findings.

At the concentrations in the bioassay, EF and PA, alone or together, did not affect IL-6-induced replication of B9 cells (data not shown). It was still possible, however, that B9 cells were influenced by an undetected interaction with ET. To further exclude this possibility, supernatant fluids from additional cultures of cells treated with LPS or ET were analyzed for IL-6 by ELISA (Fig. 1B). These data confirmed the results obtained by bioassay, demonstrating a dose-related increase in immunoreactive IL-6 production in response to ET. The amount of IL-6 released after treatment with ET varied from experiment to experiment. In 12 experiments in which monocytes were treated with either LPS or ET, IL-6 production induced by ET was 96 ± 60% (mean ± standard deviation) of that induced by LPS, with a range of 9 to 221%. These data indicate that ET is a strong inducer of IL-6 production, with a maximal stimulating capability similar to that of LPS in some individuals but with a highly variable response of individuals to either stimulus.

Since both ET and LPS induced IL-6 production, we asked whether treatment with ET and LPS simultaneously might have synergistic or antagonistic effects. The treatment of monocytes with dilutions of ET neither enhanced nor inhibited LPS-induced IL-6 production (Fig. 2). In a total of nine experiments, monocytes treated with maximally stimulatory concentrations of ET in combination with 1 μg of LPS per ml released 122% ± 93% of the IL-6 released by cells treated with LPS alone. In only two of nine experiments did ET reduce LPS-induced IL-6 release by more than 15%. IL-6 production was enhanced by more than 15% in four of nine experiments. These data indicate that treatment with ET does not inhibit and may modestly stimulate LPS-induced IL-6 production.

In sharp contrast to its consistent ability to induce substantial IL-6 production by human monocytes, ET induced little or no TNF-α release (Fig. 3, lowest line) (Table 1). In five of nine

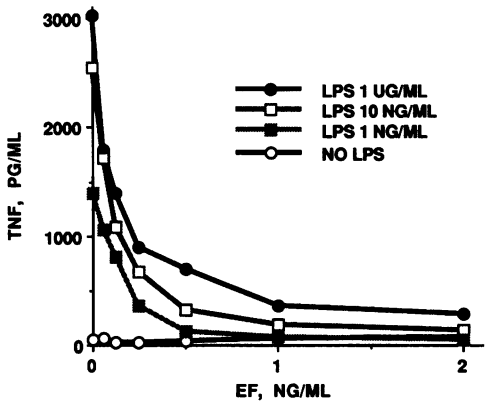

FIG. 3. Inhibition of LPS-induced TNF-α production by treatment with ET. Monocytes were treated simultaneously with ET (0 to 2 ng of EF per ml with 1 μg of PA per ml) and LPS (0 to 1 μg/ml). Culture supernatant fluids were collected 18 h after treatment and assayed for TNF-α content.

separate experiments, ET-induced release (range, 0 to 85 pg/ml) was less than or equal to TNF-α released by cells cultured in medium without ET (range, 0 to 93 pg/ml) and was less than or equal to 3% of maximal LPS-induced release. In the other four experiments, ET-induced TNF-α release ranged from 75 to 1,300 pg/ml, compared with 0 to 17 pg/ml released by cells cultured in medium alone, but never exceeded 10% of the levels of TNF-α induced by LPS. The mechanism of this modest and variable stimulation of TNF-α release by ET is not known. It is unlikely to be due to contamination with LPS, since the addition of PA alone or EF alone did not induce TNF-α production (experiment 2 in Table 1).

The modulation of LPS-induced TNF-α production by ET also sharply contrasted with its effect on LPS-induced IL-6 production. ET had cooperative or indifferent effects on LPS-induced IL-6 release (Fig. 2) but dramatically inhibited LPS-induced TNF-α production (Fig. 3). Inhibition occurred whether cells were stimulated with high or low concentrations of LPS and was maximal at EF concentrations of ≥2 ng/ml. EF alone, without PA, had no effect on spontaneous or LPS-induced TNF-α production (data not shown). These findings were highly reproducible. In a total of nine experiments, ET-mediated inhibition of LPS-induced TNF-α production ranged from 83 to 100%, with a mean ± standard deviation of 93% ± 6%. Given this profound inhibitory activity of ET, we asked whether the toxin acted early after the cytokine-inducing stimulus to inhibit TNF-α production. Cells were cultured with 1 μg of LPS per ml, and ET (5 ng of EF per ml and 1 μg of PA per ml) was added from 0 min up to 4 h after LPS was added. Supernatant fluids were harvested 20 h after the addition of LPS for the determination of TNF-α levels. Maximal ET-mediated inhibition of LPS-induced TNF-α production occurred when ET was added immediately after LPS (83% inhibition; Table 2). If the addition of ET was delayed for 2 h, ET-mediated inhibition decreased to 40%; it further decreased to 32% if the addition of ET was delayed for 4 h. In a second experiment (data not shown), inhibition was 93, 90, and 64% when ET was added 0, 15, and 120 min, respectively, after the addition of LPS.

TABLE 1. Effects of ET and dBcAMP on IL-6 and TNF-α production[a]

Experiment	Cells treated with:	TNF-α (pg/ml)	IL-6 (U/ml)
1	Medium	41 ± 4	0
	PA (1 μg/ml)	34 ± 0	0
	EF (5 ng/ml)	19 ± 1	0
	LPS (10 ng/ml)	3,420 ± 24[b]	2,200 ± 474[b]
	ET (EF [5 ng/ml] + PA [1 μg/ml])	35 ± 1	1,900 ± 659[c]
	dBcAMP (100 μM)	6 ± 0	1,200 ± 168[b]
	LPS + ET	99 ± 1[d]	2,200 ± 131[b]
	LPS + dBcAMP	63 ± 1[d]	2,900 ± 538[b]
2	Medium	9 ± 0	3 ± 0
	PA (1 μg/ml)	1 ± 0	4 ± 1
	EF (2 ng/ml)	2 ± 0	0
	LPS (1 μg/ml)	7,960 ± 183[b]	1,500 ± 172[b]
	ET (EF [2 ng/ml] + PA [1 μg/ml])	395 ± 0[b]	790 ± 45[b]
	dBcAMP (100 μM)	335 ± 7[b]	2,300 ± 387[c]
	LPS + ET	998 ± 12[d]	1,300 ± 458[b]

[a] Data are means ± the standard errors of the means.
[b] $P < 0.01$ versus cells treated with medium alone.
[c] $P < 0.05$ versus cells treated with medium alone.
[d] $P < 0.01$ versus cells treated with LPS alone.

TABLE 2. Time-dependent inhibition of LPS-induced TNF-α production by ET[a]

Cells treated at 0 h with:	Cells treated with ET at (h):	TNF-α (pg/ml)[b]	% Inhibition of LPS-induced TNF-α
Medium	None	7 ± 1	
LPS[c]	None	6,580 ± 338	
LPS	0	1,120 ± 15	83
LPS	2	3,930 ± 9	40
LPS	4	4,500 ± 254	32

[a] ET, 5 ng of EF per ml and 1 μg of PA per ml.
[b] Data are means ± the standard errors of the means.
[c] 1 μg/ml.

These findings were consistent with an effect of ET on early events in the synthesis of TNF-α. EF has adenylyl cyclase activity, and inducers of cAMP are known to induce the production of IL-6 and to inhibit LPS-induced production of TNF-α. To determine whether ET might mediate its regulatory effects via the enhancement of cAMP levels, we measured the levels of intracellular cAMP 1 h after the treatment of monocytes with ET. Parallel cultures were treated with LPS and ET or with ET alone, and supernatants were collected for cytokine determinations 20 h later. As little as 0.5 ng of EF per ml induced a detectable increase in cAMP content (Fig. 4). Treatment with higher concentrations of EF led to further increases in intracellular cAMP levels. This increase in cAMP levels paralleled the induction of IL-6 production and inhibition of LPS-induced TNF-α production. These findings indicate that the inhibition of LPS-induced TNF-α production occurs in association with much lower cAMP levels than does the induction of IL-6 synthesis. One should also note that intracellular cAMP levels continued to increase when the EF concentration was increased from 10 to 20 ng/ml, but the effects of ET on TNF-α and IL-6 production reached a plateau at 2 and 10 ng/ml, respectively. This observation is consistent with unregulated adenylyl cyclase activity by the internalized ET and suggests that at supraphysiologic levels of cAMP, no further effect on cytokine production occurs.

To verify that the magnitude of the regulatory effects we observed by the treatment of cells was consistent with a cAMP effect, monocytes were cultured with ET, 100 μM dBcAMP,

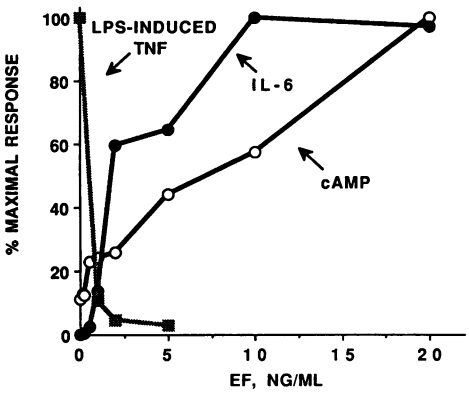

FIG. 4. Levels of cAMP in the cell monolayers and cytokines of supernatant fluids from cultures treated with ET. Monocytes were treated with dilutions of EF in combination with PA (1 μg/ml). After 1 h, the cAMP levels in cell extracts were determined. Parallel cultures were treated with identical concentrations of ET with or without simultaneous addition of LPS. Culture supernatant fluids were collected 20 h after ET treatment and assayed for TNF-α (cultures treated with LPS) by ELISA or for IL-6 (cultures not treated with LPS) by bioassay. Data have been normalized to the maximal response for each parameter measured. 100% cAMP = 6.2 pmol/10^6 cells; 100% LPS-induced TNF-α = 3,415 pg/ml; 100% IL-6 = 2,800 laboratory units per ml.

LPS, LPS with ET, or LPS with dBcAMP. Control cultures received medium, PA, or EF alone. Supernatant fluids were collected 18 h later for analysis of their IL-6 and TNF-α contents (Table 1). The effect of dBcAMP on cytokine production was comparable to that of ET. These data further suggested that ET-mediated regulatory events occurred via the enhancement of cAMP levels.

Previous reports have demonstrated that cAMP-mediated effects on IL-6 and TNF-α production are associated with alterations in the accumulation of cytokine-specific mRNA (2, 45). Our finding that the inhibition of TNF-α production by ET was diminished by about 50% if ET addition was delayed for 2 h was consistent with an effect on early events in the production pathway, such as mRNA synthesis. To examine this possibility, we cultured cells for 24 h to allow the decay of mRNA potentially induced by adherence to plastic surfaces (16) and then treated cultures with ET (5 ng of EF per ml and 1 μg of PA per ml) with or without 1 μg of LPS per ml. RNA was isolated from cells 4 h later and assayed for cytokine-specific mRNA by reverse transcription PCR. The results of these experiments were consistent with the data obtained by analysis of cytokine levels in culture supernatant fluids. Cells cultured in medium alone constitutively expressed little or no IL-6 message (Fig. 5 and 6). Treatment with ET induced or enhanced IL-6-specific mRNA accumulation. The addition of ET to LPS did not inhibit LPS-induced IL-6 message accumulation. For TNF-α, little or no constitutive message accumulation was observed, and in contrast to IL-6 message, none was induced by treatment with ET. Treatment with LPS induced abundant message accumulation. The addition of ET strongly inhibited the effect of LPS treatment (72% reduction in message in both experiments). These experiments indicated that the regulatory effects of ET on TNF-α and IL-6 production are demonstrable at the level of mRNA accumulation and further support the concept that ET-induced regulatory events result from the toxin's adenylyl cyclase activity.

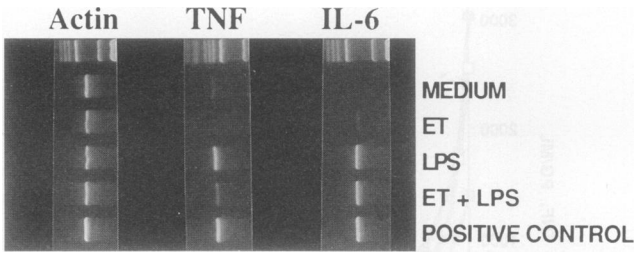

FIG. 5. Accumulation of cytokine mRNA in monocytes treated with ET. Cells were treated with medium, LPS (1 μg/ml), ET (5 ng of EF per ml and 1 μg of PA per ml), or LPS and ET. RNA was harvested 4 h later, and cytokine or β-actin mRNA was determined by reverse transcription PCR. Photographs of ethidium bromide-stained gels of the indicated reaction products were made under UV illumination. The top lane is a 1-kb DNA ladder standard. The positive control was derived from another donor's LPS-stimulated monocytes and was run in parallel to ensure the integrity of the PCR assay.

DISCUSSION

These studies demonstrate for the first time that anthrax ET induces the accumulation of cAMP in human monocytes and that these augmented cAMP levels are associated with profound effects on monocyte production of IL-6 and TNF-α. The effects of ET-induced increases in cAMP levels are qualitatively similar to the effects of increased cAMP levels in human monocytes induced by cAMP analogs or other stimuli (2).

ET is unique among the bacterial toxins that induce cAMP in that the complete toxin reaches the intracellular environment by receptor-mediated endocytosis. Once internalized, the intrinsic adenylyl cyclase activity of EF directly augments intracellular cAMP levels. Another toxin, *Bordetella pertussis* adenylyl cyclase toxin, has intrinsic adenylyl cyclase activity like ET. In contrast to ET, which enters the cell by receptor-

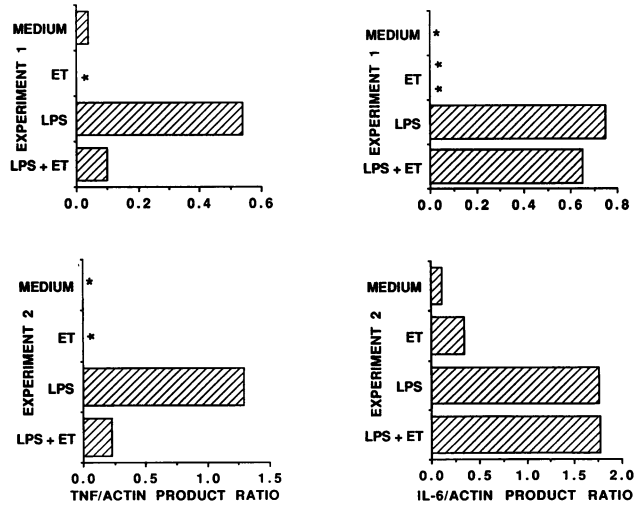

FIG. 6. Quantitation of ET effects on cytokine mRNA accumulation. The intensities of DNA product bands from two experiments were determined densitometrically. The intensity of each cytokine product was normalized to the intensity of the β-actin band derived from parallel amplification of the same cDNA sample with primers specific for β-actin. *, the absence of a visually detectable band. **, the presence of a visually detectable band that was below the threshold for detection by the scanner. Experiment 1 data were derived from the experiment depicted in Fig. 5.

mediated endocytosis, pertussis adenylyl cyclase directly enters the cell by toxin-induced pore formation (13). A number of other bacterial toxins enhance intracellular cAMP levels by directly or indirectly modulating the eucaryotic adenylyl cyclase system. Cholera toxin, also a two-component (A and B subunit) toxin, has no intrinsic adenylyl cyclase activity but induces adenylyl cyclase via activation of a Gs protein (6). In contrast, pertussis toxin (distinct from the *B. pertussis* adenylyl cyclase toxin noted above) ADP-ribosylates the Gα subunit of a Gi protein and thus inactivates it. Adenylyl cyclase activity is enhanced by pertussis toxin only if a positive stimulus for the induction of adenylyl cyclase is present, perhaps through a Gs protein (28). Interestingly, pertussis toxin does not inhibit LPS-induced TNF-α production by mouse peritoneal macrophages, presumably because LPS does not provide a positive signal for cAMP generation (20). ET thus provides a unique tool to investigate the role of cAMP in the regulation of intracellular biochemical events.

One should note that low concentrations (2 to 5 ng/ml) of EF induced maximal production of IL-6 and the inhibition of LPS-induced TNF-α production, even though treatment with higher concentrations of toxin continued to increase intracellular cAMP levels. Intracellular cAMP levels induced by effective concentrations of ET in our study were similar to cyclic nucleotide concentrations associated with regulatory effects on cytokine production in other reports (20, 35) in which cAMP levels were elevated by treatment with intravenous immune globulin, cholera toxin, or prostaglandin E_2. The failure of higher concentrations of cAMP induced either by treatment with higher concentrations of ET or by 100 μM dBcAMP to induce greater cytokine responses is consistent with a physiologic role for cAMP in the regulation of these responses. It is interesting that the effects on the production of TNF-α were reproducibly severalfold more sensitive to low concentrations of ET than were the effects on IL-6 production. This observation illustrates the potential for subtle, differential regulation of cytokine production by small changes in intracellular second-messenger levels well below those that induce a maximal response.

The enhancement of IL-6 mRNA and secretion in human monocytes by elevated cAMP levels is consistent with previous reports. Human fibroblasts (45), astrocytoma cells (19), monocytes (2), and rat pituitary folliculostellate cells (39) and thyrocytes (44) all secrete IL-6 in response to inducers of intracellular cAMP. In those studies in which the question was examined, increased expression of mRNA expression was also found. In all these studies but that of Kasahara et al. (19), the augmentation of cAMP levels without additional stimuli was sufficient to induce IL-6 secretion. We also observed that neither ET nor dBcAMP requires additional cofactors to cause IL-6 protein secretion or mRNA expression. One should note, however, that monocytes sometimes constitutively expressed mRNA and secreted low levels of IL-6 under the culture conditions we used. Since the production of IL-6 is the result of numerous signals that act by a variety of transduction pathways, one cannot clearly distinguish regulation from primary induction of cytokine production. The capability of these diverse signals to converge on IL-6 production is determined, at least in part, by the presence of a regulatory element for the IL-6 gene that responds to a number of cytokines and second-messenger agonists (1, 31, 32). Our demonstration that ET and LPS at optimal concentrations induced equivalent levels of IL-6 and that optimal concentrations of ET did not substantially enhance LPS-induced IL-6 production are consistent with a common pathway of gene activation.

The profound inhibition of LPS-induced TNF-α secretion and message accumulation by ET is consistent with the reported effects of cAMP. Prostaglandin E_2 was shown to inhibit LPS-stimulated TNF-α production (24), presumably via the enhancement of cAMP levels (33). Indeed, cAMP analogs or various agents that induce cAMP have consistently been observed to inhibit LPS-induced TNF-α secretion and mRNA accumulation (2, 20, 35). We noted that monocytes treated with ET in the absence of LPS were induced to secrete TNF-α in certain experiments, but the TNF-α levels were much lower than those induced by treatment with LPS. ET-induced TNF-α production was not due to an effect of either the EF component or PA component alone and was also occasionally observed with dBcAMP. These findings raise the possibility that cAMP may be a weakly positive signal for the induction of TNF-α production. We are presently investigating this observation further.

These in vitro studies raise the questions of whether and how cAMP-mediated cytokine regulation by ET plays a role in the pathophysiology of anthrax. As reflected by its name, ET induces local edema when it is injected into the skin of experimental animals. Moreover, skin lesions and the mediastinum of patients with cutaneous and inhalation anthrax, respectively, are characterized by prominent swelling. cAMP-mediated events may play a role in this process. Subcutaneous injection of IL-1, which is also produced by monocytes treated with ET (18) and RAW cells treated with LT (14), induces local accumulation of polymorphonuclear leukocytes (5). This accumulation of polymorphonuclear leukocytes is associated with increased microvascular permeability and limited extravasation of fluid to the extravascular compartment. In the presence of prostaglandin E_2 or calcitonin gene-related peptide, two activators of adenylyl cyclase, edema is markedly potentiated (5), possibly as a consequence of local vasodilation. Alternatively, however, other effects of cAMP augmentation on monocytes or other cells at the site of IL-1 injection may be important determinants of fluid collection. Our studies were not designed to test whether IL-6 and/or TNF-α plays an important direct role in this process. Since interactions of these and other monokines are exceedingly complex, the disruption of the normal regulatory mechanisms by uncontrolled synthesis of an important second messenger like cAMP in monocytes may well play a role in the development of edema. Similarly, ET's dysregulation of cytokine production may diminish host response to anthrax infection. TNF-α enhances the microbicidal activity of macrophages and helps protect against infection with intracellular organisms (4, 8, 21). The ability of ET to inhibit monocyte TNF-α production induced by LPS suggests that ET could also block induction of TNF-α by undefined components of the anthrax bacillus during infection. Such a block might inhibit macrophage antimicrobial activity and might represent an additional mechanism by which ET acts as a virulence factor.

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