Inhibitors of Receptor-Mediated Endocytosis Block the Entry of *Bacillus anthracis* Adenylate Cyclase Toxin but Not That of *Bordetella pertussis* Adenylate Cyclase Toxin

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Bordetella pertussis and *Bacillus anthracis* produce extracytoplasmic adenylate cyclase toxins (AC toxins) with shared features including activation by calmodulin and the ability to enter target cells and catalyze intracellular cyclic AMP (cAMP) production from host ATP. The two AC toxins were evaluated for sensitivities to a series of inhibitors of known uptake mechanisms. Cytochalasin D, an inhibitor of microfilament function, abrogated the cAMP response to *B. anthracis* AC toxin (93%) but not the cAMP response elicited by *B. pertussis* AC toxin. *B. anthracis*-mediated intoxication of CHO cells was completely inhibited by ammonium chloride (30 mM) and chloroquine (0.1 mM), whereas the cAMP accumulation produced by *B. pertussis* AC toxin remained unchanged. The block of target cell intoxication by cytochalasin D could be bypassed when cells were first treated with anthrax AC toxin and then exposed to an acidic medium. These data indicate that despite enzymatic similarities, these two AC toxins intoxicate target cells by different mechanisms, with anthrax AC toxin entering by means of receptor-mediated endocytosis into acidic compartments and *B. pertussis* AC toxin using a separate, and as yet undefined, mechanism.

Bordetella pertussis is a small, nonmotile, gram-negative coccobacillus which causes whooping cough. *Bacillus anthracis* is a large, disseminating, gram-positive bacillus which causes anthrax. Despite the major phenotypic and taxonomic differences, these two organisms have a novel feature in common: they both produce extracytoplasmic adenylate cyclase toxins (AC toxins) which enter target cells and are activated by host calmodulin to catalyze the formation of cyclic AMP (cAMP) from endogenous ATP.

AC toxin from *B. anthracis* appears in the culture medium during growth as two separate polypeptides: protective antigen (PA) (M_r, 85,000) and edema factor (EF) (M_r, 89,000) (14, 19). Individually, these proteins have no biological effects. When present together, PA functions as the binding subunit which acts to catalyze the entry of EF, the adenylate cyclase enzyme, into the target cell (13). This mechanism is consistent with the A-B model described by Gill (7). A third component from *B. anthracis*, lethal factor (M_r, 83,000) can also enter target cells with PA acting as its binding component (6, 13).

Structure-function relationships for the *B. pertussis* AC toxin are less clear. Column purification of bacterial extract has revealed at least two species, one with both enzymatic activity (in an adenylate cyclase assay) and toxin activity (shown by the ability to increase cAMP levels in target cells) and a second with only enzymatic activity (8, 11). Although these data suggest that the AC toxin from *B. pertussis* also conforms to the A-B subunit model, proof of this will require isolation of individual functional components.

Little is known about the binding and entry of these AC toxins into target cells. In his studies of lethal factor action, Friedlander (6) showed that intoxication of macrophages

could be blocked by chloroquine or ammonium chloride. Confer et al. (2) and Hanski and Farfel (8) stated that the same agents had no effect on pertussis AC toxin but showed no data. In the present experiments, the AC toxins from *B. pertussis* and *B. anthracis* were compared in a sensitive cell line to determine whether they interact with and penetrate target cells by using the same or different entry mechanisms. The results indicate that distinct mechanisms are used.

MATERIALS AND METHODS

Chloroquine, ammonium chloride, and cytochalasin D were obtained from Sigma Chemical Co., St. Louis, Mo., and F12 culture medium was obtained from GIBCO Laboratories, Grand Island, N.Y. Cytochalasin D was prepared as a 1-mg/ml stock solution in dimethyl sulfoxide. Buffer containing equivalent concentrations of solvent was used as a control. F12 was supplemented with either 10 or 1% fetal bovine serum and 1% penicillin-streptomycin (GIBCO Laboratories, Chagrin Falls, Ohio). Dulbecco phosphate-buffered saline was also obtained from GIBCO.

Toxins. *B. pertussis* BP338 was grown in modified Stainer-Scholte medium for 16 to 24 h (10). Growth was monitored by optical density at 650 nm. To prepare AC toxin, the bacteria were harvested at an opacity of approximately 0.7 by centrifugation (9,000 × *g* for 40 min) and the bacterial pellet was extracted with 4 M urea and stirring at 4°C for 18 h. The bacterial pellet was removed by centrifugation (18,000 × *g* for 1 h), and the supernatant was dialyzed against 500 volumes of Tricine (10 mM, pH 8.0; Sigma) with EDTA (0.5 mM) and EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; 0.5 mM] before use. The enzymatic activity of the preparation used in this study was 1,480 nmol of cAMP formed per min per mg. The other major toxin of *B. pertussis*, pertussis toxin, which alters hormone responsiveness of target cells by ADP-
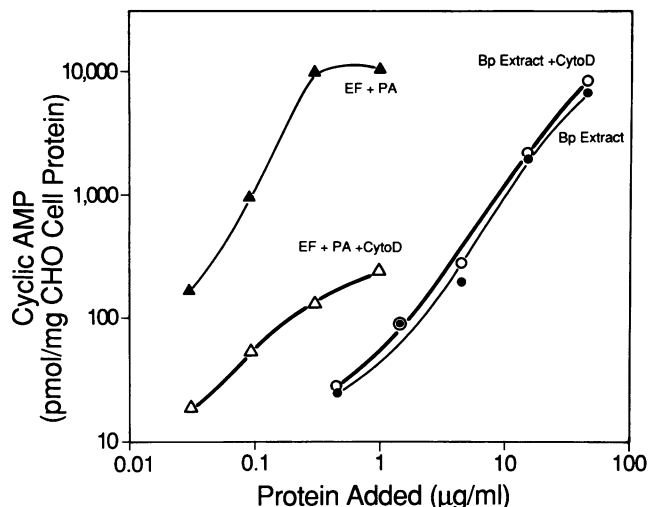
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FIG. 1. Effect of cytochalasin D treatment on cAMP response in CHO cells. CHO cells were treated with 10 μg of cytochalasin D per ml for 45 min at 37°C and then exposed to toxin for 1 h. Symbols: ●, *B. pertussis* (Bp) extract alone; O, *B. pertussis* extract plus cytochalasin D (CytoD); ▲, EF plus PA alone; △, EF plus PA plus cytochalasin D. Toxin and toxin-plus-inhibitor values were analyzed at each toxin concentration by using the paired *t* test, and differences were found to be significant ($P < 0.025$) for anthrax toxin and not significant for *B. pertussis* extract.

ribosylation of G proteins, was also present in the urea extract. Within the time constraints of the assay, pertussis toxin at equal or higher concentrations than were present in this *B. pertussis* extract had no effect on CHO cell cAMP levels or cell morphology (9).

EF and PA were prepared from the Sterne strain of *B. anthracis* by the method of S. H. Leppla (Methods Enzymol., in press).

Cell culture. CHO cells were maintained in culture flasks in F12 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Twenty-four hours before use, the cells were trypsinized, suspended in F12 containing 1% fetal bovine serum and 1% penicillin-streptomycin, and plated into 24-well culture plates (Costar, Cambridge, Mass.) at a density producing a confluent monolayer. Assays were performed directly in the culture wells.

cAMP assay. To assay for toxin-mediated cAMP generation, test materials were added to monolayers containing approximately 10^6 cells. The medium was changed between treatments. The cells were incubated at 37°C in a 5% CO_2 incubator for the designated time. Intracellular cAMP was extracted with 1 ml of 0.1 N HCl at 25°C for 30 min and measured by automated radioimmunoassay (1). Protein was extracted with 0.2 N NaOH and was quantitated by the method of Lowry et al. (15).

Data analysis. The data presented are from representative experiments repeated two to four times. Statistical analysis was performed using the paired Student *t* test, comparing control samples with drug-treated samples.

RESULTS AND DISCUSSION

In these experiments, the AC toxins from *B. pertussis* and *B. anthracis* were compared and contrasted to begin to characterize the mechanisms by which they enter target cells. Since a differential sensitivity of target cells to these

toxins has been described (E. L. Hewlett, H. J. Anderson, G. A. Myers, M. R. Conboy, A. A. Weiss, V. G. Reddy, R. D. Pearson, G. G. Wright, and S. H. Leppla, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, B-9, p. 25), the CHO cell, which is equally sensitive (same maximal cAMP accumulation) to the two toxins, was used. Since differential target cell sensitivities to the two toxins could reflect different routes of entry into the cells, characterization of the intoxication process was undertaken using a series of inhibitors of known uptake mechanisms. CHO cells were treated with cytochalasin D (10 μg/ml), a potent inhibitor of microfilament function. This compound inhibited by 93% the cAMP accumulation produced by EF plus PA but had no effect on intoxication by pertussis AC toxin (Fig. 1). Because cytochalasin D interferes with both phagocytosis and endocytosis (16), agents more specific for the well-studied receptor-mediated endocytosis pathway were used next.

Chloroquine, at a concentration as low as 100 μM, or ammonium chloride (30 mM) inhibited intoxication of CHO cells with EF plus PA by >99% (Fig. 2). Again, the action of pertussis AC toxin was unaffected by these compounds. These data suggest that acidification of endosomes is required for anthrax AC toxin entry but does not play a role in pertussis AC toxin entry.

It should be possible, therefore, to bypass the cytochalasin D-induced block of intoxication by EF plus PA by transiently exposing the cells to an acidic medium after the toxin (EF plus PA) has been allowed to bind. This approach has been used by a number of investigators, including Sandvig and Olsnes (18), Draper and Simon (4), and Didsbury et al. (3), for analysis of diphtheria toxin entry and by Friedlander (6) for intoxication by lethal factor. Acidification of the medium to pH 5.4 and below allowed full intoxication of cytochalasin D-treated cells by anthrax AC toxin (Fig. 3). Acidification of the exterior of target cells after treatment with ammonium chloride or chloroquine also bypassed the block and permitted the intoxication by EF plus PA (data not shown). Conversely, exposure of target cells to medium at pH 5.4 after treatment with pertussis AC toxin did not

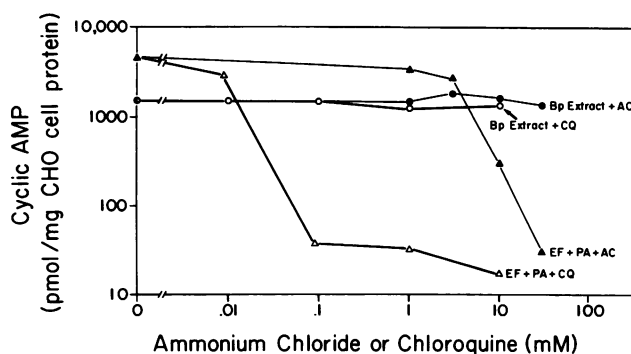Ammonium Chloride or Chloroquine (mM)

FIG. 2. Effect of chloroquine or ammonium chloride on cAMP response in CHO cells. CHO cells were treated with chloroquine or ammonium chloride for 1 h at 37°C and then exposed to toxin. Symbols: O, *B. pertussis* (Bp) extract (23 μg/ml) plus chloroquine (CQ); ●, *B. pertussis* extract (23 μg/ml) plus ammonium chloride (AC); △, EF plus PA (0.3 μg of each per ml) plus chloroquine; ▲, EF plus PA (0.3 μg of each per ml) plus ammonium chloride. Cell viability after drug treatment was confirmed by trypan blue exclusion. Toxin and toxin-plus-inhibitor values were analyzed at each toxin concentration by using the paired *t* test, and differences were found to be significant ($P < 0.025$) for anthrax toxin and not significant for *B. pertussis* extract.
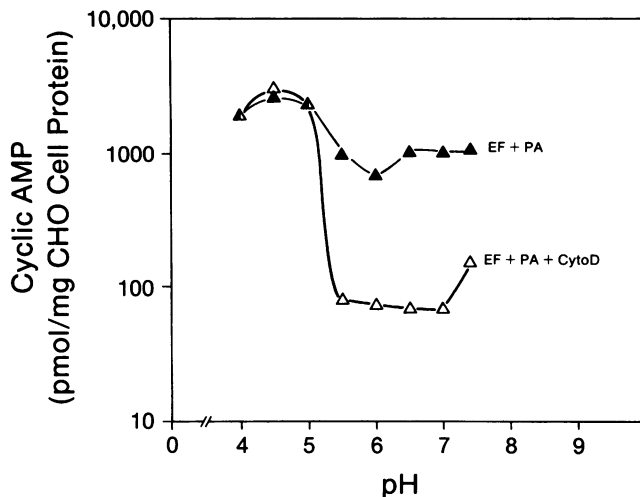
FIG. 3. Effect of pH on cAMP accumulation elicited by EF plus PA in control and cytochalasin D-treated CHO cells. CHO cells were incubated with 10 μg of cytochalasin D per ml for 45 min at 37°C. EF plus PA (1 μg of each per ml) were added for 15 min. The pH was shifted by replacing the medium for 5 min with phosphate-buffered saline adjusted to the indicated pH with H_3PO_4. The phosphate-buffered saline was in turn replaced with F12 (pH 7.4), and the cells were incubated for an additional 40 min at 37°C. Symbols: ▲, EF plus PA alone; △, EF plus PA plus cytochalasin D (CytoD). For toxin alone or for toxin plus inhibitor, cAMP values at pH 7.4 were compared with values obtained at lower pHs, using the paired t test. Differences were found to be significant ($P < 0.025$) at pH values below 5.4.

enhance cAMP accumulation (data not shown), further supporting the conclusion that this toxin enters by a different pathway which is not acidification dependent.

The data presented here clearly demonstrate that agents which interfere with endocytosis (cytochalasin D) or that prevent acidification of endosomes (ammonium chloride or chloroquine) block intoxication of CHO cells by anthrax AC toxin, whereas intoxication by pertussis AC toxin is unaffected. Leppla (13) originally stated that anthrax toxin enters CHO cells with no lag period. The first time point, however, was measured at 15 min, and a shorter lag period may have been missed. Results from recent experiments indicate that between 0 and 10 min of lag time exists for intoxication by EF plus PA (data not shown). Farfel et al. (5) measured the entry of crude pertussis AC toxin into human erythrocytes and found no lag period. Preliminary results with CHO cells and other cell types confirm this observation (data not shown). These findings are consistent with anthrax AC toxin using a vesicle-dependent entry mechanism and the hypothesis that pertussis AC toxin penetrates the plasma membrane directly (5). Thus, it appears that despite the enzymatic similarities of these two toxins, they show differential target cell sensitivities (Hewlett et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1986) and enter target cells by separate mechanisms.

These findings suggest an analogy with other bacterial toxins. Diphtheria toxin and pseudomonas exotoxin A are produced by organisms that are very different taxonomically. These two toxins, which do not cross-react immunologically or share any DNA sequence homology, catalyze an identical enzymatic reaction, ADP-ribosylation of elongation factor-2 (12), yet apparently interact with different receptors

(20) and have different target cell specificities (17). These studies and the data presented here for AC toxins raise intriguing questions about the evolution of bacterial toxins, as well as providing insights into the mechanisms by which they act.

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