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Effects of Anthrax Toxin Components on Human Neutrophils

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The virulence of *Bacillus anthracis* has been attributed to a tripartite toxin composed of three proteins designated protective antigen, lethal factor, and edema factor. The effects of the toxin components on phagocytosis and chemiluminescence of human polymorphonuclear neutrophils were studied in vitro. Initially, it was determined that the avirulent Sterne strain of *B. anthracis* (radiation killed) required opsonization with either serum complement or antibodies against the Sterne cell wall to be phagocytized. Phagocytosis of the opsonized Sterne cells was not affected by the individual anthrax toxin components. However, a combination of protective antigen and edema factor inhibited Sterne cell phagocytosis and blocked both particulate and phorbol myristate acetate-induced polymorphonuclear neutrophil chemiluminescence. These polymorphonuclear neutrophil effects were reversible upon removal of the toxin components. The protective antigen-edema factor combination also increased intracellular cyclic AMP levels. These studies suggest that two of the protein components of anthrax toxin, edema factor and protective antigen, increase host susceptibility to infection by suppressing polymorphonuclear neutrophil function and impairing host resistance.

Keppie et al. (7) found that a crude mixture of three toxin components of *Bacillus anthracis* (protective antigen [PA], edema factor [EF], and lethal factor) decreases host resistance to an in vivo *B. anthracis* challenge and inhibits phagocytosis in vitro. The authors concluded that the toxins are virulence factors in *B. anthracis* pathogenesis. Until recently, there has been little research done to define these toxic effects.

Leppla (9) has recently reported the preparation of highly purified anthrax toxin factors and their effects on cultured eucaryotic cells. The availability of these toxin components presented us with an opportunity to reinvestigate the early work on their effects (7). Leppla (9) found EF to be an inactive adenylate cyclase, probably activated by eucaryotic cell calmodulin. In intact Chinese hamster ovary cells, PA and EF cause a rapid increase of intracellular cyclic AMP (cAMP); PA is apparently required to facilitate EF entry into the cells (9). The adenylate cyclase activity of EF is potentially important to the virulence of *B. anthracis*; it has been reported (5, 6) that an increase in the cAMP concentration in polymorphonuclear neutrophils (PMN) is associated with an inhibition of phagocytosis.

In the present study, the serum opsonic factors required for the phagocytosis of the avirulent Sterne of *B. anthracis* by human PMN were investigated. Also studied were the effects of the toxin components on PMN phagocytosis and oxidative metabolism as measured by chemiluminescence (CL). A combination of PA and EF inhibited phagocytosis, blocked both particulate and soluble receptor-mediated CL, and increased PMN intracellular cAMP levels.

MATERIALS AND METHODS

Leukocyte preparation. All experiments were done with human PMN prepared from blood obtained by venipuncture of healthy volunteers. The cells were isolated by dextran sedimentation and gradient centrifugation as described previously (11), except the final cell stock,which was suspended at 10^6 cells per ml in a modified barbital (Veronal) buffer (10).

Bacteria preparation. *B. anthracis* (Sterne strain) was grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% sodium bicarbonate in sealed screw-top flasks. Cultures were incubated at 37°C for 12 h. The number of CFU was determined via a 10-fold dilution in Hanks balanced salt solution and the growth of a culture on tryptic soy agar at 37°C overnight. The remainder of the original culture was diluted to 5% with glycerol, divided into 15-ml portions, placed on ice, and gamma irradiated (3×10^6 rads). Sterility was confirmed by plating an irradiated sample on blood agar. Sterile cells were stored at −70°C.

Sterne cell wall preparation. Frozen Sterne strain cells were thawed and plated on blood agar medium and inoculated into 1 liter of Casamino Acids medium (Difco Laboratories), supplemented with 0.4% glucose. After incubation at 37°C for 18 h, the culture was transferred to a 20-liter fermentor containing the same medium. After 4 h of incubation at 37°C with agitation at 150 rpm, glucose was added to 0.4%, and incubation was continued for 2 h. Cells were harvested via flowthrough centrifugation at 10,000 × *g*, suspended in water at 0.2 g (wet weight) per ml, and sonicated (5/8-in probe, 85% output) on ice for 45 min (nine 5-min bursts). After centrifugation at 10,000 × *g* for 15 min, the pellet was washed once with 40 ml of water and centrifuged at 27,000 × *g* for 10 min. The pellet was suspended in 1% sodium dodecyl sulfate to 0.25 g (wet weight) per ml and sonicated for 1 min (5/8-in probe, 50% output). The sample was heated to 85°C and centrifuged at 17,000 × *g* for 10 min. The supernatant was discarded, and the 1% sodium dodecyl sulfate treatment was repeated twice. The pellet was washed three times with water (85°C). After centrifugation of each extraction, the dark material at the bottom of the cell pellet was discarded, and only the upper white material was retained. The final cell wall material was lyophilized. This product was determined to be free of detectable protein, lipid, and nucleic acid by high-pressure liquid chromatography.

Sera. Antiserum to Sterne cell wall peptidoglycan material was obtained after weekly intramuscular vaccinations of 100 μg of this material into a female New Zealand white rabbit. Titers were determined after heat treatment at 56°C for 30

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min by microagglutination with Falcon microtiter plates in which diluted antisera (50 μl) were mixed with 50 μl of sonicated Sterne cell wall material in saline (100 μg/ml). Titers determined after overnight incubation at 4°C were considered the last titration which produced agglutination. Samples of the serum were stored at −70°C.

Sera from nonimmunized rabbits were collected and divided. Some sera were stored at −70°C as a source of complement, and others were heat treated at 56°C for 30 min for use as complement-free sera.

Opsonization. Samples of either Sterne cells (3×10^9 CFU per ml) or 1.0 ml of a boiled solution of zymosan (2 mg/ml) (Sigma Chemical Co., St. Louis, Mo.) in saline were added to 2.0 ml of barbital buffer and centrifuged at 400 × *g* for 10 min. The supernatant was discarded, leaving approximately 100 μl of buffer over the pellet. Sera that had been previously designated for use in opsonization were added (50 μl), and the tubes were incubated for 30 min at 37°C. Treatment was ended by adding 2.0 ml of barbital buffer and centrifuging the sample for 10 min at 400 × *g*. The resulting pellet was suspended to 0.5 ml in barbital buffer, except for the zymosan, which was suspended to 5.0 ml.

CL. CL was determined at ambient temperature, under incadescent lighting, in an LS 6800 scintillation counter (Beckman Instruments, Inc.; Irvine, Calif.) by using the single photon monitor accessory. Assays were performed in plastic scintillation vials containing 1.8 ml of barbital buffer and 0.1 ml of 0.25 μM luminol. Luminol was stored as a 25 mM stock in dimethyl sulfoxide and diluted with barbital buffer just before use. PMN suspension (20 μl) was added to each vial, and CL intensity (counts per minute) was determined by 0.1-min measurements at 20-min intervals. After two counting cycles to establish a uniform background luminescence, PMN CL was initiated by adding 0.1 ml of opsonized bacteria or zymosan or 25 μl of phorbol myristate acetate (PMA). The PMA was stored as a 40 μM stock in dimethyl sulfoxide. The final concentration of bacilli varied from 30×10^9 to 1.5×10^9 CFU per ml, zymosan concentration was 20 μg/ml, and PMA was at 5.0 μM. CL, measured at 20-min intervals, was determined over 120 min, and all values were corrected for background CL. When there had been a pretreatment period with the anthrax toxin factors, CL was initiated at the end of that period by the addition of the CL inducers.

cAMP assay. For the assay of cAMP, human PMN from normal donors were collected by dextran sedimentation and Ficoll-Hypaque centrifugation. Residual erythrocytes were lysed in NH_4Cl buffer, and the PMN were suspended in 1 ml of RPMI 1640 medium with 10% serum at 10^7 cells per ml. The PMN were incubated with PA and EF at concentrations of 1 μg/ml each for 2 h at 37°C. After being centrifugated and washed twice with Hanks balanced salt solution, the cells were extracted with 0.2 ml of 0.1 M NH_4Cl, and cAMP was measured by radioimmunoassay after acetylation (2), using an assay kit (New England Nuclear Corp., Boston, Mass.).

Microscopy. PMN were microscopically evaluated to determine whether phagocytosis was occurring. One series of samples was concentrated by gentle centrifugation (50 × *g* for 5 min) and observed under oil immersion phase-contrast microscopy. In other samples, phagocytosis was stopped after 1 h by adding paraformaldehyde to a final concentration of 2%. Cells were pelleted, fixed in Karnovsky fixative (K-1965) for 1 h at room temperature, embedded in agar, and postfixed for 1 h in 1% OsO_4. Pellets were then incubated in 0.5% uranyl acetate, dehydrated through a graded ethanol series, and embedded in Epon 812. Thin sections stained with uranyl acetate and lead citrate were observed with a JEOL JEM 100B electron microscope at 80 kV.

Anthrax toxin components. The anthrax toxin components were prepared as described previously (9). Samples of each factor (1 mg/ml in 10 mM HEPES [*N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 2 mM B-merceptoethanol) were stored at −70°C. Before use, portions were rapidly thawed, diluted in barbital buffer, and supplemented with 0.1% bovine serum albumin. The stock preparations were not refrozen but kept for 3 weeks on ice. After that time, activity decreased and fresh stocks were prepared.

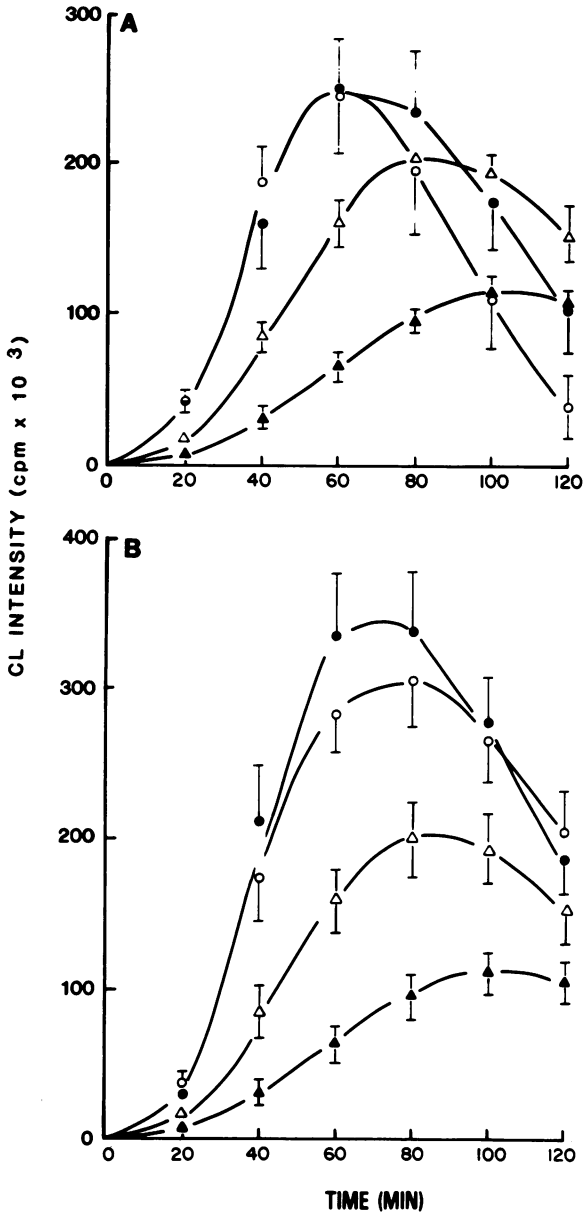

FIG. 1. CL intensity versus time of exposure of human PMN to different concentrations of *B. anthracis* Sterne opsonized with either (A) complement or (B) antibody. The CFU-to-PMN ratio was 30:1 (○), 15:1 (●), 7.5:1 (△), and 1:1 (▲). Each data point is the mean ± standard error of the mean determined from five separate experiments, each of which were performed with PMN from a different individual.

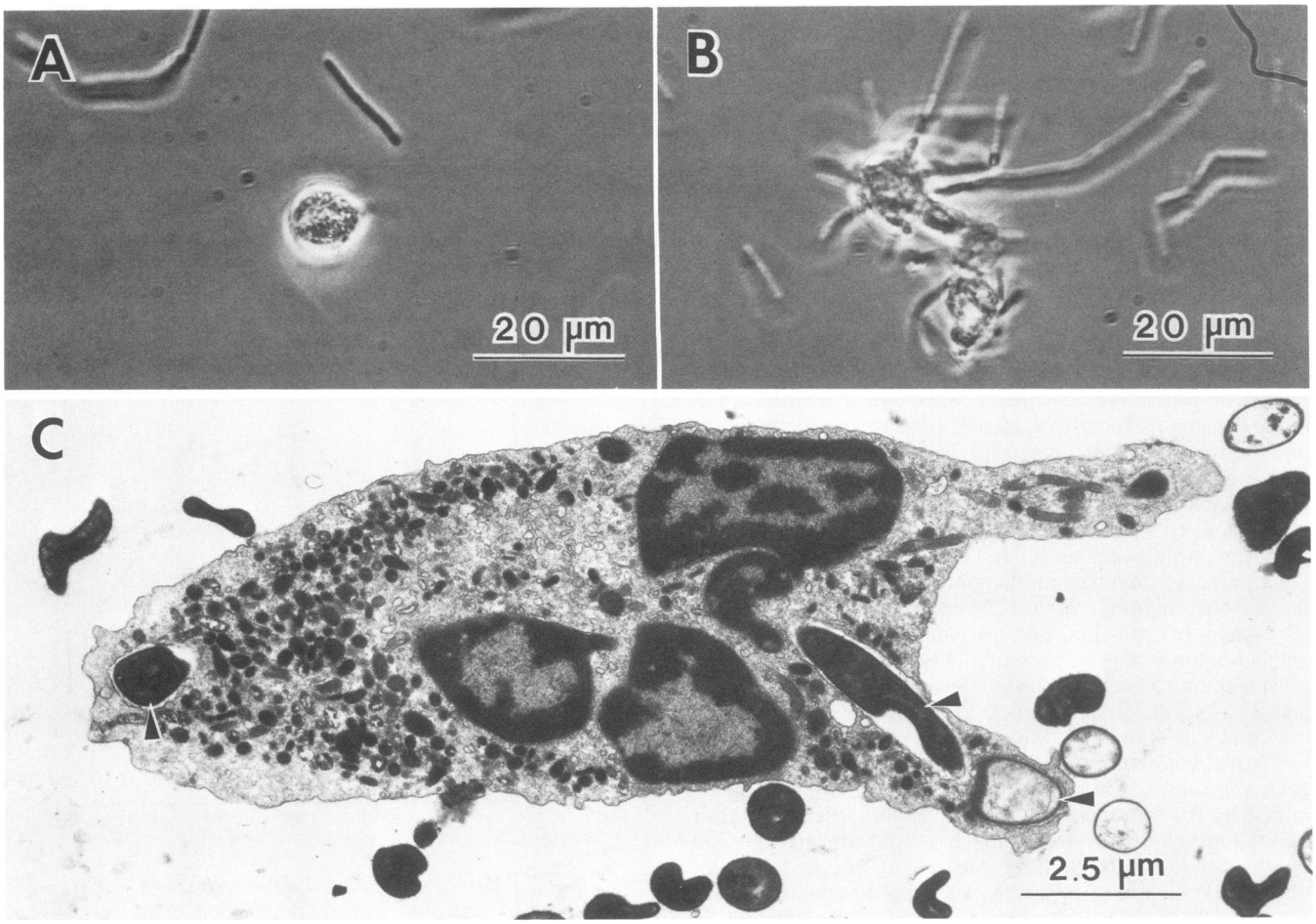

FIG. 2. Phagocytosis of *B. anthracis* Sterne by human PMN. (A) Control PMN, under phase microscopy, exposed to bacteria treated with heat-inactivated nonimmune serum (no opsonins), which showed no stimulation of CL activity. (B) Phase microscopy of PMN exposed to bacteria oposonized with complement at the time of maximum CL induction. (C) Electron micrograph of PMN from the same sample as shown in Fig. 2B. Arrows denote internalized bacilli. The assay conditions were the same as in those described in the legend to Fig. 1. The CFU-to-PMN ratio was 30:1.

RESULTS

Our initial experiments characterized the particulate-induced CL response of human PMN exposed to the avirulent Sterne strain of *B. anthracis*. Sterne cells treated with heat-inactivated nonimmune sera were unable to induce a CL response in human PMN and served as the control for background CL. Bacterial cells treated with either fresh nonimmune sera (complement opsonized) or heat-treated immune sera (antibody opsonized) induced dose- and time-dependent increases in PMN CL (Fig. 1).

The highest CL response represented the maximun response of the PMN, since CL response was not increased by adding fourfold more bacteria or using fourfold more sera (Fig. 1). The importance of establishing these maximum response conditions in studying phagocytosis has been discussed (12). The radiation-killed Sterne preparation, the optimal concentration of which was determined as shown in Fig. 1, was used in all subsequent experiments at a CFU-to-PMN ratio of approximately 30:1. The preparation gave reproducible results for 1 year when stored in portions at −70°C. Other lots of Sterne cells have since been used with results similar to those reported here, although each lot had to be separately characterized for the optimal CFU-to-PMN ratio.

To determine the relationship between induction of CL and phagocytosis, samples were collected directly from the scintillation vials at the height of the CL response. The PMN controls, in which CL was not being induced, were found under phase-contrast microscopy (×1000) to be rounded and unassociated with bacilli (Fig. 2A). However, the PMN exposed to complement-opsonized Sterne cells had an irregular, distorted shape and were associated with multiple bacilli (Fig. 2B). Electron microscopic studies on the same samples revealed internalized bacilli (Fig. 2C), and the controls were negative.

The three anthrax toxin components were tested separately and in combination to determine their effects on the induction of PMN CL with complement-opsonized Sterne cells. When bacteria and toxin component(s) were added simultaneously, a normal CL response, similar to that shown in Fig. 1, was obtained. However, when the opsonized Sterne were added 60 min after the addition of toxin components, a mixture of PA and EF dramatically suppressed CL (Fig. 3). Inhibition was not found with any of the toxin components individually or in other combinations. Although

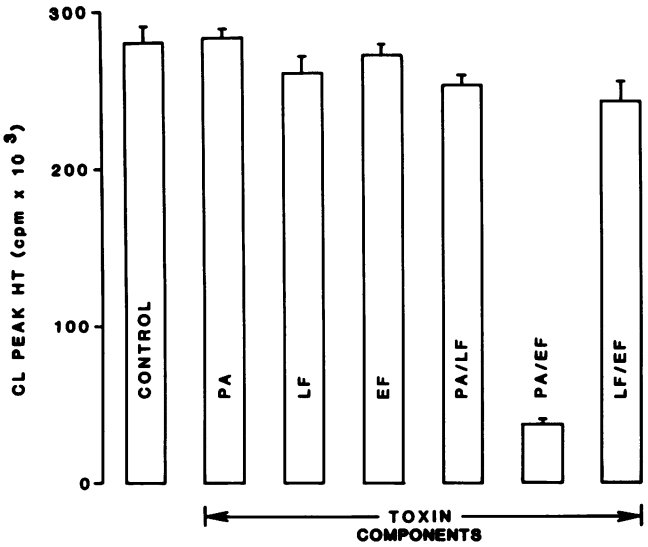

FIG. 3. Effect of anthrax toxin components on CL induced in human PMN with complement-opsonized *B. anthracis* Sterne. The toxin components (1 μg/ml) were introduced to the PMN 1 h before the addition of the bacteria. The CL response is reported as the maximum CL intensity (counts per minute) ± the standard error of the mean on triplicate determinations. Assay conditions were the same as those described in the legend to Fig. 1. The CFU-to-PMN ratio was 30:1.

not shown, lethal factor was without effect on the PA and EF inhibition of CL when all three components were at 1 μg/ml.

The PA-EF effect was greatest after a 60-min pretreatment with the toxin combination. Studies with phase-contrast microscopy showed that the PA-EF-treated PMN, which had shown suppressed CL response to opsonized Sterne bacilli, were spherical and unassociated with bacteria; these findings indicate that phagocytosis was inhibited. In addition, we found that when, after a 60-min exposure of PMN to PA and EF, the cells were suspended for an additional hour in buffer without toxins, a normal CL response was induced by opsonized Sterne cells. Thus the effects of the toxin components were readily reversed.

The effect of the PA-EF combination on the PMN CL response to inducers other than complement-opsonized Sterne cells was determined. Suppression of CL induction was found when PMN were exposed to either Sterne cells opsonized with antibody or to the soluble CL-inducer PMA (Fig. 4). Complement-opsonized zymosan gave similar results (data not shown).

Since EF has been reported to be an adenylate cyclase (9), studies were designed to determine the effect of PA and EF on PMN cAMP concentrations. PA together with EF caused a significant increase in intracellular cAMP in PMN from a control value of 3.7 ± 0.2 to 23.2 ± 0.8 pmol per 10^7 cells. The increase varied from 2- to 13-fold in three separate experiments with three different donors. There was no effect when either factor was added individually.

DISCUSSION

The Sterne strain of *B. anthracis* is used as a live vaccine in domestic animals. Its avirulence has been attributed to the absence of the anti-phagocytic capsule found on fully virulent strains (7, 13). It has been reported that the opsonized virulent Vollum strain of *B. anthracis* will not induce a CL response in human neutrophils, regardless of the opsonins used (11). The results reported here are the first to demonstrate that the opsonized Sterne strain is phagocytized, and that opsonization is readily accomplished with either complement or Sterne strain cell wall antibodies.

The observation of Keppie et al. (7), upon which these studies were based, is that a mixture of the three toxin components of *B. anthracis* inhibited guinea pig PMN phagocytosis in vitro. In the present study, we have further defined phagocytosis inhibition by using highly purified toxin components, and we have found that the antiphagocytic activity is due to a combination of PA and EF and is associated with an increase in cellular cAMP.

The effects of the toxin combination appear to be at the cellular level. Although little is known of the mechanism of phagocytosis induced PMN CL, it is obvious that if phagocytosis is inhibited, there will be no phagocytosis-induced CL response. It is not evident, however, why the receptor-mediated nonphagocytic PMA induction of CL is inhibited by the toxin combinations. Although PMA and the particulate inducers of CL have similarities (4), they involve different mechanisms. These differences are demonstrated by the observation that although cytochalasin B blocks both

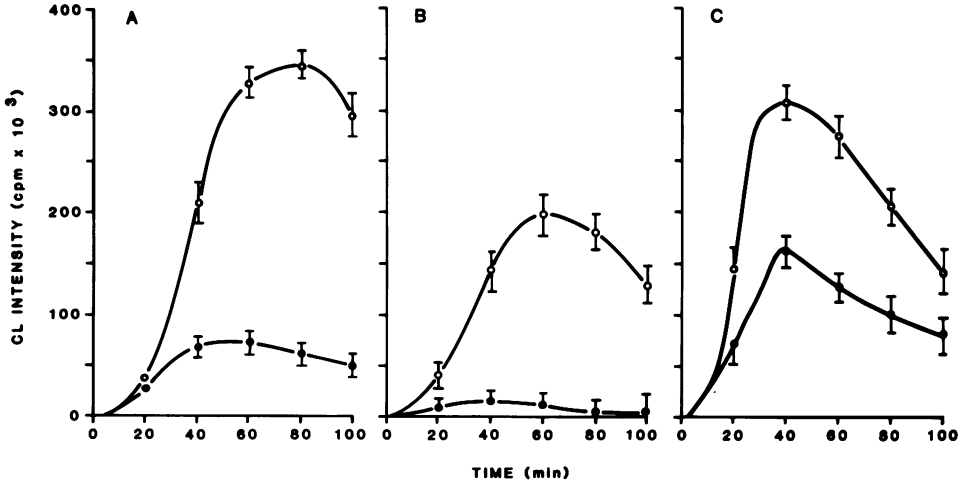

FIG. 4. Effect of PA and EF on the stimulation of human PMN CL with different inducers. PMN were pretreated with the toxin components for 1 h before addition of the CL inducer. Induction of CL was with antibody-opsonized Sterne Cells (A), complement-opsonized Sterne cells (B), and PMA (C). Symbols; ○, samples without toxin; ●, samples with toxin. Data points are means of triplicate determinations ± the standard error of the mean. Assay conditions were as in Fig. 1. The CFU-to-PMN ratio was 30:1.

zymosan-induced phagocytosis and the corresponding CL response, it does not inhibit PMA-induced CL (15). Also, although Fc receptor immune complexes cause increases in neutrophil cytosolic calcium and cAMP (8), neither of these responses were found with PMA. We do not know whether PA and EF inhibit phagocytosis and CL separately or on a common pathway. It is possible that the block of PMA-induced CL is due to inhibition of uptake of PMA since PA and EF inhibit pinocytosis in macrophages (A. Friedlander, unpublished observations).

In a previous study, EF in the presence of PA was shown to act as an adenylate cyclase in Chinese hamster ovary cells (9). It has been proposed that the stimulation of intracellular PMN cAMP leads to an inhibition of phagocytosis (5, 6). An exception is the effect of cholera toxin, which has been shown to augment cAMP levels in PMN yet does not inhibit phagocytosis (1). It has been reported that *Bordetella pertussis* produces an adenylate cyclase that decreases PMN bactericidal activity (3). Here we report that PA and EF increase PMN cAMP, which coincides with phagocytic inhibition. In view of the disparities between the effects of cholera toxin and other augmenters of cAMP, it is clear that the exact relationship between phagocytosis, cAMP levels, and CL remains to be determined. Although fatal anthrax is associated with suppressed host immune defense (7, 13), little is known concerning the in vivo production and involvement of the anthrax toxin proteins (14). This study suggests, however, that EF in the presence of PA may increase host susceptibility to infection by suppressing PMN function.

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