**Bacillus anthracis** Edema Toxin Impairs Neutrophil Actin-Based Motility

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Inhalation anthrax results in high-grade bacteremia and is accompanied by a delay in the rise of the peripheral polymorphonuclear neutrophil (PMN) count and a paucity of PMNs in the infected pleural fluid and mediastinum. Edema toxin (ET) is one of the major Bacillus anthracis virulence factors and consists of the adenylate cyclase edema factor (EF) and protective antigen (PA). Relatively low concentrations of ET (100 to 500 ng/ml of PA and EF) significantly impair human PMN chemokinesis, chemotaxis, and ability to polarize. These changes are accompanied by a reduction in chemoattractant-stimulated PMN actin assembly. ET also causes a significant decrease in Listeria monocytogenes intracellular actin-based motility within HeLa cells. These defects in actin assembly are accompanied by a >5-fold increase in intracellular cyclic AMP and a >4-fold increase in the phosphorylation of protein kinase A. We have previously shown that anthrax lethal toxin (LT) also impairs neutrophil actin-based motility (R. L. During, W. Li, B. Hao, J. M. Koenig, D. S. Stephens, C. P. Quinn, and F. S. Southwick, J. Infect. Dis. 192:837-845, 2005), and we now find that LT combined with ET causes an additive inhibition of PMN chemokinesis, polarization, chemotaxis, and FMLP (N-formyl-met-leu-phe)-induced actin assembly. We conclude that ET alone or combined with LT impairs PMN actin assembly, resulting in paralysis of PMN chemotaxis.

Inhalation anthrax can lead to sepsis and death within days if not diagnosed early and treated effectively (21). Epidemiological analyses of the anthrax bioterrorist attacks in 2001 indicated a mean duration of 4.5 days between exposure and symptom onset in the six inhalation anthrax cases for whom the exposure dates could be determined. Analyses of the clinical findings from 10 of the 11 inhalation anthrax cases revealed normal or minimally elevated peripheral polymorphonuclear neutrophil (PMN) counts at the time of hospital admission, despite high-level Bacillus anthracis bacteremia (22). Furthermore, heavily infected pleural fluid demonstrated a paucity of white blood cells. In the fatal cases, mediastinal infection was associated with marked edema and hemorrhage but minimal infiltration by acute inflammatory cells (16). Similarly, experimental inhalation anthrax in monkeys was associated with edema and hemorrhage of the mediastinum and pulmonary interstitium, with absent or modest infiltration by neutrophils (37). These findings suggest impaired delivery of neutrophils to the sites of infection during the early stages of systemic B. anthracis infection.

*B. anthracis* produces three exotoxins, protective antigen (PA), edema factor (EF) and lethal factor (LF), that account for many of the clinical manifestations of this deadly pathogen. PA binds to the widely distributed host cell receptors and then self-associates into heptamers and ushers LF and EF into the cytoplasm of cells (4). The anthrax toxins have been termed AB toxins, PA combined with LF being called lethal toxin (LT), and PA combined with EF termed edema toxin (ET). LF is a Zn2+-dependent metalloprotease that cleaves mitogen-activated protein kinase kinases (12). EF is a calcium calmodulin-dependent adenylate cyclase, an enzyme that converts ATP to cyclic AMP (cAMP) and pyrophosphate (17) and increases intracellular cAMP levels (26).

Neutrophils constitute the first line of defense against bacterial infections. These phagocytic cells are able to quickly crawl, or chemotax, to the site of infection, and defects in neutrophil chemotaxis compromise the innate immune response. Chemotaxis is accompanied by shape changes that are mediated by rapid assembly and disassembly of actin filaments. We have previously shown that anthrax LT impairs neutrophil chemotaxis and chemokinesis by reducing the formation of actin filaments in response to the chemoattractant N-formyl-met-leu-phe (FMLP) (14). To better understand the full consequences of *B. anthracis* infection on neutrophil motile function, we have now focused on the effects of ET alone and in combination with LT. Although a prior study suggested that ET enhanced chemotaxis (38), we find that ET impairs neutrophil chemotaxis and when combined with LT has additive inhibitory effects. The findings demonstrate that anthrax toxins can induce near-complete paralysis of neutrophil actin-based motility, and these effects may explain the meager neutrophil response that accompanies early stages of systemic anthrax.

**MATERIALS AND METHODS**

**Toxin purification.** EF was expressed and purified from Escherichia coli as previously described (33). PA and LF were purified from Bacillus anthracis as previously described (28).

**PMN isolation and treatment with ET.** Human neutrophils were purified by using a Ficoll-Hypaque gradient as previously described (14). The study followed U.S. Department of Health and Human Services guidelines and was approved by the Institutional Review Board at the University of Florida. Healthy volunteer
donors (total of seven subjects) ranged in age from 24 to 58 years and included both males and females of Caucasian and Asian descent. Purified neutrophils were resuspended in RPMI medium with t-glutamine (Mediatech) and adjusted for \(1 \times 10^6\) cells/ml. Neutrophils were treated with various concentrations of EF plus PA, LF plus PA, or PA plus LF plus EF for 2 h at 37°C while being gently rotated to prevent clumping or cell activation. For experiments with ET and LT, a 1:1 weight ratio of PA to EF was used. We found that a weight ratio for PA to EF of 2:1 had effects identical to those of a 1:1 ratio. For experiments combining all three toxins, the PA concentration was increased to 1 \(\mu\)g/ml to assure sufficient binding sites for both EF and LF. For the majority of experiments, control cells were incubated with buffer alone. In addition, to assure the specificity of our findings, for each experimental condition cells were incubated with EF, LF, and PA alone. Neutrophils were studied immediately after the 2 h of incubation, and experiments were completed within 4 to 5 h of blood drawing.

**Cell culture.** HeLa cells were grown in Dulbecco’s modified Eagle’s medium with 4.5 g/liter glucose supplemented with 10% fetal bovine serum and 5% penicillin and streptomycin (Cellgro.) Cells were grown to 70 to 80% confluence.

**Annexin V staining, analysis for necrosis, and NBT assay.** Annexin V staining was performed on neutrophils by using an annexin V-Fluos staining kit (Roche) combined with propidium iodide to assess necrosis, and 10,000 cells were analyzed by fluorescence-activated cell sorting (FACS) as previously described (13). To further assess necrosis, in separate experiments cells were mixed in a 1:1 ratio with propidium iodide (Sigma) at 37°C for 2 h. Samples were then loaded onto a hemocytometer and allowed to sit for 2 min, and the intracellular content of trypan blue determined by light microscopy. Two hundred cells were analyzed for each condition. The nitroblue tetrazolium (NBT) assay was performed before and after stimulation with a final concentration of 200 ng/ml of phorbol myristate acetate (Sigma), in accordance with the manufacturer’s protocol. One hundred cells were analyzed for each condition.

**Phosphorylation, chemokinesis, chemotaxis, and polarization.** Neutrophil chemokinesis, chemotaxis, and polarization assays were performed as previously described (14). Briefly, untreated and ET-, LT-, and ET-plus-LT-treated PMNs (1 \(\times\) 10^6 cells in 2 ml of RPMI medium) were added to a 35-mm glass-bottom microwell dish (Matek) coated in 0.1% fibronectin (Sigma). For chemokinesis experiments, 1 \(\mu\)M FMLP was added to each plate 5 min prior to time-lapse phase-contrast video imaging. Images were captured at 10 s intervals using an inverted Zeiss microscope and a cooled charge-coupled-device camera (model CSI85; Hamamatsu). The velocity of PMNs was determined by using the MetaMorph software Track Objects application (Universal Imaging). The percentage of polarized PMNs (having a distinct lamellapod and uropod) was assessed 15 min after the addition of FMLP. Chemotaxis toward a gradient was assessed by using a FemtoJet needle (0.5-\(\mu\)m tip; Eppendorf) containing a concentration of 10 \(\mu\)M FMLP. The tip of the needle was placed just inside the visual field, and chemotactic buffer was infused into buffer solution at a pressure of 15 lb/in 2 using an injection micropump (Ismatec). The velocity of movement toward the needle was measured by time-lapse video microscopy at 10 s intervals. In addition to annexin V, in selected experiments, cells were treated with 10 \(\mu\)M of forskolin (Sigma-Aldrich) and 100 \(\mu\)M 3-isobutyl-1-methylnitrate (IBMX; Sigma-Aldrich) for 15 min at 37°C.

**Whole-cell cAMP levels.** cAMP levels in human neutrophils and HeLa cells were determined by using an enzyme-linked immunoassay (Amersham Biosciences) as previously described (6). Neutrophils were cultured in RPMI medium with 10% fetal bovine serum and 5% penicillin and streptomycin (Cellgro.) Cells were grown to 70 to 80% confluence.

**PMN phallolidin and CD11/CD18 staining and FACS analysis.** Phallolidin staining was performed as previously described (14). Briefly, after incubation for 2 h at 37°C with PA plus EF (ET), PA plus LF (LT), or both toxins (PA plus LF plus EF), neutrophils were exposed to 1 \(\mu\)M FLMPL (Sigma-Aldrich) for 0, 5, 10, 15, 30, and 60 s. Cells were fixed at these time points with a final concentration of 1 \(\mu\)M paraformaldehyde, followed by permeabilization with 0.2% Triton X-100 and staining with Alexa 488-phallolidin stain (Invitrogen-Molecular Probes). In separate experiments, PMNs were stained with CD11/CD18 primary antibody (Abcam) at 2.5 \(\mu\)g/ml followed by secondary antibody conjugated with Alexa 488-phallolidin at a 1:100 dilution (Invitrogen). Immediately following staining, cells were subjected to FACS analysis.

**Measurement of phosphorylated PKA.** Protein kinase A (PKA) phosphorylation was determined by using a PFA assay kit (Upstate Cell Signaling Solutions) with \([\gamma-32P]ATP\) (PerkinElmer). Neutrophils were treated with 500 ng/ml of ET and incubated at 37°C for 2 h or treated with the positive control \(\alpha\)-butyryl-cAMP at 100 \(\mu\)M for 1 h (Biolog Life Science Institute.) Cells were lysed by using lysis buffer (1% Triton X-100, 50 mM Tris-Cl, 150 mM KCl, 50 mM EDTA, 0.2% Na2, 200 mM imidazole, 100 mM NaFl, 100 mM Na3VO4) and a complete mini-protease inhibitor cocktail tablet (Roche). As previously described (3), phosphorylation of PKA was determined by using 25 \(\mu\)g of radio-

**RESULTS**

**Anthrax ET is active in human neutrophils.** ET entered both human neutrophils and HeLa cells, as evidenced by a concentration-dependent rise in cAMP levels. Neutrophils and HeLa cells exposed to increasing concentrations of ET (50 to 1,000 ng/ml of a 1:1 weight ratio of EF and PA; Fig. 1A legend) for 2 h demonstrated a progressive rise in intracellular cAMP, reaching a maximum ratio of >50-fold the level in untreated cells (Fig. 1A to D). In neutrophils, this marked increase in cAMP was similar in magnitude to that induced by the combined cAMP agonists forskolin and IBMX (Fig. 1A). In HeLa cells, ET caused a greater rise in cAMP levels than these agonists (Fig. 1C). These effects were shown to be time dependent in neutrophils and HeLa cells, prolonged exposure being associated with a progressive accumulation of cAMP (Fig. 1B and D). Exposure of human neutrophils and HeLa cells to PA, EF, or LF alone had no effect on cAMP levels, the resulting concentrations being identical to those in cells exposed to buffer alone (data not shown).

**Cellular cAMP’s main downstream effector protein is PKA** (20). For assessment of PKA phosphorylation, cell extracts from untreated neutrophils were compared to extracts from cells treated with 6-db-cAMP (positive control) or 500 ng/ml of ET. ET treatment of neutrophils resulted in a fourfold increase in \(\beta^3P\) incorporation into PKA (Fig. 1E). This is the first time that ET-induced \(\beta^3P\) incorporation in PKA has been demonstrated in neutrophils.

**Effects of ET on apoptosis, necrosis, and NBT reduction.** To ensure that ET’s effects on motility were not the result of apoptosis, we compared annexin V staining in ET-treated cells to the staining in those exposed to buffer. Propidium iodide exclusion was also measured to assess necrosis. As observed previously with LT (14), exposure to concentrations of up to 500 ng/ml of ET did not significantly increase neutrophil apoptosis and resulted in only low levels of necrosis compared to exposure to buffer alone (Table 1). Similarly, these concentrations of ET had minimal effects on HeLa cell apoptosis or necrosis (Table 1). To further assess cell viability, the ability of control, ET, and ET-plus-LT-treated cells to exclude trypan blue was assessed. Under control conditions, 99% of PMNs excluded trypan blue. After ET treatment (100 to 500 ng/ml, 1:1 weight ratio), 96 to 97% of cells excluded trypan blue, and following ET-plus-LT treatment (50 ng to 500 ng/ml of ET and LF combined with 1 \(\mu\)g of PA), 93 to 94% of cells excluded the dye. To further assure that PMN functions unrelated to actin-based motility remained intact, we compared the ability of control and ET-treated, as well as ET-plus-LT-treated, PMNs to reduce NBT. The reduction of this dye to a blue precipitate reflects the generation of superoxide. We found comparable
described for panel B. Error bars indicate the SEMs of the results of cAMP levels in HeLa cells. Conditions were identical to those the effects of incubation time on the ET-induced rise in intracellular (500 ng/ml) had no effect on cAMP levels. (D) Bar graph showing experiments. As observed with neutrophils, PA alone and EF alone (final concentrations, 500 ng/ml) failed to alter cAMP levels, the resulting concentrations being identical to those in cells incubated in buffer. PA alone and EF alone (final concentrations, 500 ng/ml) failed to alter cAMP levels, the resulting concentrations being identical to those in cells incubated in buffer. (B) Bar graph showing the effects of incubation time on the ET-induced rise in intracellular cAMP levels in human neutrophils. Cells incubated with ET (PA and EF in a 1:1 weight ratio, e.g., 50 ng/ml ET = 50 ng/ml PA + 50 ng/ml EF) for 2 h at 37°C were compared to cells incubated in buffer. The far right bar shows the results for neutrophils treated with the cAMP agonists forskolin (10 mM) and IBMX (100 mM) for 15 min at 37°C. Error bars indicate the SEMs of the results of three separate experiments. PA alone and ET alone failed to alter cAMP levels, the resulting concentrations being identical to those in cells incubated in buffer. (B) Bar graph showing the effects of incubation time on the concentration dependence of ET-induced intracellular cAMP levels in human neutrophils. As observed with neutrophils, PA alone and EF alone (final concentrations, 500 ng/ml) failed to alter cAMP levels, the resulting concentrations being identical to those in cells incubated in buffer. (C) Bar graph showing the concentration dependence of ET-induced intracellular cAMP levels in human neutrophils. Cells incubated with ET (PA and EF in a 1:1 weight ratio, e.g., 50 ng/ml ET = 50 ng/ml PA + 50 ng/ml EF) for 2 h at 37°C were compared to cells incubated in buffer. The far right bar shows the results for neutrophils treated with the cAMP agonists forskolin (10 mM) and IBMX (100 mM) for 15 min at 37°C. Error bars indicate the SEMs of the results of three experiments. (E) Bar graph showing the effects of incubation time on the ET-induced rise in intracellular cAMP levels in HeLa cells. Experimental conditions were identical to those described for panel A. Error bars indicate the SEMs of the results of three experiments. (D) Bar graph showing the concentration dependence of ET-induced intracellular cAMP levels in HeLa cells. Experimental conditions were identical to those described for panel A. Error bars indicate the SEMs of the results of three experiments. (E) Bar graph showing the effects of incubation time on the ET-induced rise in intracellular cAMP levels in HeLa cells. Experimental conditions were identical to those described for panel A. Error bars indicate the SEMs of the results of three experiments. As observed with neutrophils, PA alone and EF alone (500 ng/ml) had no effect on cAMP levels. (D) Bar graph showing the effects of incubation time on the ET-induced rise in intracellular cAMP levels in HeLa cells. Conditions were identical to those described for panel B. Error bars indicate the SEMs of the results of three experiments. (E) Bar graph showing the effects of ET on PKA phosphorylation (P-PKA). Neutrophils were exposed to buffer, treated with 6-db-cAMP (100 mM) for 1 h, or treated with 500 ng/ml ET for 2.5 h. A 4-fold increase in phosphorylated PKA was observed after ET treatment. Error bars indicate the SEMs of the results of three experiments. Ctl, control (buffer treated); Fsk, forskolin.

TABLE 1. Effects of ET on cell necrosis and apoptosis

<table>
<thead>
<tr>
<th>Cell type and treatment</th>
<th>No. (%) of cells</th>
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<tbody>
<tr>
<td></td>
<td>Viable</td>
</tr>
<tr>
<td>Neutrophils</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8,502 (96.7)</td>
</tr>
<tr>
<td>50 ng/ml ET</td>
<td>8,324 (89.9)</td>
</tr>
<tr>
<td>300 ng/ml ET</td>
<td>8,541 (95.8)</td>
</tr>
<tr>
<td>500 ng/ml ET</td>
<td>8,558 (92.0)</td>
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<tr>
<td>HeLa cells</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8,604 (93.9)</td>
</tr>
<tr>
<td>50 ng/ml ET</td>
<td>7,476 (98.5)</td>
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<td>500 ng/ml ET</td>
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* Cells were treated with ET for 2 h and stained with annexin V and propidium iodide, followed by FACS analysis (see Materials and Methods). HeLa cells were scraped from tissue culture dishes, explaining the relatively high percentage of necrosis in the control HeLa cells.

percentages of NBT-positive cells in phorbol myristate acetate-stimulated PMNs, as follows: control, 91%; ET treated, 89% (500 ng/ml PA plus 500 ng/ml EF); and ET plus LT treated, 91% (1 μg/ml PA plus 500 ng/ml EF plus 500 ng/ml LF).

**PMN chemokinesis and chemotaxis.** We have previously shown that LT exposure significantly impairs neutrophil chemotaxis and chemokinesis (14). However, during systemic anthrax infection, neutrophils are exposed to both LT and ET. Therefore, we have now examined the effects of ET alone, as well as the combination of ET and LT, on human neutrophil motility. The velocity of chemokinesis was significantly reduced by treatment with ET, a reduction in velocity being observed at concentrations of 50 ng/ml, with maximal reduction in velocity of 40% being observed at 300 to 500 ng/ml (Fig. 2A). Dual treatment with ET and LT had additive inhibitory effects on the velocity of chemokinesis, causing a maximal inhibition of 80% at a concentration of 250 ng/ml for both toxins (Fig. 2B). When the concentration of ET combined with LT was raised to 300 ng/ml, neutrophils no longer adhered to the surface. Therefore, for our chemokinesis and chemotaxis experiments combining both toxins, the individual concentrations of EF and LF did not exceed 250 ng/ml. ET and LT alone at concentrations of up to 1,000 ng/ml had no measurable effect on adherence, indicating that impaired adherence required the activities of both toxins (data not shown).

In control neutrophils, exposure to FMLP resulted in a distinctly polarized morphology, a high percentage of cells forming broad lamellipodia at the leading edge and small uropodia at the rear (Fig. 2C and E). Exposure to ET alone markedly impaired the ability of neutrophils to polarize, maximum inhibition being seen at the same concentrations that maximally slowed chemokinesis, 300 to 500 ng/ml (Fig. 2E). Exposure of neutrophils to dual toxin treatment resulted in greater inhibition than for either toxin alone, maximum reductions in the percentage of polarized cells being observed at 250 ng/ml (Fig. 2D and F). ET treatment also reduced the speed of neutrophil-directed migration, or chemotaxis, treatment with 300-ng/ml or-higher concentrations of ET reducing mean chemotactic velocity by 50% (Fig. 2G). Under these same conditions, LT also caused a concentration-dependent reduction in chemotaxis velocity, and a maximum inhibition of nearly 50% was
FIG. 2. Effects of anthrax toxins on neutrophil chemokinesis, polarity, and chemotaxis. (A) Bar graph showing the mean relative velocity of human neutrophil chemokinesis after treatment with buffer, increasing concentrations of ET (PA plus EF, 1:1 weight ratio; see Fig. 1), or forskolin-IBMX (10 nM/100 nM). A final concentration of 1 μM FMLP was added to the solution, and velocities measured between 5 and 15 min after addition. Error bars indicate the SEMs of the results of seven experiments. Incubation with PA, EF, or LF alone (500 ng/ml) resulted in velocities that were identical to those of neutrophils incubated in buffer. Differences were highly significant (P < 0.0001). (B) Bar graph showing the effects of the combination of PA, EF, and LF on neutrophil chemokinesis. Conditions were identical to those described for panel A except that cells were incubated with 1 μg of PA and increasing concentrations of 1:1 weight ratios of EF and LF (e.g., 50 ng = 1 μg PA + 50 ng/ml EF + 50 ng/ml LF). Error bars indicate the SEMs of the results of three experiments. Differences were highly significant (P < 0.0001). (C) Phase-contrast micrograph of control PMNs 5 min after the addition of 1 μM FMLP. PMNs show broad lamellipodia at the head and narrow uropods at the back. The arrows point in the direction of polarity and movement of each cell. Bar = 10 μm. (D) Phase-contrast micrograph of PMNs treated with PA,
observed at concentrations of between 250 and 500 ng/ml (Fig. 2H). Dual toxin treatment also inhibited chemotaxis, a maximal inhibition of 80% being observed at 250 ng/ml of the combination. (Fig. 2I). Thus, as observed for chemokinase and polarity, the combination of LT and ET had additive inhibitory effects on chemotaxis. PA, EF, or LF alone at a concentration of 500 ng/ml had no significant effect on chemokinesis or chemotaxis, the resulting velocities being identical to those of control cells (data not shown).

**CD11/CD18 expression in neutrophils.** Signaling via the adhesion molecules of the β2 integrin family, CD11b/CD18, plays an essential role in PMN recruitment and activation during inflammation. ET-induced reductions in chemotaxis and chemokinesis could in part be mediated by a change in the surface expression of these adherence molecules. Therefore, we examined the effects of treatment with 500 ng/ml ET and forskolin-IBMX on the PMN surface marker expression of CD11b/CD18. No significant differences in surface expression were observed compared to the level of expression in control neutrophils (Fig. 3A). Similarly, LT at concentrations of up to 500 ng/ml had no effect on the surface expression of CD11b/CD18 (Fig. 3A). However, when EF, LF, and PA were combined, a concentration-dependent decrease in receptor surface expression was observed (Fig. 3B). Minimal effects were seen at 100 ng/ml; however, a 50% reduction in expression was observed at 300 ng/ml. PA, LF, and EF alone (500 ng/ml) had no effect on surface expression (data not shown).

**Effects of ET alone and ET combined with LT on neutrophil actin assembly.** Neutrophil chemotaxis requires rapid assembly of actin filaments at the leading edge, and ET-induced impairment of chemotaxis could be mediated by inhibition of neutrophil actin assembly. As assessed by Alexa-phalloidin staining of filamentous actin and by FACS analysis, ET treatment resulted in a delay in FMLP-stimulated actin assembly and a reduction in the maximum actin filament content (Fig. 4A), and the extent of inhibition was comparable to that observed for treatment with 500 ng/ml of LT (Fig. 4A) (14). The combination of PA plus EF plus LF (ET plus LT) resulted in an additive reduction in F-actin content, suggesting that EF and LF impair neutrophil actin assembly by different signaling pathways (Fig. 4A). PA, EF, or LF alone at 500 ng/ml had no significant effect on FMLP-stimulated actin assembly, the resulting F-actin content being nearly identical to that of neutrophils incubated in buffer (data not shown).

To further explore the effects of anthrax toxins on neutrophil actin assembly, we compared fluorescence micrographs of Alexa-phalloidin-stained adherent neutrophils following exposure to 1 μM FMLP. Control neutrophils were polarized and demonstrated a high content of F-actin at the leading edge in lamellipodia (Fig. 4B). LT-treated adherent neutrophils appeared rounded, with considerably reduced F-actin content (Fig. 4C). ET- and ET-plus-LT-treated neutrophils demonstrated a distribution of filamentous actin that was distinctly different from that in control cells or cells treated with LT alone (Fig. 4D and E). Small discrete regions of increased

**FIG. 3. Effects of anthrax toxins on CD11/CD18 surface expression by neutrophils.** (A) Neutrophils were treated with buffer or 500 ng/ml ET or LT (500 ng/ml PA plus 500 ng/ml EF or LF) for 2 h or forskolin (Fsk)-IBMX (10 mM/100 mM) for 15 min, followed by surface staining and FACS analysis of 10,000 cells. In comparison to the level of expression in cells in buffer alone, no significant differences in receptor expression were observed in toxin- or forskolin-IBMX-treated cells. (B) Neutrophils were treated with 1 μg/ml of PA plus increasing concentrations of a 1:1 weight ratio of EF and LF (identical to conditions described for Fig. 2F) for 2 h, followed by staining and sorting as described above. A 50% reduction in the CD11/CD18 surface receptor expression was observed with 300 ng/ml (1 μg/ml PA plus 500 ng/ml EF plus 300 ng/ml LF). Error bars indicate the SEMs of the results of three experiments (P < 0.001). x axis, relative fluorescence intensity.
FIG. 4. Effects of anthrax toxins on FMLP-induced neutrophil actin assembly. (A) Graph showing the effects of ET (500 ng/ml PA plus 500 ng/ml EF; open circles) and LT (500 ng/ml PA plus 500 ng/ml LF; open squares), as well as the combination of ET and LT (1 μg PA plus 500 ng/ml EF plus 500 ng/ml of LF; closed squares), on actin filament content of neutrophils measured as relative fluorescence (see Materials and Methods) compared to that of cells incubated in buffer (Control; closed circles). Cells were treated for 2 h with toxin or buffer and then exposed to 1 μmol/liter of FMLP at time zero. Cells were formalin fixed at the times indicated, permeabilized, and stained with Alexa-phalloidin. The median fluorescence intensity was determined by FACS analysis of 10,000 cells for each time point. ET and LT slowed the onset of actin assembly both alone and in combination, and the combination resulted in an additive reduction in peak F-actin content of 34%, compared to the reduction with ET (15% reduction) and LT (15% reduction) alone (P < 0.001). Error bars show the SEMs of the results of three experiments. (B) Fluorescence micrograph of human neutrophils incubated with buffer for 2 h, allowed to attach to fibronectin-coated glass slides, and then exposed to 1 μM FMLP for 10 min, followed by formalin fixation, permeabilization, and staining with Alexa-phalloidin. Note the high concentrations of filamentous actin at the leading edge of the polarized neutrophils. Bar = 10 μm. (C) Fluorescence micrograph of human neutrophils incubated with 500 ng/ml LT (500 ng/ml PA plus 500 ng/ml LF) for 2 h and then treated as described for panel B. Note the reduction in fluorescence intensity, indicative...
F-actin content were noted throughout the horizontal plane of the cells. By shifting the Z-plane of focus in 0.5-μm steps beginning at the top of the cell, these F-actin structures were found to be in the lowest focal plane, adjacent to the slide surface (Fig. 4F).

**Effects of ET alone and combined with LT on Listeria monocytogenes and Shigella flexneri actin-based motility.** Listeria monocytogenes (7, 36) and Shigella flexneri (2) both hijack the actin-regulating system of host cells to induce the assembly of actin filaments. Both organisms bypass many of the signal transduction mechanisms required for receptor-mediated actin assembly, and we have used these model systems to further assess the effects of ET and the combination of PA plus EF plus LF on in vivo actin assembly. The velocity of bacterial movement directly correlates with the rate of actin assembly and, assuming that the rate of actin disassembly is constant, the length of each actin filament tail also directly correlates with the assembly rate of actin filaments within the tail (30, 35). Therefore, we examined the effects of these toxins on bacterial intracellular velocities and on actin tail lengths. Exposure of HeLa cells to 500 ng/ml of ET resulted in a reduction of nearly 50% in Listeria velocity, and treatment with forskolin-IBMX also reduced Listeria velocity (Fig. 5A). Listeria actin tail lengths were reduced comparably (Fig. 5B to D). The combination of PA plus EF plus LF (ET plus LT) also impaired Listeria actin-based motility, maximum inhibition occurring at a concentration of 50 to 100 ng/ml (Fig. 5E and F). The maximal inhibition of the combined toxins was similar to that of ET or LT alone. PA, EF, or LF alone at 500 ng/ml had no significant effect on Listeria intracellular actin-based motility, the resulting velocities and tail lengths being identical to those in cells treated with buffer alone (data not shown).

Treatment of Shigella flexneri-infected HeLa cells with 500 ng/ml of ET minimally slowed the velocity of intracellular movement (mean control velocity ± standard error of the mean [SEM], 0.125 ± 0.007 μm/s, n = 270, versus 0.109 ± 0.007 μm/s for ET-treated cells, n = 315; P = 0.07). This finding is consistent with previous observations that Shigella and Listeria actin-based motilities utilize different actin-regulating proteins and signal transduction pathways (5).

**DISCUSSION**

Inhalation anthrax is a rapidly progressive disease that is associated with a level of mortality of 85%, death occurring on average 4 to 5 days after the onset of symptoms (19). The fulminating nature of this illness, combined with autopsy findings of hemorrhagic necrosis and a general paucity of acute inflammatory cells, indicates that systemic anthrax is accompanied by profound impairment of the innate immune response (1, 16). The toxins produced by virulent *B. anthracis* are likely to play a critical role in paralyzing the innate immune system. Neutrophils are a primary component of the innate immune response and are the earliest responders to invasion by bacterial pathogens. Investigations of the anthrax toxins’ effects on neutrophil function promise to provide insight into the pathogenesis of systemic, as well as cutaneous, anthrax. Recently, we investigated the effects of LT on neutrophil motile function and discovered that relatively low concentrations of LT (50 to 100 ng/ml) impair neutrophil chemotaxis and chemotractant-induced actin assembly (14). Both LT and ET have been shown to markedly impair the activation of neutrophil NADPH oxidase activity, thus disarming the powerful superoxide bactericidal system (6).

Less is known about the effects of ET on cell motility. More than 2 decades have passed since the effects of ET on human neutrophil chemotaxis were last examined (38). Recently, investigators have been able to express ET in *E. coli* and have shown that this purified recombinant protein has binding affinity and biological activity comparable to those of toxin purified from *B. anthracis* (24, 33). This advance has allowed us to reexamine the biological effects of this calcium-sensitive, calmodulin-dependent adenylate cyclase on neutrophil motility. Unlike the original study that noted a doubling of directed neutrophil migration in response to ET, as well as to the combination of PA, EF, and LF (38), we find that ET treatment results in a concentration-dependent reduction in neutrophil chemotaxis (Fig. 2G). We utilized a different assay for chemotaxis, video microscopy of neutrophils adherent to a fibronectin-coated surface rather than migration through agarose, and our utilization of this assay may account for our contradictory findings. As further support for ET-mediated impairment of chemotaxis, we find that ET treatment also reduces chemokinesis (Fig. 2A) and the ability of neutrophils to polarize in response to the chemotractant FMLP (Fig. 2E).

These findings suggested that ET may globally impair neutrophil actin assembly, and our assessment of filament assembly kinetics using Alexa-phalloidin staining revealed that ET slows both the onset and extent of chemotractant-stimulated neutrophil actin assembly (Fig. 4A). The ET-mediated reduction in actin filament content is accompanied by a distinct change in the actin filament localization. Rather than homogeneously concentrating at the leading edge in lamellipodia as observed in untreated neutrophils exposed to FMLP, actin filaments in ET-treated neutrophils concentrate in discrete small foci dispersed throughout the cell near the adherent membrane surface. This staining pattern is reminiscent of actin filament clusters associated with focal contacts in neutrophils adhered to uncoated plastic surfaces, a condition
shown to stimulate actin assembly in the absence of FMLP (25, 34), and suggests that ET may act through similar signal transduction pathways.

Because systemic anthrax infection would be expected to expose neutrophils to the combination of LF, EF, and PA, we also examined the effects of this combination. We find that inhibition of chemotaxis, chemokinesis, and polarization, as well as FMLP-induced actin assembly, are additive. These findings suggest that LF and EF act by different pathways to impair actin-based motility. In addition, the combination of both toxins can reduce the surface expression of the adherence receptor CD11b/CD18 (Fig. 3), and this effect may contribute to the
poor delivery of neutrophils to the sites of infection. Given the close association between the cytoskeleton and integrins, it is likely that the marked reduction in actin filament assembly induced by dual toxin treatment may contribute to the reduction in CD11b/CD18 surface expression. ET-mediated activation of PKA would be expected to phosphorylate and activate the actin regulatory protein VASP, as well as to activate the G proteins CDC42 and Rac (10), while LT would be expected to block the activation of extracellular signal-regulated kinase, a necessary step in early focal contact formation (15). Our future experiments will focus on assessing the contributions of these pathways to adherence and actin assembly. It is of interest that in HeLa cells, this same combination does not result in an additive reduction in Listeria actin-based motility, each individual toxin, as well as the combination, resulting in a similar level of inhibition (Fig. 5). However, Listeria bypasses many of the signal transduction pathways required for FMLP-induced actin assembly, and therefore, the pathways by which these two toxins inhibit Listeria may differ from those of receptor-induced actin assembly.

What are the mechanisms underlying anthrax toxin-mediated inhibition of host cell actin assembly? We have recently discovered that one of the primary downstream targets for LT is the actin monomer sequestering heat shock protein 27 (Hsp27). The inability of LT-treated cells to phosphorylate Hsp27 prevents the shuttling of actin monomers to the leading edge of motile cells (13). We are presently beginning to explore the pathway or pathways by which ET interferes with actin assembly. As previously reported (6, 38), we find that ET induces a rise in cAMP levels, and under the conditions of our experiments, we observe a >50-fold rise of cAMP in neutrophils. Our findings are consistent with previous observations of neutrophils and T lymphocytes showing that agents inducing a rise in cAMP can impair actin assembly and chemotaxis (11, 18, 29, 31, 39, 40). However, a simple quantitative relationship between cAMP levels and alterations in chemotaxis has not been observed, suggesting that additional signal transduction pathways can modify the effects of cAMP (18, 29, 40).

We document for the first time that the ET-induced rise in cAMP is accompanied by the phosphorylation of PKA in human neutrophils, a condition that would be expected to activate this kinase. Our findings are consistent with previous observations that ET treatment increases cAMP levels and activates PKA in many other cell types (8, 9, 23, 27). Our experiments utilizing the intracellular bacterium Listeria monocytogenes help to narrow the potential downstream targets for ET and activated PKA. Listeria requires the bacterial surface protein ActA, and this protein directly activates the host cell Arp2/3 complex. ActA also attracts the actin-regulating protein VASP. On the other hand, Shigella flexneri requires the bacterial surface protein IcsA. IcsA directly attracts and activates the host cell protein N-WASP, and this protein in turn activates the Arp2/3 complex. Also Shigella, unlike Listeria, does not require VASP for intracellular actin-based motility (5). Finally, Listeria requires phosphatidylinositol 3-kinase activity, while Shigella does not (32). Thus, the ability of ET to impair Listeria but not Shigella actin-based motility points to three potential mechanisms of ET action: (i) impairment of ActA-induced activation of the Arp2/3 complex, (ii) inhibition of VASP binding or activation by ActA, and (iii) inhibition of phosphatidylinositol 3-kinase activity. It is also possible that an additional previously unappreciated difference in the pathways by which Listeria and Shigella utilize the actin-regulatory protein pathways of the host cell accounts for our observations. We are presently exploring all three of these possibilities. Although many of the actin-regulatory proteins and pathways for Listeria-induced actin assembly have proven to be applicable to non-muscle cell actin-based motility, it will be important to relate our specific findings for Listeria to the regulation of actin assembly in neutrophils. Further investigations will also be required to determine if ET specifically affects Listeria and neutrophil function independently of its ability to raise cAMP levels.

Understanding the mechanisms by which anthrax toxins impair actin assembly promises to not only provide a new understanding of anthrax pathogenesis, but also provide new insights into how immune cells regulate actin filament assembly in order to change shape and crawl toward the sites of active infection.

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