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Bacillus anthracis Toxins Inhibit Human Neutrophil NADPH Oxidase Activity[1]

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***Bacillus anthracis*, the causative agent of anthrax, is a Gram-positive, spore-forming bacterium. *B. anthracis* virulence is ascribed mainly to a secreted tripartite AB-type toxin composed of three proteins designated protective Ag (PA), lethal factor, and edema factor. PA assembles with the enzymatic portions of the toxin, the metalloprotease lethal factor, and/or the adenylate cyclase edema factor, to generate lethal toxin (LTx) and edema toxin (ETx), respectively. These toxins enter cells through the interaction of PA with specific cell surface receptors. The anthrax toxins act to suppress innate immune responses and, given the importance of human neutrophils in innate immunity, they are likely relevant targets of the anthrax toxin. We have investigated in detail the effects of *B. anthracis* toxin on superoxide production by primary human neutrophils. Both LTx and ETx exhibit distinct inhibitory effects on fMLP (and C5a) receptor-mediated superoxide production, but have no effect on PMA nonreceptor-dependent superoxide production. These inhibitory effects cannot be accounted for by induction of neutrophil death, or by changes in stimulatory receptor levels. Analysis of NADPH oxidase regulation using whole cell and cell-free systems suggests that the toxins do not exert direct effects on NADPH oxidase components, but rather act via their respective effects, inhibition of MAPK signaling (LTx), and elevation of intracellular cAMP (ETx), to inhibit upstream signaling components mediating NADPH oxidase assembly and/or activation. Our results demonstrate that anthrax toxins effectively suppress human neutrophil-mediated innate immunity by inhibiting their ability to generate superoxide for bacterial killing. *The Journal of Immunology,* 2006, 176: 7557–7565.**

Bacillus anthracis is a Gram-positive bacterium whose endospores enter the body through skin abrasions, inhalation, or ingestion to cause the often fatal disease anthrax. Once infected, the *B. anthracis* spores germinate into vegetative bacilli and start secreting multiple virulence factors, including a toxin and an antiphagocytic poly-D-glutamic capsule (1). The action of the toxin is thought to play a critical role in the pathogenesis of anthrax. Purified toxin preparations cause death in laboratory animals (2), and *B. anthracis* strains missing genes that encode for toxin are less virulent (3).

Anthrax toxin is a classic AB toxin, which is composed of three polypeptides. The B component, protective Ag (PA),[3] binds to two alternative catalytically active A components, lethal factor (LF) and edema factor (EF), to produce lethal toxin (LTx) and edema toxin (ETx), respectively (reviewed in Ref. 1). For the toxins to gain entry into cells, PA first binds to cell surface receptors, two of which have been characterized, tumor endothelial marker 8 and capillary morphogenesis protein 2 (4, 5). Receptor-bound PA is then cleaved by cell surface proteases, leaving a 63-kDa fragment, which assembles into a heptameric prepore complex (6, 7). Up to three molecules of LF and/or EF bind to the prepore complex, and the entire toxin complex undergoes receptor-mediated endocytosis (8, 9). As the complex traffics through the endocytic pathway, it is exposed to increasingly acidic pH, which initiates pore formation by the PA heptamer, ultimately enabling EF and LF to translocate into the cytosol (10, 11).

LF is a zinc-dependent metalloprotease that cleaves and inactivates six of the seven MEKs (12, 13). These enzymes are direct activators of the MAPK pathways, which include ERK, p38, and JNK. MAPKs are key signaling mediators involved in proliferation, differentiation, inflammation, stress response, and cell survival (14). EF is a calcium- and calmodulin-dependent adenylyl cyclase that increases intracellular levels of cAMP (15, 16), subsequently activating protein kinase A (PKA), cyclic nucleotide-gated ion channels, and the Epac (exchange proteins directly activated by cAMP) Rap1 guanine nucleotide exchange factors (17). Increased cAMP has been shown to cause edema, and impair phagocyte microbicidal activity and migration (18–20).

Clinical findings in individuals infected with *B. anthracis* suggest an early inhibition of the innate immune system (21, 22). Considerable research has centered on the effects of LTx upon macrophages, which are clearly critical for the etiology of anthrax; however, there is little information on the effects of the *B. anthracis* toxin on other cells of the innate immune system, particularly in humans. LTx causes death of sensitized murine macrophages (23–25), human peripheral mononuclear leukocytes (26), and endothelial cells (27). LTx has also been shown to modulate cytokine release from macrophages (28–31). In addition, both ETx and LTx have been found to impair the function of dendritic cells (32, 33) and T cells (34, 35). Despite the fact that neutrophils constitute one of the most powerful host defenses against bacteria and are among the first cells recruited to sites of anthrax infection (36, 37), there is only a limited amount of research investigating the effects of anthrax toxins on human neutrophil innate immune responses, and much of this work has reported contradictory results.

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[3] Abbreviations used in this paper: PA, protective Ag; LF, lethal factor; EF, edema factor; LTx, lethal toxin; ETx, edema toxin; PKA, protein kinase A; Epac, exchange proteins directly activated by cAMP; ROS, reactive oxygen species; IBMX, 3-isobutyl-1-methylxanthine; GSP, neutrophil membrane fraction; GSS, neutrophil cytosol fraction; PGB, phosphate glucose BSA.

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Neutrophils are recruited to inflammatory foci through the generation of chemoattractants at these sites. Two early studies reported that both ETx and LTx increased neutrophil chemotaxis toward the peptide chemoattractant fMLP (38, 39). However, it has been recently shown that LTx potently inhibits neutrophil chemotaxis (40). Once recruited to the inflammatory site, neutrophils phagocytically ingest bacteria and induce bacterial killing via the generation of superoxide anion and other reactive oxygen species (ROS), as well as by release of antimicrobial peptides and proteases into the phagocytic vesicle. Neutrophil ROS are generated by the phagocyte NADPH oxidase, and include the primary species, superoxide anion ($O_2^{-\cdot}$), a precursor of hydroxyl radical ($OH^{\cdot}$), H_2O_2, and hyperchlorous acid (reviewed in Ref. 41). The importance of ROS production in host defense is exemplified by the immune disorder chronic granulomatous disease. Chronic granulomatous disease patients are unable to produce ROS and suffer from life-threatening bacterial and fungal infections due to genetic defects in NADPH oxidase components (reviewed in Ref. 42).

The human neutrophil NADPH oxidase is a multicomponent enzyme consisting of membrane-associated cytochrome b_{558}, made up of 91- and 22-kDa subunits, and the cytosolic regulatory components $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and the Rac2 GTPase. These proteins are induced by appropriate neutrophil stimuli to assemble at the phagosomal or plasma membrane to form a functional NADPH oxidase. Much of the biochemistry of the NADPH oxidase has been elucidated using cell-free systems in which native and/or recombinant regulatory components can be manipulated (reviewed in Ref. 43).

Early studies on the ability of *B. anthracis* LTx to induce cell death in host macrophages suggested that LTx itself induced an increase in leukocyte ROS production, leading to cell lysis (30). Data on the effects of the *B. anthracis* toxins on human neutrophil-mediated ROS generation remains limited. ETx, but not LTx, was reported to inhibit opsonin-dependent ROS production; however, this was associated with a reduction in phagocytosis, precluding any conclusions as to whether there were direct NADPH oxidase inhibitory effects (16). ETx was also reported to inhibit PMA-induced ROS formation by neutrophils (16). However, subsequent studies have reported that neither LTx nor ETx have effects on this pathway (Refs. 39, 40, see also this study). Finally, both LTx and ETx were shown to inhibit fMLP-mediated superoxide production in LPS and muramyl dipeptide-primed neutrophils (39). Due to the importance of neutrophil ROS production in human host resistance and bacterial killing, we have investigated in detail the effects of *B. anthracis* toxins on ROS production by primary human neutrophils. We report in this study that both LTx and ETx exhibit distinct inhibitory effects on fMLP (and C5a) receptor-mediated ROS production, but have no effect on nonreceptor-dependent PMA-mediated ROS production. Our results indicate that anthrax toxins effectively suppress neutrophil-mediated innate immunity by inhibiting their ability to generate ROS for bacterial killing.

Materials and Methods

Isolation of human neutrophils

This study received ethical approval from The Scripps Research Institutional (TSRI) Review Board. Human blood was collected from the TSRI Normal Blood Donor Service donor pool by venipuncture and anticoagulated with acid citrate dextrose. Neutrophils were isolated using dextran sedimentation and Ficoll gradient centrifugation as described previously (44) with minor changes. Briefly, RBC were lysed in lysis buffer containing 0.15 M ammonium chloride (NH_4Cl), 12 mM sodium bicarbonate ($NaHCO_3$), and 100 μM EDTA (Na_2EDTA) for 5 min on ice. Cells were also treated with 2.7 mM diisopropyl fluorophosphate (Sigma-Aldrich) for 10 min on ice. Following isolation, neutrophils were resuspended at 2×10^7cells/ml in PBS containing 1 mM $CaCl_2$, 20 mM glucose, and 0.25% BSA (phosphate glucose BSA, PGB buffer). Neutrophil preparations were routinely >95% pure and >99% viable. Experimental data was obtained from separate donors.

Analysis of MAPK kinase and NADPH oxidase proteolysis by LTx

Neutrophils were either left untreated or treated with PA (1 μg/ml, final) and/or LF (0.3 μg/ml, final) at 37°C with continuous rotation for the indicated periods of time. Both toxins were provided by S. Leppla (National Institutes of Health, Bethesda, MD). After incubation, treated samples were spun down and resuspended in 100 μl of PGB buffer. Cells were then lysed with 100 μl of lysis buffer (50 mM Tris, 200 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1% Nonidet P-40, and 10% glycerol with 1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin added fresh). Total protein concentrations of the lysates were determined with a bicinchonic acid protein assay (Pierce). Lysates were diluted 1/1 in 4× SDS sample buffer, boiled for 5 min, then equal amounts of total protein were separated by 12% SDS-PAGE and electrotransferred onto polyvinylidene fluoride membrane (Millipore). MEK2 Western blots were washed in blocking buffer (10% 10 mM HEPES (pH 7.4), 500 mM NaCl, and 3% BSA) overnight at 4°C. MEK2 was detected with an anti-N-terminal polyclonal Ab (anti-MEK2 N20, 1/1000 dilution; Santa Cruz Biotechnology). MEK3 Western blots were washed in TBS with 5% BSA overnight at 4°C. MEK3 was detected with an anti-C-terminal polyclonal Ab (anti-MEK3 C19, 1/1,000 dilution; Santa Cruz Biotechnology). $p67^{phox}$ was detected using an anti-$p67^{phox}$ Ab 3958, 1/10,000 dilution, provided by B. Babior (TSRI). $p47^{phox}$ was detected using an anti-$p47^{phox}$ Ab 039, 1/5,000 dilution, in house; Rac2 was detected using an anti-Rac2 Ab R785, 1/2,000 dilution, in house; $gp91^{phox}$ was detected using an anti-$gp91^{phox}$ Ab 54.1, 1/500 dilution, provided by M. Quinn (Montana State University, Bozeman, MT).; $p22^{phox}$ was detected using an anti-$p22^{phox}$ Ab 44.1, 1/1,000 dilution, provided by M. Quinn. Blots were developed using SuperSignal West Pico chemiluminescent substrate according to the manufacturer's instructions (Pierce).

Measurement of whole cell cAMP and superoxide production

cAMP levels in human neutrophils were determined using the cAMP Biotrak Enzymeimmunoassay System (Amersham Biosciences). Human neutrophils were suspended at 6.25×10^6 cells/ml in DMEM and were either left untreated or were treated with the indicated amounts of toxins for 3 h at 37°C. Positive controls were treated with forskolin (10 μM, final) plus 3-isobutyl-1-methylxanthine (IBMX) (100 μM, final) for 15 min at 37°C. Neutrophils (1×10^6) from each group were assayed in duplicate on 96-well tissue culture plates and lysed using the supplied lysis buffer. Supernatants were then collected and cAMP concentrations determined according to the manufacturer's instructions.

During the review of this manuscript, we were made aware by a reviewer that the EF used throughout these studies, obtained from List Biological Laboratories, contains an amino acid mutation that reduces its enzymatic activity. We had originally determined that we were using the List Biological Laboratories' EF at concentrations that we observed to effectively elevate neutrophil cAMP in our experiments. Native wild-type EF (provided by Wei-Jen Tang, University of Chicago, IL) gave similar maximum levels of neutrophil cAMP, but was active at ~10-fold lower concentrations. We have subsequently verified our experimental results using maximally effective concentrations of recombinant wild-type EF.

Superoxide production in human neutrophils was measured using the cytochrome *c* reduction assay (45). Neutrophils with or without preincubation at 37°C with continuous rotation with PA (1 μg/ml), LF (0.3 μg/ml), and/or EF (0.3 μg/ml; List Biological Laboratories) were aliquoted into wells of a 96-well plate, 2×10^6 cells total. Cytochrome *c* (1 mg/ml, final; Sigma-Aldrich) was added, and the reaction was brought to a final volume of 200 μl with PGB buffer without BSA. The reactions were incubated at 37°C for 10 min followed by stimulation with fMLP (2 μM, final; Sigma-Aldrich), C5a (50 nM, final; Sigma-Aldrich), or PMA (2 μg/ml, final; Sigma-Aldrich); control wells contained superoxide dismutase (0.1 mg/ml, final; Sigma-Aldrich). Upon stimulation, the rate and extent of cytochrome *c* reduction by superoxide was monitored for 8–15 min at 550 nm using a Versamax microplate reader (Molecular Devices). All experiments were performed at least in duplicate, and initial rates were used to calculate V_{max} using Softmax Pro software (Molecular Devices).

Measurement of superoxide production in experiments with cAMP-elevating agents, cAMP analogs, PKA/Epac1-specific activators, and MAPK inhibitors were preformed as described above with or without preincubation with forskolin (10 μM, final; Sigma-Aldrich); IBMX (100 μM, final; Sigma-Aldrich); PGE_1 (10 μM, final; Sigma-Aldrich); PGE_2 (10 μM, final; Sigma-Aldrich); the cAMP analogs adenosine 3′,5′-cyclic

monophosphate,N^6,$O^{2'}$-dibutyryl-sodium salt (db-cAMP) (2 mM, final; Calbiochem) and adenosine 3′,5′-cyclic monophosphate,8-bromo-sodium salt (br8-cAMP) (2 mM, final; Calbiochem); the Epac1-specific activator 8-(4-chlorophenylthio)-2′-*O*-methyladenosine-3′,5′-cyclic monophosphate (cPT-cAMP) (2 mM, final; Biolog); the PKA-specific activator N6-benzoyladenosine-3′,5′-cyclic monophosphate (BNZ-cAMP) (2 mM, final; Biolog); the p38 inhibitor SB203580 (10 μM, final; Calbiochem); the MEK1 and MEK2 inhibitor U0126 (30 μM, final; Calbiochem); and the JNK inhibitor (30 μM, final; Calbiochem) with appropriate DMSO, ethanol, and water controls.

Cell viability assays

Cell viability of anthrax toxin-treated neutrophils was routinely assayed using trypan blue exclusion. A sample of the untreated, LF (0.3 μg/ml), EF (0.3 μg/ml), LTx (PA = 1 μg/ml; LF = 0.3 μg/ml), ETx (PA = 1 μg/ml; EF = 0.3 μg/ml), and LTx + ETx (PA = 1 μg/ml; LF and EF = 0.3 μg/ml) treated cell suspensions were diluted 1/1.5 in PGB buffer and then further diluted 1/1 in 0.4% trypan blue solution (Sigma-Aldrich). After standing for 10 min, the total number of cells and the number of cells with disrupted membranes were determined by light microscopy. Viability results were verified using the MTT reduction assay (46), annexin V and propidium iodine staining (Vybrant apoptosis assay kit no. 2; Molecular Probes), and by morphological examination.

Cell-free NADPH oxidase system

Human neutrophil membrane fractions (GSP) and neutrophil cytosolic fractions (GSS) were prepared according to the method of Curnutte et al. (47). To assess superoxide formation using neutrophil membrane and cytosol, the cell-free system of Curnutte et al. (47, 48) was used. Briefly, neutrophil membrane and cytosol were preincubated for indicated time periods with or without PA (1 μg/ml), LF (0.3 μg/ml), and/or EF (0.3 μg/ml) at 37°C with continuous rotation. A total of 10 μg of purified neutrophil membrane and 100 μg of purified neutrophil cytosol (per reaction) were then aliquoted to wells of a 96-well plate. Cytochrome *c* (100 μM, final), guanosine 5′-(3-*O*-thio)triphosphate (GTPγS) (10 μM, final; Sigma-Aldrich), flavin adenine dinucleotide (10 μM, final; Sigma-Aldrich), and SDS (100 μM, final; Sigma-Aldrich) were then added to each reaction. Control wells also contained superoxide dismutase (0.1 mg/ml, final). After a 5-min equilibration at 25°C, the formation of superoxide was initiated by the addition of nicotinamide adenine dinucleotide phosphate, the reduced form of NADPH (400 μM, final; Sigma-Aldrich). The rate and extent of cytochrome *c* reduction was monitored for 8 min at 550 nm using a Versamax microplate reader (Molecular Devices). All experiments were done in duplicate, and initial rates were used to calculate V_{max} using Softmax Pro software (Molecular Devices).

Cell-free systems were also reconstituted using neutrophil membrane and cytosol fractions isolated from anthrax toxin-treated neutrophils (47, 48) with minor modification. Briefly, human neutrophils were isolated from human whole blood as described above. Neutrophils (5×10^8) were then left untreated, whereas 5×10^8 neutrophils were treated with either LTx (PA = 1 μg/ml; LF = 0.3 μg/ml) or ETx (PA = 1 μg/ml; EF = 0.3 μg/ml). At regular time points, NADPH oxidase activity in the treated and untreated samples was checked using the cytochrome *c* reduction assay. Once effective NADPH oxidase inhibition was established, the cells were disrupted by nitrogen cavitation, and the membrane and cytosol fractions were isolated. Cell-free systems were reconstituted as described above combining toxin-treated and untreated membrane with GSS cytosol and combining toxin-treated and untreated cytosol with GSP membrane.

Statistical analysis

Values are given in this study as mean ± SEM of at least three duplicate experiments. Statistically significant differences between sample groups were determined using the one-way ANOVA test with a Dunnett and/or Bonferroni posttest. A p value of <0.05 was considered significant.

Results

Anthrax LTx and ETx are active in human neutrophils

Prior studies have established that LTx is a zinc metalloprotease capable of cleaving the N terminus of MEK1–4, MEK6, and MEK7, whereas ETx is a calcium- and calmodulin-dependent adenylate cyclase capable of increasing intracellular cAMP levels (13, 16, 49, 50). To ensure that both LTx and ETx were able to enter and exert their catalytic activities in primary human neutrophils, MEK cleavage and intracellular cAMP levels were assayed.

Lysates were prepared from LTx-treated human neutrophils over a 7-h time course, and cleavage of MEK2 and MEK3 was examined by immunoblotting. Preliminary concentration-response studies indicated that maximum MEK cleavage took place at LF = 0.3 μg/ml, with PA = 1 μg/ml (data not shown). As shown in Fig. 1, MEK2 cleavage in the LTx-treated samples was detected by 1 h, and complete cleavage was achieved by 5 h. Similarly, MEK3 cleavage in the LTx-treated samples was seen to begin as early as 1 h, with complete cleavage observed by 5 h. When neutrophils were treated with both LTx and ETx (EF = 0.3 μg/ml), the time course and extent of MEK2 and MEK3 cleavage was no different from that observed with LTx alone (data not shown). No MEK2 or MEK3 cleavage was seen in untreated or LF only controls, and there was no cleavage of the endogenous Rac2 GTPase used as a protein control.

ETx was also effectively taken up and was active in primary human neutrophils. As reported previously (15), neutrophils treated with ETx (PA = 1 μg/ml; EF = 0.3–1 μg/ml) demonstrated a >2-fold increase in cAMP levels within 3 h of treatment, and this elevation was observed to persist at least out to 7 h of treatment (Fig. 2). Maximum elevation of cAMP occurred at EF concentrations of 0.3 μg/ml or above (data not shown). Although modest, this level of cAMP elevation was similar to the maximum achieved by treatment with the potent cAMP-elevating agents, forskolin plus IBMX. These results indicate that MEK cleavage and intracellular elevations in cAMP were induced by *B. anthracis* LTx and ETx, respectively, and that this required the action of the PA pore complex to enable entry into the cell.

Anthrax LTx and ETx inhibit receptor-stimulated, but not PMA-stimulated, superoxide production by human neutrophils

Inherent to the neutrophil's ability to kill ingested microbes is the activity of the NADPH oxidase system, which is responsible for the generation of superoxide and subsequently formed ROS. The importance of MEKs in the upstream activation of the NADPH oxidase system, as well as existing literature indicating that increases in intracellular cAMP can inhibit superoxide production, suggested that LTx and ETx may potentially inhibit NADPH oxidase-mediated ROS production, thereby promoting evasion of this

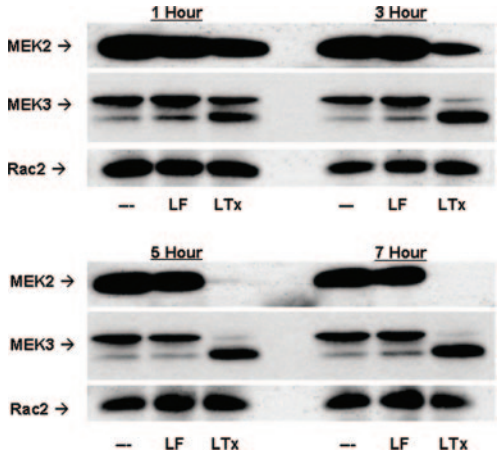

FIGURE 1. Cleavage of MEK2 and MEK3 in LTx-treated human neutrophils. Human neutrophils (2×10^7/ml) were either left untreated or treated with LF (0.3 μg/ml) or LTx (PA = 1 μg/ml; LF = 0.3 μg/ml) at 37°C for indicated amounts of time. Total MEK2 levels (*top panels*), MEK3 levels (*middle panels*), and Rac2 levels (loading control, *bottom panels*) were determined by Western blot analysis. A representative experiment from three separate experiments is shown.

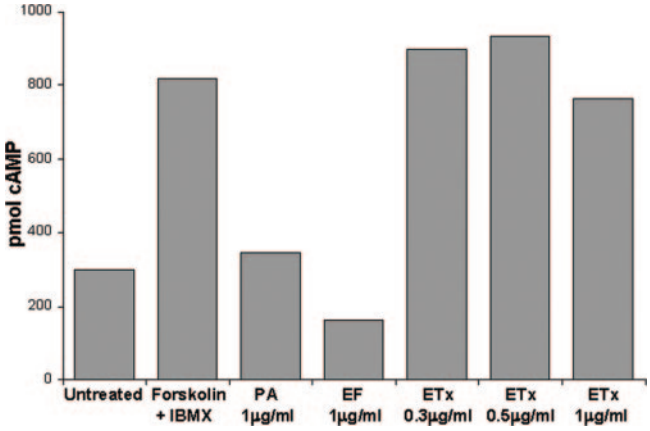

FIGURE 2. Elevation of human neutrophil cAMP by ETx. Human neutrophils (1×10^6/well) that had either been left untreated or treated with PA only (1 μg/ml), EF only (1 μg/ml), or various concentrations of ETx (PA = 1 μg/ml; EF = 1 μg/ml or 0.5 μg/ml or 0.3 μg/ml) at 37°C for 3 h or treated with forskolin (10 μM) plus IBMX (100 μM) for 15 min at 37°C. Total cellular cAMP concentrations were determined as described in *Materials and Methods*.

innate immune killing mechanism (51–53). To evaluate this possibility, neutrophils were treated with maximally effective concentrations of anthrax toxins (PA = 1 μg/ml; LF and/or EF = 0.3 μg/ml) for various times, and the production of superoxide was determined.

Fig. 3*A* shows that LTx began to inhibit fMLP-stimulated superoxide production by 3 h, with an increasing degree of inhibition through 7 h as compared with untreated or LF only controls. Consistent with its rapidity of action to increase intracellular cAMP, ETx-treated neutrophils were significantly inhibited in fMLP-mediated superoxide production within 1 h, and this inhibition persisted through the 7 h of incubation as compared with untreated and EF only controls. This rapid onset of inhibition by ETx was observed both with EF (obtained from List Biological Laboratories) and recombinant wild-type EF (see *Materials and Methods*). Upon exposure of neutrophils to both LTx and ETx together, the onset of inhibition occurred by 1 h, and the severity of inhibition was similar to that of the cells treated with ETx alone. By 7 h, the inhibition seen in the LTx plus ETx-treated samples reached a maximum of ~80%. Similar levels of inhibition were observed whether V_{max} (initial rates) or the extent (at 300 s) of superoxide formation was considered.

Inhibition of superoxide formation induced by fMLP was not due to any reduction in fMLP receptor levels after toxin treatment as determined by flow cytometry analysis of the binding of the fluorescein-labeled fMLP analog, formyl-Nle-Leu-Phe-Nle-Tyr-Lys (data not shown). We also observed that superoxide production in response to a second receptor-mediated activator of NADPH oxidase, the chemoattractant C5a, was effectively inhibited by similar concentrations of LTx and/or ETx (Fig. 3*B*).

In contrast with fMLP and C5a receptor-mediated NADPH oxidase activity, PMA-induced, receptor-independent superoxide production was not inhibited by LTx, ETx, or LTx plus ETx at any time within the 7 h of incubation (Fig. 3*C*). No effect of these toxins on this nonreceptor-mediated stimulus was observed even at submaximal PMA concentrations near or below the IC_{50} (~40 ng/ml). Neither the fMLP, C5a, nor PMA-stimulated superoxide production was inhibited in the LF only (0.3 μg/ml) or EF only (0.3 μg/ml) controls, indicating that the toxins were acting intracellularly.

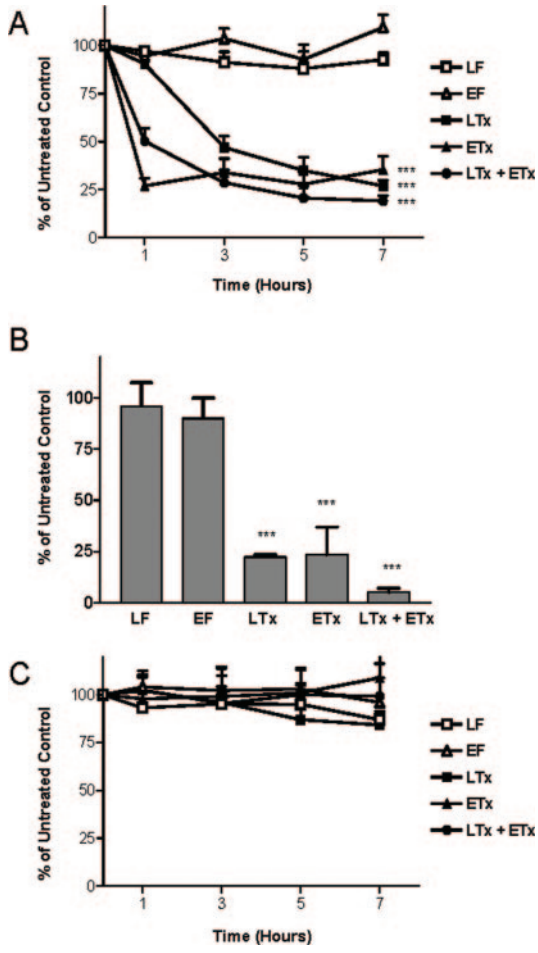

FIGURE 3. Anthrax toxin inhibits fMLP-mediated superoxide production. Human neutrophils (2×10^7/ml) were either left untreated or treated with LF (0.3 μg/ml); EF (0.3 μg/ml); LTx (PA = 1 μg/ml; LF = 0.3 μg/ml); ETx (PA = 1 μg/ml; EF = 0.3 μg/ml) or LTx and ETx (PA = 1 μg/ml; LF and EF = 0.3 μg/ml) at 37°C for indicated amounts of time. Neutrophils were tested for fMLP-stimulated superoxide production (*A*); C5a-stimulated superoxide production at 3 h (*B*); or PMA-stimulated superoxide production (*C*) as described in *Materials and Methods*. Results are expressed as percentage of superoxide production of the untreated control at 300 s and are the mean of six separate experiments (*A*), two separate experiments (*B*), and three separate experiments (*C*). Essentially identical inhibition curves were obtained when the initial rates (V_{max}) of superoxide production were plotted (data not shown). ***, $p < 0.001$.

Anthrax LTx and ETx do not affect human neutrophil viability

Previous literature has established that LTx is effective in inducing apoptotic cell death in macrophages (23–25). To exclude cell death as the reason for inhibition of superoxide production observed upon treatment with *B. anthracis* toxins, neutrophil viability was examined. After 7 h of incubation with the anthrax toxins (PA = 1 μg/ml; LF and/or EF = 0.3 μg/ml), cells from each treatment group were stained with trypan blue and visualized microscopically for viability. As shown in Fig. 4, treatment with LTx, ETx, or both toxins together had no significant effect on neutrophil viability over a 7-h period as compared with untreated, LF only and EF only controls. The lack of effect of the anthrax toxins on cell viability was confirmed with annexin V and propidium iodide staining as measured by flow cytometry, with the MTT assay for viability and proliferation, and by morphological examination (data not shown).

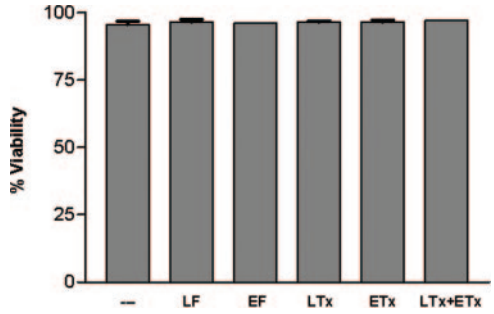

FIGURE 4. Anthrax toxin does not cause cell death in human neutrophils. Human neutrophils (2×10^7/ml) were either left untreated or treated with LF (0.3 μg/ml); EF (0.3 μg/ml); LTx (PA = 1 μg/ml; LF = 0.3 μg/ml); ETx (PA = 1 μg/ml; EF = 0.3 μg/ml) or LTx and ETx (PA = 1 μg/ml; LF and EF = 0.3 μg/ml) at 37°C for 7 h before viability was measured using trypan blue exclusion. Results are expressed as percentage of viability and are the mean of three separate experiments. (Note: Similar levels of viability were determined by MTT reduction, annexin V binding, and morphological criteria.)

Agents that increase cAMP mimic the inhibitory effect of ETx on human neutrophil superoxide production

In an effort to determine whether the effects of ETx on fMLP-stimulated superoxide production in human neutrophils might be due to the ability of ETx to elevate intracellular cAMP, the effects of other cAMP-elevating agents on human neutrophil NADPH oxidase activity were examined. The receptor-mediated cAMP-elevating autocoids PGE_1 and PGE_2 both significantly inhibited superoxide production (Fig. 5*A*). Elevation in cAMP using the direct adenylate cyclase activator, forskolin, and the cAMP phosphodiesterase inhibitor, IBMX, were also able to individually reduce superoxide production (Fig. 5*B*). When neutrophils were treated with both forskolin and IBMX simultaneously, inhibition was even greater than when either agent was used alone. The cAMP analogs bromo8-cAMP and dibutyryl-cAMP were also examined for their ability to inhibit superoxide production in human neutrophils (Fig. 5*C*). Both cAMP analogs inhibited superoxide production; however, dibutyryl-cAMP was significantly more effective in its inhibitory effects. These results are consistent with the idea that ETx is likely inhibiting human neutrophil NADPH oxidase activity through its ability to act as an intracellular adenylate cyclase.

Studies have now established that cAMP can activate two major downstream target proteins in most cells: cAMP-dependent protein kinase (or PKA) and the Epacs. To determine whether intracellular rises in cAMP were inhibiting fMLP-mediated NADPH oxidase activity through a PKA or Epac-mediated mechanism, PKA and Epac1 were specifically activated using target-selective cAMP analogs (54). As shown in Fig. 5*D*, the specific activation of PKA by BNZ-cAMP inhibited the fMLP-mediated superoxide response of human neutrophils. In comparison, cPT-cAMP, a specific activator of Epac1, was not capable of inhibiting superoxide production. These data strongly indicate that ETx is likely acting to inhibit NADPH oxidase activity through the downstream activation of PKA. We attempted to verify this conclusion using the PKA selective inhibitor H89 to attempt to attenuate the inhibitory effects of ETx. However, although we observed reversal when neutrophils were pretreated with concentrations of H89 from 5 to 10 μM, we also observed direct stimulatory effects of H89 on superoxide formation in control cells, precluding any definitive conclusions (data not shown). The effects of all cAMP-elevating agents were examined for their effects on PMA-stimulated human neutrophil superoxide production, and all were found to have no effect on

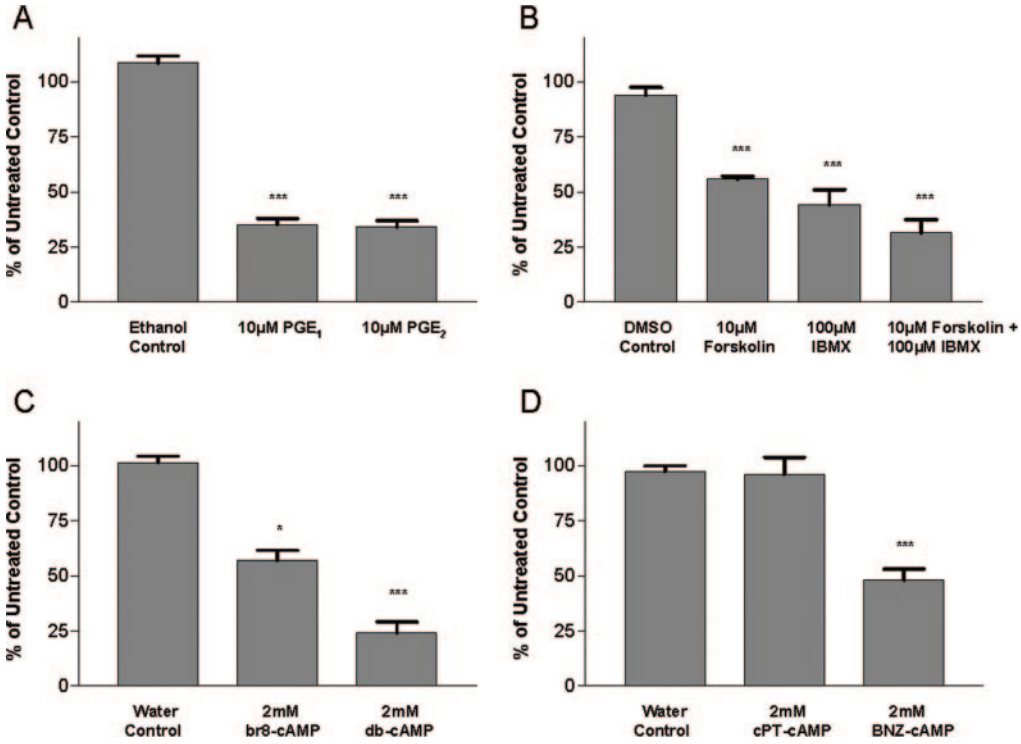

FIGURE 5. Agents that elevate intracellular cAMP selectively inhibit fMLP-mediated superoxide production. Human neutrophils (2×10^7/ml) were either left untreated or treated with the indicated agent at 37°C for the times indicated. *A*, Effects of receptor agonists PGE_1 or PGE_2 after 20-min incubation. *B*, Effects of the direct adenylyl cyclase activator forskolin ± the phosphodiesterase inhibitor IBMX after 15-min incubation. *C*, Effects of cell-permeable cAMP analogs after 20-min incubation. *D*, Effects of selective cAMP mimetics. Appropriate ethanol, DMSO, and water controls are included. Results are expressed as percentage of superoxide production of untreated control at 300 s and are the mean of three separate experiments. *, $p < 0.05$; ***, $p < 0.001$.

activation through this nonreceptor-mediated mechanism, as observed with ETx (data not shown).

The inhibition of p38 MAPK mimics the inhibitory effect of LTx on human neutrophil superoxide production

LTx proteolytically degrades MEK family members, resulting in defective activity of the downstream ERK, p38, and JNK pathways (55, 56). To gain insight into the possible mechanism of LTx inhibition of fMLP-mediated NADPH oxidase activity, the effects of a MEK1/2 inhibitor capable of blocking ERK activation, and selective p38 and JNK inhibitors on fMLP-induced superoxide production by human neutrophils were examined (57). Fig. 6 shows that the p38 inhibitor, SB203580, significantly inhibited fMLP-stimulated superoxide production. Inhibition was concentration dependent and occurred at concentrations previously shown to inhibit p38 selectively (data not shown). The MEK1/2 inhibitor, U0126, also reduced fMLP-mediated superoxide production, whereas the JNK II inhibitor had no effect. Inhibition observed when all three drugs were added together was comparable to inhibition produced by SB203580 alone (data not shown). All three inhibitors had no effect on PMA-induced superoxide production at various concentrations in human neutrophils (data not shown).

Anthrax LTx and ETx do not inhibit superoxide production in a human neutrophil cell-free system

To gain additional insights into NADPH oxidase inhibition by anthrax LTx and ETx, we used a cell-free NADPH oxidase system to investigate whether these toxins exerted any direct effects on the assembly or function of the NADPH oxidase. Human neutrophil membrane and cytosol were either left untreated or treated with LTx (PA = 1 μg/ml; LF = 0.3 μg/ml) or ETx (PA = 1 μg/ml; EF = 0.3 μg/ml) for varying amounts of time before being used in a reconstituted cell-free system. The data from these experiments are summarized in Table I. ETx did not inhibit cell-free superoxide production at any of the time points assayed. The inclusion of 2 mM ATP, a required substrate for the production of cAMP, did not promote inhibitory activity. Similarly, LTx was not capable of inhibiting superoxide production in the cell-free system, even though we established that MEK2 cleavage had taken place by the 7-h time point. Consistent with the lack of inhibitory effect of LTx in this cell-free system, we also observed no LF-induced proteolysis of the NADPH oxidase components, gp91^{phox}, p22^{phox}, p47^{phox}, p67^{phox}, or Rac2 (Fig. 7). No effects in the cell-free assay were detected even at higher EF and LF concentrations, up to 1 μg/ml.

Table I. *LTx and ETx do not inhibit neutrophil superoxide production in a cell-free system*[a]

Group	Initial rate of O_2^- production (nmol O_2^-/min∗mg Mb protein)
Untreated, 2 h	115.88 ± 1.96[b]
Lethal toxin, 2 h	118.81 ± 3.65
Edema toxin, 2 h	130.26 ± 3.02
Untreated, 7 h	148.78 ± 2.27
Lethal toxin, 7 h	147.34 ± 3.61
Edema toxin, 7 h	164.59 ± 5.96

[a] Human GSP and GSS were preincubated with or without PA (1 μg/ml), LF (0.3 μg/ml), and/or EF (0.3 μg/ml) at 37°C with continuous rotation for indicated amounts of time. The GSS and GSP were then used in the reconstitution of the cell-free system as described in *Materials and Methods.*

[b] Shown are the mean V_{max} (nmol O_2^-/min∗mg membrane protein) ± SEM for three independent experiments.

To determine whether the anthrax toxin-mediated NADPH oxidase inhibition established in whole cell assays would be maintained once the cells were broken, human neutrophils were treated with LTx or ETx before the isolation of cytosol and membrane fractions. The untreated and toxin-treated membrane and cytosol fractions were then combined with membranes and cytosols (GSP and GSS, respectively) derived from normal neutrophils for mix-and-match experiments. As summarized in Table II, neither LTx

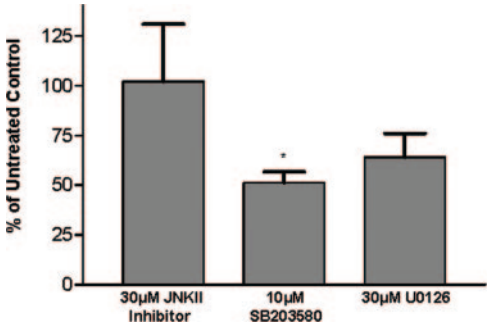

FIGURE 6. Effects of inhibitors of MAPK pathways on superoxide production. Human neutrophils (2×10^7/ml) were either left untreated or treated with the p38 inhibitor SB203580, the MEK1 and MEK2 inhibitor U0126, or the JNK inhibitor II with appropriate DMSO and ethanol controls. After 15 min of incubation at 37°C, the cells were tested for fMLP-mediated superoxide production as described in *Materials and Methods*. Results are expressed as the percentage of superoxide production of untreated control at 300 s and are the mean of three separate experiments. ∗, $p < 0.05$.

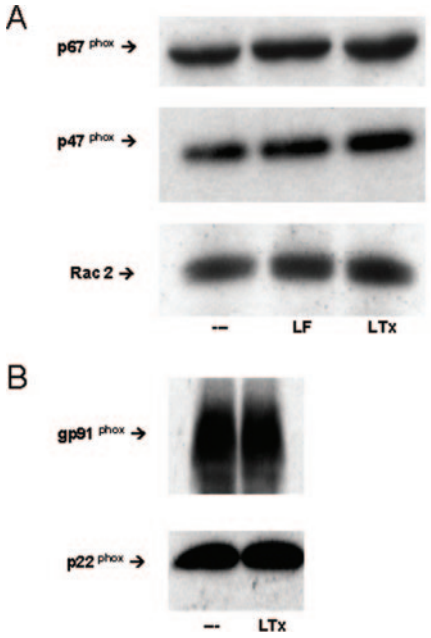

FIGURE 7. Anthrax LTx does not cleave cytosolic or membrane components of the NADPH oxidase complex. *A*, Human neutrophils (2×10^7/ml) were either left untreated or treated with LF alone (LF = 0.3 μg/ml) or LTx (PA = 1 μg/ml; LF = 0.3 μg/ml) at 37°C for 7 h. *A*, total p67^{phox} levels (*top panel*), p47^{phox} levels (*middle panel*), and Rac2 levels (*bottom panel*) were determined by Western blot analysis. A representative experiment from three separate experiments is shown. *B*, Human neutrophils (2×10^7/ml) were either left untreated or treated with LTx (PA = 1 μg/ml; LF = 0.3 μg/ml) at 37°C for 7 h. Membrane fractions were then isolated, and total gp91^{phox} levels (*top panel*) and p22^{phox} levels (*bottom panel*) were determined by Western blot analysis. A representative experiment from two separate experiments is shown.

Table II. *Anthrax-mediated oxidase inhibition is not stable through membrane and cytosol isolation*[a]

Group	Initial rate of O_2^- production (nmol O_2^-/min*mg Mb protein)
Untreated cytosol + GSP	94.86 ± 6.42[b]
LTx-treated cytosol + GSP	93.53 ± 6.95
Untreated membrane + GSS	55.22 ± 3.14
LTx-treated membrane + GSS	60.05 ± 8.14
Untreated cytosol + GSP	106.62 ± 23.24
ETx-treated cytosol + GSP	93.39 ± 19.76
Untreated membrane + GSS	43.45 ± 2.36
ETx-treated membrane + GSS	46.34 ± 1.53

[a] Cell-free systems were reconstituted using neutrophil membrane and cytosol fractions isolated from anthrax toxin-treated neutrophils. Cells were either left untreated or treated with LTx (PA = μg/ml; LF = 0.3 μg/ml) or ETx (PA = 1 μg/ml; EF = 0.3 μg/ml) and once oxidase inhibition was established, the cells were used in membrane and cytosol isolation. Cell-free systems were reconstituted by combining toxin-treated and untreated membrane with GSS cytosol and combining toxin-treated and untreated cytosol with GSP membrane.

[b] Shown are the mean V_{max} (nmol O_2^-/min*mg membrane protein) ± SEM for three independent experiments.

nor ETx-treated membranes were deficient in their ability to support cell-free superoxide production in the presence of normal cytosol. Similarly, neither LTx nor ETx-treated cytosols were deficient in their ability to support cell-free superoxide production in the presence of normal membranes. These results support the idea that the anthrax toxins do not directly affect NADPH oxidase components, and that these toxins may exert their effects upstream of NADPH oxidase assembly.

Discussion

As major virulence factors of *B. anthracis*, LTx and ETx have been thought to play critical roles in the ability of this organism to evade innate immune responses of the host. We show in this study that both toxins at relatively low concentrations are capable of inhibiting one of the major microbicidal pathways of human neutrophils, the NADPH oxidase. Interestingly, both toxins effectively inhibited NADPH oxidase activation by chemoattractant (fMLP or C5a) receptor-mediated stimulation, but not activation by the nonreceptor activator, PMA. These inhibitory effects of the anthrax toxins were concentration-dependent. Unlike the effects of anthrax LTx to induce an apoptotic response in macrophages, the NADPH oxidase inhibition we observed in human neutrophils was not associated with significant cell death (Fig. 4), indicating that NADPH oxidase inhibition is not a secondary consequence of decreased cell viability. This is in agreement with the recent report by During et al. (40), which showed that anthrax LTx is also able to inhibit human neutrophil chemotactic responses to the chemoattractant fMLP. The combined inhibition of neutrophil chemotaxis to infectious sites, coupled with inhibition of the oxidative killing response, would effectively enable *B. anthracis* to avoid innate immune clearing. This would allow a systemic infection to be established before an adaptive immune response could be achieved.

Our studies clearly demonstrate the ability of both LTx and ETx individually to inhibit human neutrophil NADPH oxidase activity initiated through receptor-mediated stimulation. A single prior study over 20 years ago using fMLP as a stimulus in human neutrophils primed with bacterial LPS or muramyl dipeptide reported inhibition by both LTx and ETx individually is essentially in agreement with our findings (39). We observed no effect of LTx and ETx on PMA-stimulated ROS formation at all PMA concentrations examined. Our results differ from those reported by O'Brien et al. (16), in which chemiluminescence induced by the phorbol ester PMA was reported to be inhibited by ETx. We note that this effect was only observed in the aforementioned study after very long times of stimulation with this agonist. The same study reported that phagocytic uptake and chemiluminescence induced by the avirulent Sterne strain of *B. anthracis* was inhibited by ETx.

NADPH oxidase inhibition by anthrax LTx is well correlated with the entry of the toxin into primary human neutrophils and the resulting proteolytic cleavage of the MEKs (Fig. 1). Indeed, the effect of LTx is mimicked by inhibitors of the p38 MAPK pathway, suggesting that LTx is likely to induce NADPH oxidase inhibition through this mechanism. Previous reports have shown that inhibition of p38 MAPK can decrease NADPH oxidase output (58, 59). It has been shown that p38 MAPK phosphorylates the cytosolic regulatory component, $p47^{phox}$, and that this phosphorylation is likely to be necessary for chemoattractant-initiated assembly and activation of the NADPH oxidase (60, 61).

The inhibition of NADPH oxidase activity by anthrax ETx also appears to be a direct result of its activity as an adenylate cyclase (Fig. 2). The inhibitory effects of ETx were mimicked by agents that elevated cAMP in neutrophils through both receptor-mediated and nonreceptor-mediated mechanisms (Fig. 5). Through the use of selective cAMP analogs, we were able to show that inhibition was dependent upon the ability to activate the downstream mediator cAMP-dependent protein kinase or PKA. Activation of Epacs had no inhibitory effect, indicating that the effects of ETx were not mediated through the activation of Rap1 exchange factors. These were significant observations, because Rap1 has been shown to associate with components of the phagocyte NADPH oxidase and had been suggested as a possible mechanism for the inhibitory action of cAMP (62, 63).

The molecular basis for the effects of the anthrax toxins on NADPH oxidase activity was investigated in a cell-free assay system (Table I). We observed no direct inhibitory effects on cell-free NADPH oxidase activity, nor proteolysis of NADPH oxidase regulatory components by the LTx protease. This is consistent with the inability of LTx to inhibit PMA-stimulated oxidant formation, contrary to what would be expected if regulatory components had been degraded. Similarly, there was no change in the levels of these components after treatment with ETx. We attempted to localize the effect of the LTx or ETx to either a membrane-associated NADPH oxidase component or a cytosolic regulatory component by performing mix-and-match experiments with materials isolated from toxin-pretreated cells. However, we could detect no defect in either fraction after toxin treatment (Table II). These results can be interpreted in several ways: first, the inhibitory effects of the toxins could be reversible, and not maintained after breakage of the neutrophils and isolation of subcellular fractions; second, the intrinsic nature of the cell-free system, in which activation must be achieved through the addition of anionic amphiphiles (e.g., SDS), may obviate the inhibitory effect of the toxins. Indeed, it is clear that the use of such in vitro-activating agents bypasses many of the normal signaling events (e.g., phosphorylation) required for normal NADPH oxidase activation. Thus, it may also be that the toxins act on upstream signaling pathways necessary for NADPH oxidase assembly and activation.

In conclusion, we have shown that both anthrax LTx and ETx are individually able to effectively block NADPH oxidase-initiated formation of microbicidal oxidants in human neutrophils. These effects are potent and are likely to aid in the evasion of innate immune clearing mechanisms by *B. anthracis*, thereby contributing to the establishment and severity of the resulting anthrax infection.

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Disclosures

The authors have no financial conflict of interest.

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