ANTHRAX TOXIN BLOCKS PRIMING OF NEUTROPHILS BY LIPOPOLYSACCHARIDE AND BY MURAMYL DIPEPTIDE

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Anthrax toxin, a critical virulence factor of *Bacillus anthracis* (1–3), consists of three protein components: protective antigen (PA)[1], edema factor (EF), and lethal factor (LF). PA, the major antigen of acquired immunity (4, 5) evidently combines with susceptible cells, forming a receptor for EF and LF (6). EF, initially recognized by its ability to produce edema in tissues, has been identified as an adenylate cyclase, which, in combination with PA, forms adenosine 3′,5′-monophosphate (cAMP) in susceptible cells; EF alone forms cAMP in a reconstituted cell-free system (6, 7). LF is identified by its acute lethality in animals when injected in combination with PA; its mode of action is unknown despite extensive studies (4, 8, 9). None of the factors by itself produces acute toxic reactions. Keppie et al. (10) presented evidence that the toxin was antiphagocytic and antibactericidal by virtue of an action on phagocytic cells. Despite the significance of these effects for the further understanding of pathogenesis, only recently have efforts been made to elucidate their mechanism.

By analogy with other bacterial adenylate cyclases, we anticipated that PA plus EF would inhibit chemotaxis of PMN; we found instead that pretreatment with PA plus EF or PA plus LF markedly stimulated chemotaxis (11). It seemed possible that the observed stimulation was associated with inhibition of the normal modulation of chemotaxis by oxidative or other secretory products of stimulated PMN (12, 13). PA plus EF, but not PA plus LF, was reported (14) to inhibit phagocytosis of opsonized *B. anthracis* by human polymorphonuclear neutrophils (PMN) and to block chemiluminescence induced by opsonized *B. anthracis* or by phorbol myristate acetate (PMA).

Pretreatment with toxin reduced the release of superoxide anion (O_2^-) after PMN were stimulated by *N*-formyl-methionyl-leucyl-phenylalanine (FMLP). Initially, the inhibition of O_2^- release was variable, and it was suggested that spontaneous fluctuations in the level of contamination with pyrogen were a contributory factor. A conceptual basis for the variability was provided by reports (15, 16) that PMN isolated under conditions that minimize exposure to bacterial lipopolysaccharide (LPS) produced weak oxidative responses to FMLP and

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[1] *Abbreviations used in this paper:* EF, edema factor; FMLP, formyl-methionyl-leucyl-phenylalanine; HBSS, Hanks' balanced salt solution; LF, lethal factor; MDP, muramyl dipeptide (*N*-acetylmuramyl-L-alanyl-D-isoglutamine); PA, protective antigen; PMA, phorbol myristate acetate; PMN, polymorphonuclear neutrophils; SOD, superoxide dismutase.

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certain other stimuli. Preincubation with LPS at concentrations as low as 1 ng/ml produced a severalfold increase in O_2^- release, an effect referred to as priming. The marked increase in the release of O_2^- and lysosomal enzymes associated with priming increases not only the antimicrobial potential of PMN but also their ability to damage adjacent tissue (17).

We found that unprimed PMN treated with PA plus EF or PA plus LF resist subsequent priming by LPS and priming by *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide [MDP]), a synthetic glycopeptide mitogen analogous to bacterial peptidoglycans (18). The inhibition of priming results in marked inhibition of O_2^- release relative to primed controls after stimulation with FMLP, but not after stimulation with PMA. The inhibition of priming represents a mechanism, not described previously, whereby a bacterial toxin dampens the oxidative response of PMN.

Materials and Methods

Preparation of PMN. Human blood was drawn into 1/10 vol of 3.8% sodium citrate, prepared by dilution of 46.7% sodium citrate solution (Alpha Medical Products, Providence, RI) with water for injection. PMN were isolated by dextran sedimentation and hypotonic lysis of erythrocytes and washed twice according to the method of Guthrie et al. (15), except that Hanks' balanced salt solution (HBSS) without phenol red (Whittaker M.A. Bioproducts, Walkersville, MD) was used for washing and final suspension of PMN. In exploratory experiments, PMN gave variable production of O_2^- after stimulation; this effect was associated with clumping during preincubation and was ascribed to the presence of Ca^{++} and Mg^{++} in the HBSS and the variable carry-over of citrate. The variability and clumping were overcome by addition of 2 mM sodium citrate to the HBSS used for washing and suspension of PMN; the final concentration in the test was 1 mM. The suspensions were ≥80% PMN; they were diluted to 8–10 × 10^6 cells/ml (total count). Great care was taken to avoid the uncontrolled introduction of pyrogens by use of pyrogen-free, single-use plastic- or glassware and pyrogen-free solutions, which were stored at −70°C when possible or handled under aseptic conditions and refrigerated..

Anthrax Toxin. PA, EF, and LF were supplied by Dr. S. H. Leppla of the U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD. They resembled previous preparations in purity and specific activity (6, 19). The stock solutions were diluted in HBSS to 30 μg/ml, dispensed in amounts sufficient for one experiment, and held at −70°C.

Toxin Treatment, Priming, and Stimulation of PMN. PMN were exposed to the toxin components, alone and in various combinations, for 1 h at 37°C, followed by addition of the priming substance and incubation for an additional hour at 37°C. Cytochrome *c* and stimulant were then added, and the tests were incubated for 10 min at 37°C in a shaker water bath, cooled, and centrifuged for 15 min at 3,000 *g*. Each determination consisted of two tubes, which were identical except that one tube received superoxide dismutase (SOD) and was placed in ice immediately before the addition of cytochrome *c* and stimulant. The release of O_2^- in the incubated tube was determined from the difference in absorption of supernatants of each pair of tubes at 550 nm.

Tests in a final volume of 600 μl were set up in duplicate in 12- × 75-mm plastic tubes (2054; Falcon Labware, Becton, Dickinson & Co., Oxnard, CA). The final concentrations were: PA, EF, LF, as indicated; human serum albumin, 0.2%; PMN, 4–5 × 10^6/ml; LPS and MDP, as indicated; SOD (when present), 0.1 mg/ml; cytochrome *c*, 0.12 mM; stimulant, 10^{-7} M (FMLP) or as indicated (PMA). Concentrations of toxin and primer are referred to the final 600-μl volume. SOD from bovine erythrocytes, cytochrome *c* type VI, and FMLP were obtained from Sigma Chemical Co., St. Louis, MO; PMA was from Consolidated Midland Corp., Brewster, N.Y. FMLP and PMA were dissolved in dimethyl sulfoxide at concentrations of 0.01 M and 100 and 1 mg/ml, respectively, held at −70°C

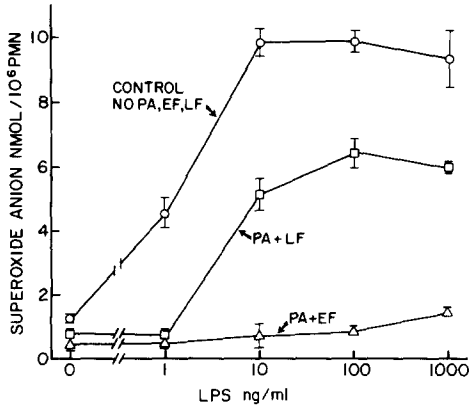

FIGURE 1. Release of O_2^- after stimulation of PMN with 10^{-7} M FMLP as a function of concentration of LPS during priming. PMN were incubated for 1 h at 37°C with control buffer, or PA plus EF (each 0.25 μg/ml), or 0.25 μg/ml PA plus 0.5 μg/ml LF. LPS was added and tests were incubated for 1 h at 37°C and stimulated with FMLP, and the O_2^- released in 10 min was measured. $n = 4$.

in small quantities, and thawed and diluted in HBSS immediately before use. LPS from *Escherichia coli* K235 (List Biological Laboratories, Campbell, CA) was suspended in HBSS at 1 mg/ml, dispersed by brief sonication, and stored at 4°C. MDP (Sigma Chemical Co.) was dissolved in HBSS, held in small quantities at −70°C, and thawed and diluted immediately before use. Human serum albumin, in 5% solution for clinical use, was obtained from Cutter Laboratories, Cutter Biological, Berkeley, CA, or the New York Blood Center. The undiluted solution gave positive tests for LPS by the *Limulus* amebocyte lystate test (using Pyrotell reagent; sensitivity, 0.006 ng of LPS; obtained from Associates of Cape Cod, Inc., Woods Hole, MA), but gave negative tests at a 1:5 dilution. There was no indication that either preparation caused priming of PMN at the 0.2% final concentration used.

Determination of O_2^- Release. The difference between each pair of absorption measurements was divided by the PMN count in millions and the extinction coefficient of 1.85×10^4 cm^2 $mmol^{-1}$ (20). This yielded O_2^- released in millimoles per 10^6 PMN. Control tests without anthrax toxin were set up in duplicate for each set of conditions, so that the effects of toxin could be determined. The patterns of inhibition of O_2^- release were consistent in repeat experiments using PMN from different donors, but the levels of O_2^- varied somewhat, which presumably reflects individual differences in the proportion of PMN that responded to FMLP (21). Accordingly, the percent changes in O_2^- release relative to the mean control value without toxin were determined, means and standard deviations were calculated for replicate experiments, and these values were reconverted to O_2^- in nanomoles per 10^6 PMN by reference to the respective mean control values. The means and standard errors are represented in the figures; the values of n, the number of replicate experiments, are given in the legends.

Results

Effects of LPS Priming and Anthrax Toxin Treatment on O_2^- Release. Human PMN isolated with minimal exposure to bacterial products released relatively small amounts of O_2^- upon stimulation with FMLP; treatment with a range of concentrations of LPS for 1 h at 37°C (priming) increased their subsequent release as much as eightfold (Fig. 1). Smaller but appreciable effects were observed with concentrations of LPS as low as 1 ng/ml. The levels of response of control PMN to low concentrations of LPS varied somewhat among cells from

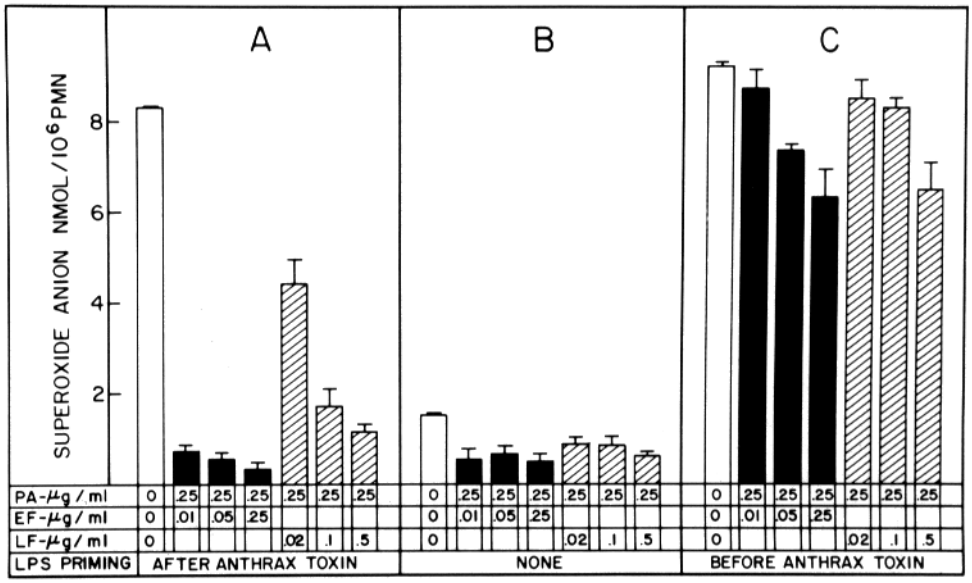

FIGURE 2. Release of O_2^- after stimulation of PMN with 10^{-7} M FMLP as a function of concentrations of PA, EF, and LF present during preincubation, and of conditions of priming with 3 ng/ml LPS. In *A*, PMN were exposed to PA, EF, or LF in the concentrations shown for 1 h at 37°C, after which LPS was added and the tests were incubated for 1 h at 37°C. The release of O_2^- in 10 min was determined after stimulation with FMLP. Dose-related inhibition of O_2^- release relative to the control without anthrax toxin is evident with both PA plus EF and PA plus LF. In *B*, tests were carried out in the same manner, except that HBSS was added instead of LPS. In the absence of priming, the small amount of O_2^- released in the control without anthrax toxin makes it difficult to detect inhibition by PA plus EF or PA plus LF. In *C*, PMN were exposed to LPS before exposure to anthrax toxin; otherwise, conditions were the same as in *A*. Inhibition by PA plus EF and PA plus LF relative to the control is much smaller than in *A*. $n = 4$–6. Tests in which PA, EF, or LF were added singly gave results essentially identical to controls without toxin, and have been omitted.

different donors, which perhaps reflects a variation in humoral immunity to LPS among individuals (22).

Pretreatment of PMN with PA plus EF for 1 h at 37°C markedly reduced the levels of O_2^- released after LPS priming; reduction was ≥90% in the range from 1 to 100 ng/ml LPS. The pretreatment produced consistent reductions in the small amount of O_2^- released from PMN not primed with added LPS. Pretreatment with PA plus LF also reduced the release of O_2^- after priming and stimulation, but the effect was less marked than the effect of PA plus EF, especially at higher concentrations of LPS. After priming with 1 ng/ml of LPS, however, the inhibition of O_2^- release was 84%.

To explore the interactions of priming and anthrax toxin treatment of PMN, experiments were set up with PA plus a range of concentrations of EF or LF, in which O_2^- release was compared without priming, with priming with LPS after exposure to toxin, and with priming before exposure to toxin (Fig. 2). PA, when present, was held constant at 0.25 μg/ml. It is evident that both PA plus EF and PA plus LF produced strong inhibition over a range of concentrations when LPS priming was carried out after exposure to anthrax toxin; reversing this order

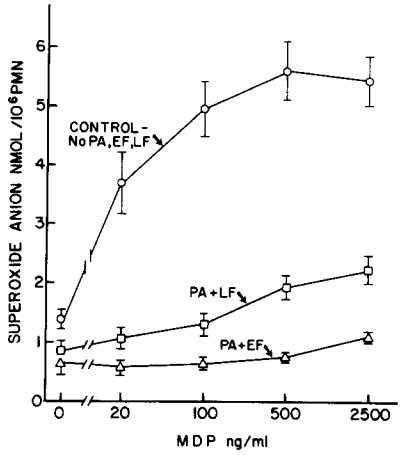

FIGURE 3. Release of O_2^- after stimulation of PMN with 10^{-7} M FMLP as a function of concentration of MDP during priming. PMN were incubated for 1 h at 37°C with control buffer, or 0.25 μg/ml PA plus 0.5 μg/ml EF, or 0.25 μg/ml PA plus 0.5 μg/ml LF. MDP was added to reach the concentrations shown, and tests were incubated for 1 h at 37°C. PMN were stimulated with FMLP and the O_2^- released in 10 min was measured. $n = 8$.

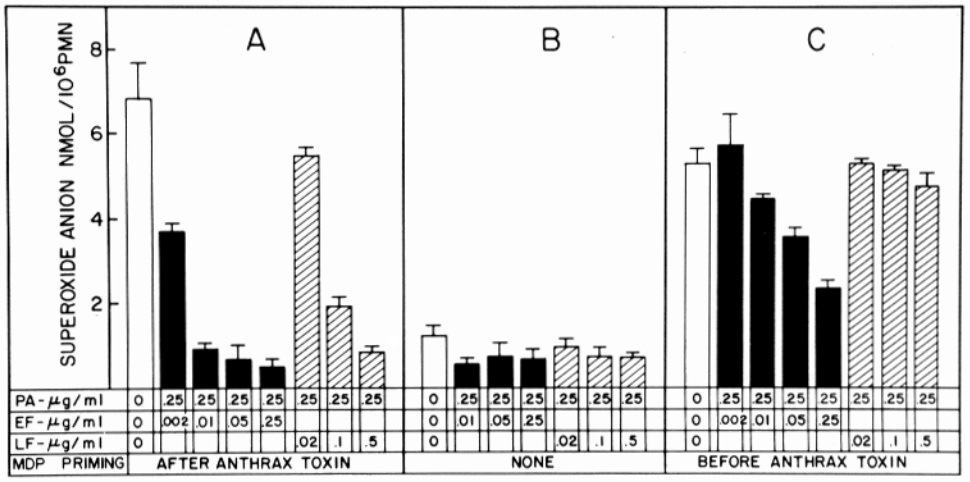

FIGURE 4. Release of O_2^- after stimulation of PMN with 10^{-7} M FMLP as a function of concentration of PA, EF, and LF present during preincubation, and of priming with 100 ng/ml MDP. The experimental design was otherwise the same as for Fig. 2. $n = 6$–10.

markedly reduced the inhibitory effect. Strong inhibition was obtained with 0.01 μg/ml of EF; LF was less active, but produced 84% inhibition of O_2^- release at 0.5 μg/ml. Without priming, toxin treatment produced slight inhibition of O_2^- release relative to the low value of the control without toxin.

Effects of Priming with MDP and Treatment with Anthrax Toxin on O_2^- Release. Experiments similar to those described above were carried out, except that MDP was used instead of LPS (Figs. 3 and 4). The priming effects were slightly smaller than with LPS, and the higher concentrations of MDP did not overcome the inhibitory effects of PA plus LF to as great a degree as did LPS.

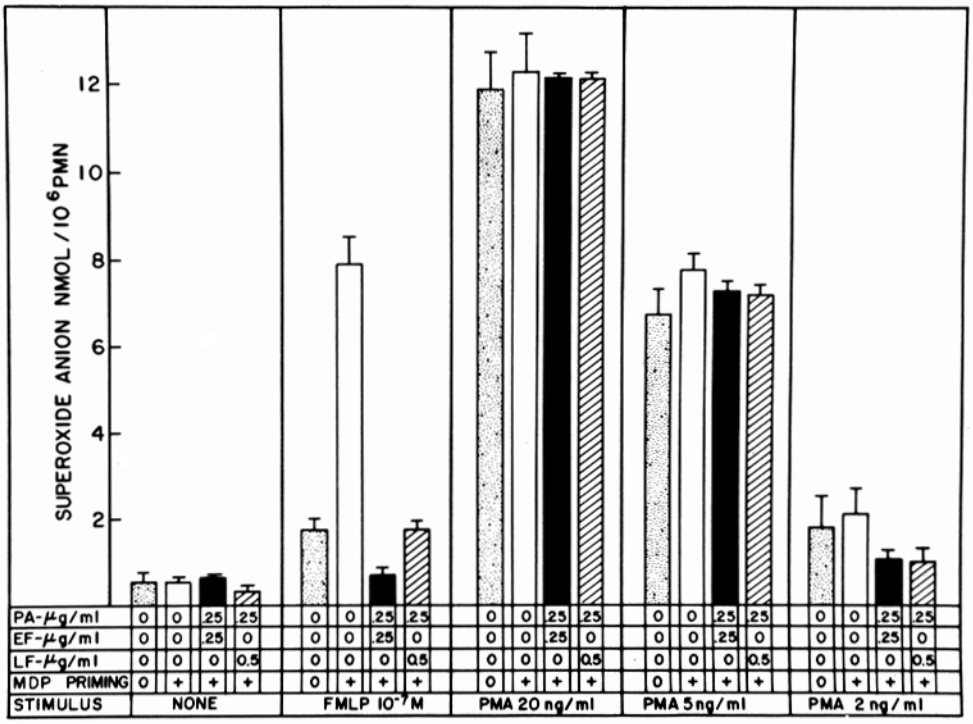

FIGURE 5. Comparison of the effects of stimulation of PMN by FMLP and three concentrations of PMA on priming effects of 100 ng/ml MDP, and on the inhibitory effects of PA plus EF and PA plus LF. PMN were exposed first to anthrax toxin components or control buffer for 1 h at 37°C, after which MDP or control buffer was added to a final concentration of 100 ng/ml and incubation continued for an additional hour at 37°C. The release of O_2^- in 10 min was measured after no stimulation, after stimulation with 10^{-7} M FMLP, or after stimulation with PMA at 20, 5, or 2 ng/ml. $n = 4$–6.

In addition, PA plus EF added after priming resulted in somewhat greater inhibition of O_2^- release than was observed with LPS priming. The overall effects were similar.

Comparison of FMLP and PMA Stimulation after Anthrax Toxin and MDP Priming. Five conditions of stimulation were compared: no stimulus, 10^{-7} M FMLP, and three concentrations of PMA: 20, 5, and 2 ng/ml. Four combinations of anthrax toxin treatment and MDP priming were investigated for each condition of stimulation: no anthrax toxin and no priming, no toxin and MDP priming, PA plus EF and MDP priming, PA plus LF and MDP priming. The results (Fig. 5) are consistent with those reported above for FMLP stimulation and MDP priming. In contrast, with PMA there was no evidence of priming by MDP, and no inhibition of O_2^- release by PA plus EF or PA plus LF.

Discussion

The marked inhibition of FMLP-induced O_2^- release produced by pretreatment of PMN with PA plus EF or PA plus LF before priming with LPS or MDP indicates that anthrax toxin alters the cells in such a manner that they resist priming. When PMN were primed first and then exposed to the toxin, only

slight inhibition was observed. These effects indicate that inhibition by toxin and priming, once established, are not readily reversed. The concept of inhibition of priming by toxin is compatible with the critical role of toxin in virulence (2, 3, 10) and the major increase in the potential to release O_2^- and lysosomal enzymes associated with priming (15–17). The release of these substances by PMN represents a major mechanism of bactericidal activity (23, 24); the possession of mechanisms for inhibiting bactericidal effects would contribute significantly to virulence.

The results in Fig. 2 provide explanations for the initial difficulties in obtaining consistent effects of anthrax toxin when LPS was not controlled, and may explain, in part, the results of others (14). Exposure of PMN to 3 ng/ml LPS, a concentration readily obtained in solutions not carefully handled to exclude pyrogens, initiated a level of priming that was not inhibited appreciably by subsequent treatment with anthrax toxin. In other experiments, the exclusion of LPS prevented priming and reduced the release of O_2^- in controls to a level at which inhibition by treatment with the toxin was difficult to detect. Only when exposure to LPS was controlled with respect to concentration and timing were clearly recognizable and consistent effects of anthrax toxin obtained.

A model system that includes priming by LPS does not reproduce fully the processes occurring during anthrax because *B. anthracis* does not produce LPS. The observation that MDP is also active in priming PMN, a possibility raised initially by analogy with activation of macrophages (25, 26), provides a closer link to *B. anthracis* because MDP is related to peptidoglycans of bacterial cell walls in structure and activity (26, 27). MDP has been reported to be inactive in priming of PMN under other conditions, however (28).

The priming of PMN by LPS not only increases the release of O_2^- in response to stimuli, but also enhances the release of lysosomal enzymes, induces spontaneous changes in shape (16), and modulates chemotactic responsiveness (16, 29). Our previous observation (11) that chemotaxis was stimulated by treatment with anthrax toxin can be explained by the assumption that control PMN were primed by LPS introduced during their isolation, resulting in reduced chemotactic responsiveness. This priming was inhibited in the presence of the toxin, producing apparent stimulation of chemotaxis. This concept appears more probable than the tentative explanation suggested previously, that anthrax toxin inhibits the secretory activities of PMN that modulate chemotaxis.

Pretreatment with amounts of anthrax toxin that produced almost complete inhibition of O_2^- release after stimulation with FMLP had no effect on O_2^- release after stimulation with PMA. PMA stimulation also did not reveal evidence of priming as a result of pretreatment with MDP or (not shown) LPS; this absence of inhibition by the toxin when priming does not occur provides additional evidence that the toxin acts to prevent priming. Guthrie et al. (15) obtained priming by LPS with PMA stimulation, but the ratio of O_2^- with LPS treatment to O_2^- without LPS was 1.64 for PMA, compared with 7.76 for FMLP. Our results with PMA suggest that priming alters the polyphosphoinositide transmembrane signal mechanism at a point proximal to activation of protein kinase C, since PMA activates protein kinase C directly (30).

Summary

We studied the pretreatment of human polymorphonuclear neutrophils (PMN) with purified preparations of the anthrax toxin components—protective antigen (PA), edema factor (EF), and lethal factor (LF)—and their effects on release of superoxide anion (O_2^-) after stimulation with the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (FMLP). PMN isolated in the absence of lipopolysaccharide (LPS) (<0.1 ng/ml) released only small amounts of O_2^- after FMLP stimulation; pretreatment with anthrax toxin had little effect. The release of O_2^- was increased fivefold by prior treatment with 3 ng/ml LPS for 1 h at 37°C, an effect referred to as priming. PMN were primed to an equivalent extent by treatment with 100 ng/ml *N*-acetyl-muramyl-L-alanyl-D-isoglutamine (muramyl dipeptide [MDP]). Pretreatment of PMN with anthrax toxin components PA plus EF or PA plus LF inhibited priming by LPS or MDP, as shown by the reduction in the release of O_2^- up to 90% relative to controls not treated with toxin; single toxin components were inactive. The inhibition was markedly reduced when priming with LPS or MDP was carried out before exposure to toxin. O_2^- release after stimulation by phorbol myristate acetate was not increased by priming, and pretreatment with toxin did not inhibit O_2^- release after this stimulus. Evidently, anthrax toxin inhibits the priming that is normally induced in PMN by bacterial products and is necessary for the full expression of antimicrobial effects.

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