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Pretreatment with anthrax toxin inhibits priming of polymorphonuclear neutrophils by lipopolysaccharide or muramyl dipeptide, resulting in reduction in release of superoxide anion when the cells are stimulated with chemotactic peptide. The resulting attenuation of critical antibacterial activities of neutrophils may represent an important mechanism of virulence in Bacillus anthracis.

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Report Number 2

STUDIES OF THE BIOLOGICAL AND MOLECULAR BASIS OF THE INHIBITION OF ACTIVITY OF PHAGOCYTIC CELLS BY ANTHRAX TOXIN

Annual Report

George G. Wright and Gerald L. Mandell

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Summary

The three-component toxin of Bacillus anthracis exerts antiphagocytic and antibactericidal effects on polymorphonuclear neutrophils (PMN), which are believed to contribute to the essential role of the toxin in virulence. Toward elucidation of these effects, we studied pretreatment of human PMN with purified preparations of the toxin components - protective antigen (PA), edema factor (EF), lethal factor (LF) - and its effect on their release of superoxide anion (O1:21;1;-1:) after stimulation with the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP). PMN isolated with minimal exposure to lipopolysaccharide (LPS) released only small amounts of O1:21;1;-1: after FMLP stimulation; O1:21;1;-1: release was increased 5.2-fold by treatment with 3 ng per ml of LPS for 1 hour at 371;o1:C (priming) prior to FMLP stimulation. PMN were primed to an equivalent extent by treatment with N-acetyl-muramyl-l-alanyl-d-isoglutamine (muramyl dipeptide-MDP) 100 ng per ml. Pretreatment of PMN with anthrax toxin components PA + EF or PA + LF inhibited priming by LPS or MDP as shown by inhibition of release of O1:21;1;-1: up to 90% relative to controls not treated with toxin; single toxin components were inactive. The inhibition was reduced markedly if priming with LPS or MDP were carried out prior to exposure to toxin. O1:21;1;-1: release after stimulation by phorbol myristate acetate was not increased by priming, and pretreatment with toxin did not inhibit O1:21;1;-1: release after this stmulus. Evidently anthrax toxin inhibits the PMN priming normally induced by bacterial products and necessary for full expression of antibacterial effects. Implications for elucidation of the mechanism of priming and its inhibition are discussed.

Foreword

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations. For the protection of human subjects the investigator(s) have adhered to policies of applicable Federal Law 45CFR46.

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Introduction

The anthrax toxin, a critical virulence factor of Bacillus anthracis, (1-3) consists of three protein components: protective antigen (PA), 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 in whole animals (4,8,9). None of the factors by itself produces acute toxic reactions.

Keppie et al. (10) presented evidence that the complete toxin was antiphagocytic and antibactericidal by virtue of an action on phagocytic cells. Despite the significance of these effects for further understanding of pathogenesis, only recently have efforts been made to elucidate their mechanism. PA + EF, but not PA + LF or individual components, was reported 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) (11).

We anticipated, by analogy with other bacterial adenylate cyclases, that PA + EF would inhibit chemotaxis of PMN; we found instead that pretreatment with PA + EF, PA + LF, or PA + EF + LF markedly stimulated chemotaxis of human PMN (12). PA + EF produced low levels of cAMP in these cells, whereas urea extracts of Bordetella pertussis which produced high levels of cAMP abolished chemotaxis (13). 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 (14, 15). Exploratory studies of the effects of pretreatment of PMN with toxin on formation of chloramines after stimulation with opsonized zymosan revealed that both PA + EF and PA + LF produced significant inhibition that developed over a two-hour period at 37° (16). Pretreatment with toxin interfered with formation of superoxide anion (O_2^-) after the PMN were stimulated by N formyl-methionyl-leucyl-phenylalanine (FMLP). Initially the effects were variable, and it was suspected that uncontrolled fluctuations in the level of pyrogen in the experimental system altered the release of O_2^-.

Support for this notion and a conceptual basis for resolution of the problem of variability were provided by reports (17,18) that PMN isolated under conditions that exclude bacterial lipopolysaccharide (LPS) responded weakly to FMLP and certain other stimuli, and could be primed to a several fold increase in response by preincubation with LPS at concentrations as low as 1ng/ml, a concentration frequently present in reagents used in conventional methods for isolation of PMN. The present paper presents evidence that human PMN isolated without appreciable exposure to LPS and treated with PA + EF or PA + LF resist subsequent priming by LPS, and also priming by N-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide-MDP), a synthetic glycopeptide mitogen analogous to bacterial peptidoglycans (19). This 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. Evidently inhibition of priming is a novel mechanism whereby a bacterial toxin dampens the PMN oxidative response.

Materials and Methods

Preparation of PMN. Human blood was drawn into 1/10 volume of 3.8% sodium citrate, prepared by dilution of 46.7% sodium citrate solution (Alpha Medical Products, Providence, R.I.) 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 (17), except that Hanks' balanced salt solution, without phenol red (HBSS) (Whittaker M.A. Bioproducts) 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 2mM sodium citrate to the HBSS used for washing and suspension of PMN; the final concentration in the test was 1mM. The suspensions were 80% or more PMN; they were diluted to 8 to 10 x 10^6 cells per ml (total count). Great care was taken to avoid 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 Components. PA, EF, and LF were supplied by Dr. S.H. Leppla of the U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland. They resembled previous preparations in purity and specific activity (6,20). The frozen stock solutions, which contained 1-3 mg per ml of the respective component, were thawed and diluted in HBSS to 30 µg per 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 hour at 37°, followed by addition of the priming substance and incubation for an additional hour at 37°. Cytochrome C and stimulant were then added, and the tests were incubated for 10 minutes at 37°C in a shaker water-bath, cooled, and centrifuged 15 minutes at 3000g. Each determination consisted of

two tubes, identical except that immediately before addition of cytochrome C and stimulant, one tube received superoxide dismutase (SOD) and was placed in ice. Release of O_2^- in the incubated tube was determined from the difference in absorption of supernatants of each pair of tubes at 550nm .

Tests were set up in duplicate in 12 x 75 mm plastic tubes (Falcon #2054); the final volume was 600 µl. Final concentrations were: PA, EF, LF, as indicated; human serum albumin, 0.2%; PMN, 4 to 5 x 10^6/ml; LPS or MDP, as indicated; SOD (when present) 0.1mg/ml; cytochrome C, 0.12mM; stimulant, FMLP 10^{-7}M, or PMA as indicated. 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., phorbol myristate acetate (PMA) from Consolidated Midland Corp. FMLP and PMA were dissolved in dimethyl sulfoxide at concentrations of M/100 and 1 mg per ml respectively, held at -70°C in small quantities, and thawed and diluted in HBSS immediately before use. Lipopolysaccharide (LPS) from Escherichia coli K235 was obtained from List Biological Laboratories; it was suspended in HBSS at 1 mg per ml, dispersed by brief sonication and stored at 4°C. N acetylmuramyl-L-alanyl-D-isoglutamine (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 or New York Blood Center. The undiluted solution gave positive tests for LPS by the Limulus Amebocyte Lystate Test (using Pyrotell Reagent, sensitivity 0.01 ng of LPS, obtained from Cape Cod Associates, Inc.), but gave negative tests at 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^-. Each pair of absorption measurements was analyzed by subtracting the value for the tube with SOD from the tube without

SOD, and dividing the result by the PMN count in millions and the extinction coefficient of 0.0185 cm^2 per nanomole (21). This yielded O_2^- released in nanomoles per 10^6 PMN. Two sets of control tests without toxin were set up in each experiment 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, presumably reflecting individual differences in the proportion of PMN that respond to FMLP (22). Accordingly, the percent changes in O_2^- formation relative to the mean control value without toxin for each experiment were determined, means and standard deviations were calculated for replicate experiments, and these values reconverted to O_2^- in nanomoles per 10^6 PMN by reference to mean control values for the respective conditions.

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^- on stimulation with FMLP; treatment with a range of concentrations of LPS for 1 hour at 37° (priming) increased their subsequent release as much as eight fold (Figure 1). Smaller but appreciable effects were observed with concentations of LPS as low as 1 ng per ml. The levels of response of control PMN to low concentrations of LPS varied somewhat between cells from different donors, presumably reflecting individual variation in humoral immunity to LPS (data not shown)(23).

Pretreatment of PMN with PA + EF for 1 hour at 37° reduced markedly the levels of O_2^- released after LPS priming; reduction was 90% or more in the range from 1 to 100 ng per ml of LPS. The pretreatment produced

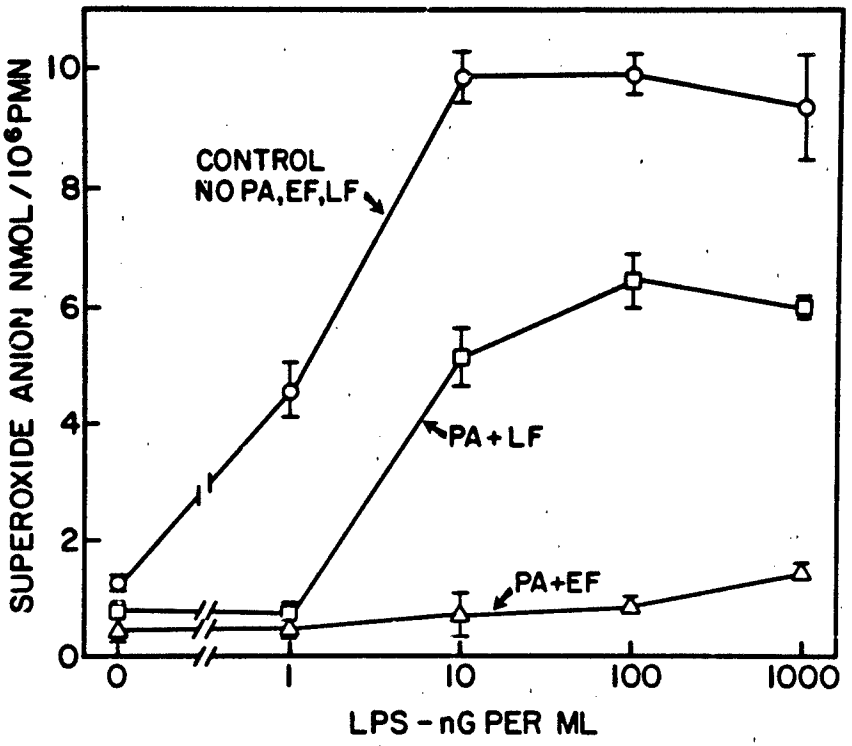

Figure 1. Release of O_2^- after stimulation of PMN with 10^{-7}M FMLP as a function of concentration of LPS during priming. PMN were incubated 1 hour at 37°C with control buffer, or with PA + EF (each 0.25 μg/ml), or with 0.25 μg/ml PA + 0.5 μg/ml LF. LPS was added and tests were incubated 1 hour at 37°, stimulated with FMLP, and O_2^- released in 10 minutes was measured. Means represent four values.

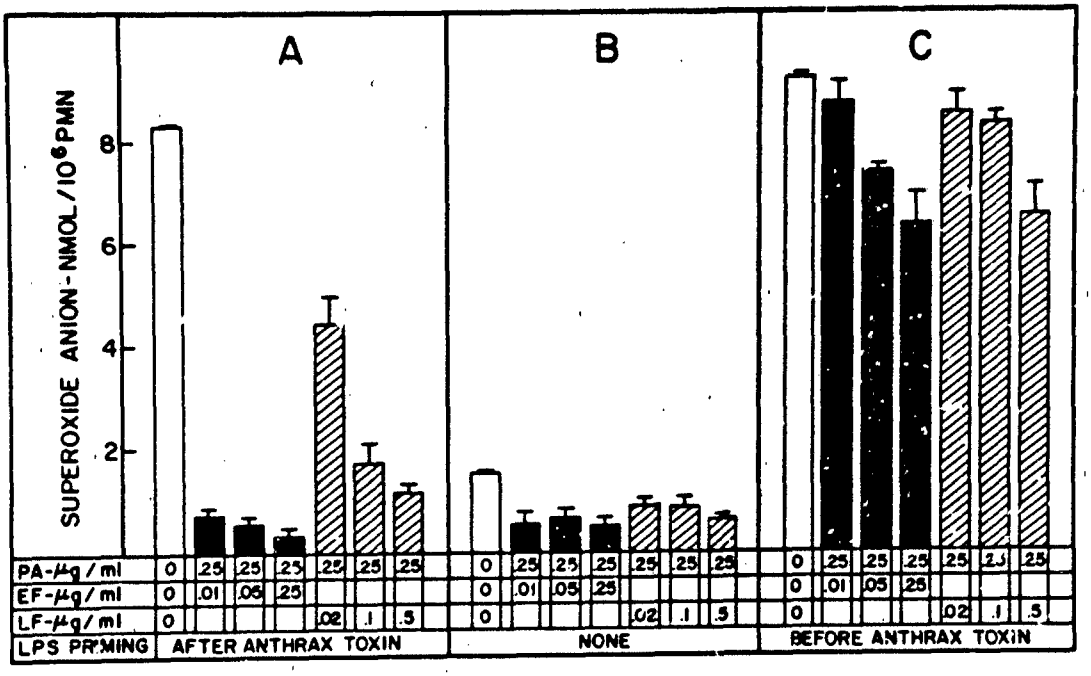

Figure 2. Release of O_2^- after stimulation of PMN with 10^{-7}M FMLP as a function of concentrations of PA, EF, and LF present during preincubation, and of conditions of priming with LPS, 3ng/ml. In section A, PMN were exposed to PA, EF, or LF in the concentrations shown for 1 hour at 37°C, after which LPS was added and the tests were incubated 1 hour at 37°C. Release of O_2^- in 10 minutes was determined after stimulation with FMLP. Dose related inhibition of O_2^- release relative to the control without anthrax toxin is evident both with PA + EF and with PA + LF. In section B, tests were carred 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 + EF or PA + LF. In section C, PMN were exposed to LPS prior to exposure to anthrax toxin; otherwise conditions were the same as in section A. Inhibition by PA + EF and PA + LF relative to the control is much less than in section A. Means represent 10 values for controls, 4 to 6 values elsewhere.

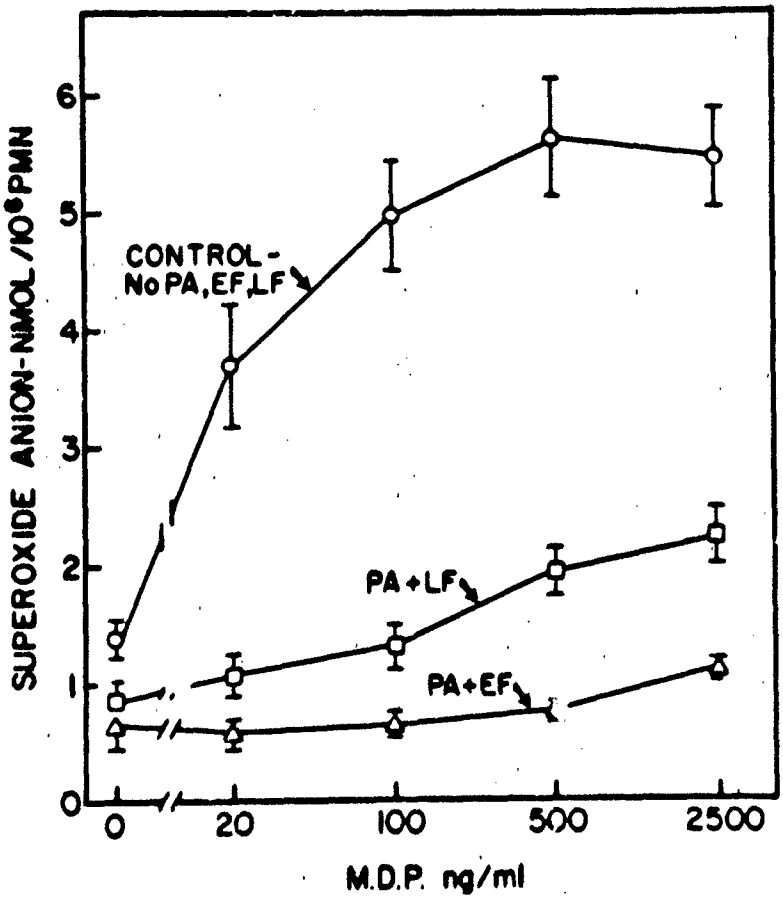

Figure 3. Release of O_2^- after stimulation of PMN with 10^{-7}M FMLP as a function of concentratin of N acetylmuramyl-L-alanyl-D-isoglutamine (MDP) during priming. PMN were incubated 1 hour at 37°C with control buffer, with 0.25 µg/ml PA + 0.5 µg/ml EF, or with 0.25 µg/ml PA + 0.5 µg/ml LF. MDP to reach the concentrations shown was added, and tests were incubated 1 hour at 37°C. PMN were stimulated with FMLP and O_2^- released in 10 minutes was measured. Means represent 8 values.

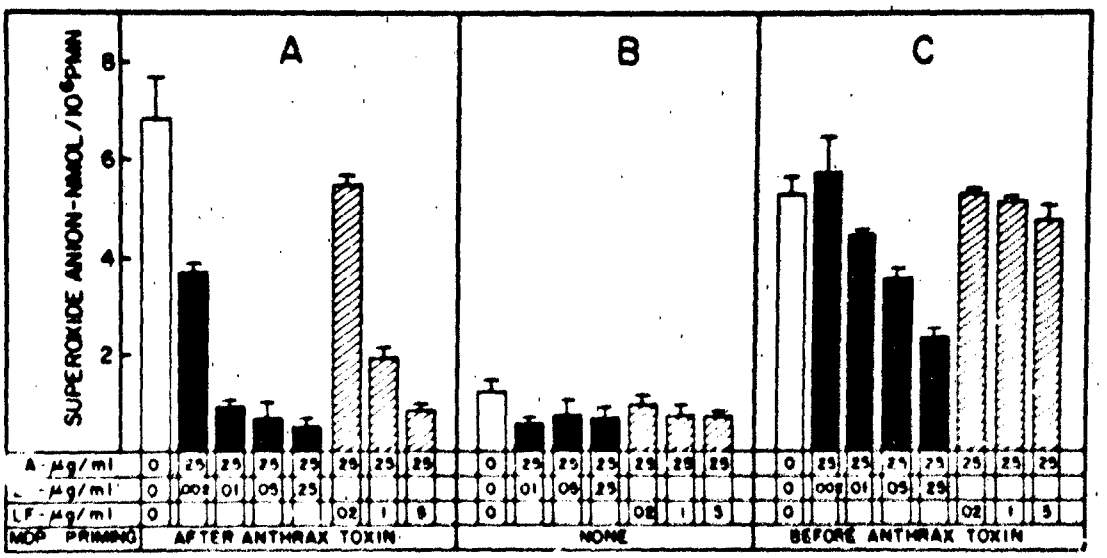

Figure 4. Release of O_2^- after stimulation of PMN with 10^{-7}M FMLP as a function of concentration of PA, EF, and LF present during preincubation, and of priming with MDP, 100 ng per ml. Experimental design was otherwise the same as for Figure 2. Means represent 6 to 10 values.

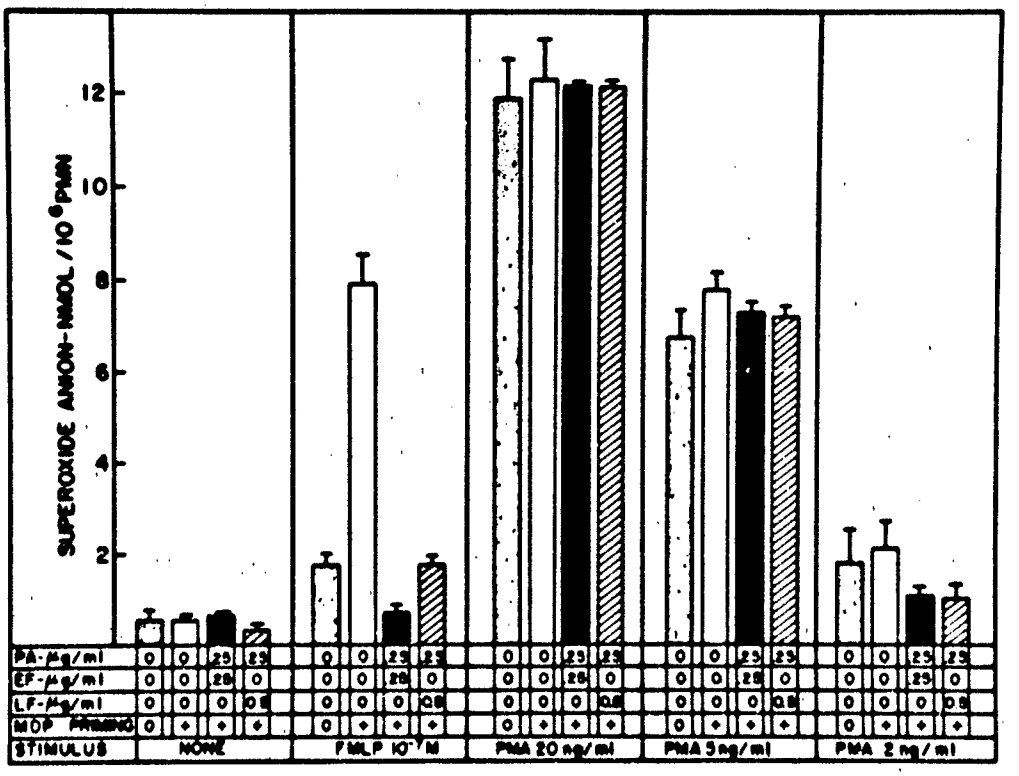

Figure 5. Comparison of the effects of stimulation of PMN by FMLP and 3 concentrations of PMA on priming effect of 100 ng per ml of MDP, and on the inhibitory effect of PA + EF and PA + LF. PMN were exposed first to anthrax toxin components or control buffer for 1 hour at 37°C, after which MDP to a final concentration of 100 ng/ml or control buffer was added and incubation continued for an additional hour at 37°C. Release of O_2^- in 10 minutes was measured after no stimulation, after stimulation with 10^{-7}M FMLP, or after stimulation with PMA at 20 ng/ml, 5 ng/ml, or 2 ng/ml. Means represent 4 to 6 values.

consistent reductions in the small amount of O_2^- released from PMN not primed with added LPS. Pretreatment with PA + LF also reduced the release of O_2^- after priming and stimulation, but the effect was less marked than the effect of PA + EF, especially at higher concentrations of LPS. After priming with 1 ng per 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 (Figure 2). PA, when present, was held constant at 0.25 ug per ml. It is evident that both PA + EF and PA + LF gave strong inhibition over a range of concentrations when LPS priming was carried out after exposure to anthrax toxin; reversing this order markedly reduced the inhibitory effect. Strong inhibition was otained with 0.01 µg per ml of EF; LF was less active, but gave 84% inhibition of O_2^- release at 0.5 µg per 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 (Figure 3 and 4). Priming effects were slightly less than with LPS, and the higher concentrations of MDP did not overcome the inhibitory effects of PA + LF to as great a degree as did LPS. In addition, PA + 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, FMLP

10^{-7}M, and three concentrations of PMA: 20, 5, and 2 ng per 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 + EF and MDP priming, PA + LF and MDP priming. The results (Figure 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 + EF or PA + LF.

Discussion

The marked inhibition of FMLP-induced O_2^- release produced by pretreatment of PMN with PA + EF or PA + LF prior to priming with LPS or MDP indicates that anthrax toxin alters the cells in a manner such that they resist priming. When PMN were primed first, 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 seems reasonable in light of 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 (17,18). Release of these substances is responsible for extracellular killing of bacteria (24, 25); possession of mechanisms for inhibiting their release would be expected to contribute significantly to virulence.

Guthrie et al (17) have shown that priming in vitro is a relatively slow process, requiring about 1 hour to approach completion at 37°C. The process did not increase binding of FMLP to receptors, nor involve synthesis of protein; it was associated with an increased V_{max} of the NADPH oxidase, suggesting that activation of oxidase was more efficient in primed PMN.

Results in Figure 2 provide explanations for initial difficulties in obtaining consistent effects of anthrax toxin when LPS was not controlled, and may explain, in part, the results of others (11). Exposure of PMN to 3 ng

per ml of 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, exclusion of LPS prevented priming, and reduced the release of O_2^- in controls to a level from 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 analogies with activation of macrophages (26,27), provides a closer link to B. anthracis, because MDP is related to peptidoglycans of bacterial cell walls in structure and activity (27,28). MDP has been reported to be inactive in priming of PMN under other conditions, however (29).

Priming of PMN by LPS not only increases the release of O_2^- in response to stimuli, but also enhances release of lysosomal enzymes, induces spontaneous change in shape (18), and modulates chemotaxic responsiveness (30,18). Previous observations that chemotaxis was stimulated by treatment with anthrax toxin (12) 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 appears more probable than the tentative explanation suggested previously, that anthrax toxin inhibits the secretory activities of PMN that modulate chemotaxis.

Pretreatment with 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. (17), obtained a priming ratio (O_2^- with priming/O_2^- without priming) of 1.64, which is markedly smaller than the ratio of 7.76 which they obtained with FMLP stimulation. The results with PMA suggest that priming alters the polyphosphoinositide transmembrane signal mechanism at a point proximal to activation of protein kinase C, since PMA bypasses receptor activation and activates protein kinase C directly (31). The concept that PA + EF inhibits priming by LPS is consistent with evidence that PA + EF forms cAMP in PMN (12), and the report that inhibition of chemotaxis by LPS, an aspect of priming, is blocked by substances that raise intracellular cAMP (30).

In platelets, cAMP and functionally related substances activate protein kinase A, resulting in phosphorylation of at least 4 endogenous proteins that are involved in regulation of cell functions (31,32). Analogous reactions, should they occur after treatment of PMN with PA + EF, could account for inhibition of priming and provide clues to its nature. Enzymatic activity has not been identified for LF, and no evident basis can be proposed for its generally similar but less marked effect on priming.

The present studies have focused on the action of anthrax toxin on PMN. However, the basic mechanisms of intracellular signaling and control are conserved over a wide range of tissues (31), and it is probable that actions of toxin closely related to those operative against PMN are involved in pathologic changes in other tissues. Terminal vascular changes in anthrax infection and toxemia are similar to changes occurring in septicemias of other etiologies (9); this provides support for the seemingly reasonable expectation that the effective virulence mechanisms attributed to anthrax toxin have been

conserved among infectious agents. Expanding knowledge of the conditions for elaboration of the toxin and for expression of its biological effects should facilitate efforts to identify additional agents with analogous actions.

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