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Stimulus-Specific Effects of Endotoxin on Superoxide Production by Rabbit Polymorphonuclear Leukocytes*

JAMES T. ROSENBAUM, M.D.,[a] AND HOLLY ENKEL, B.A.[b]

[a]*Oregon Health Sciences University, Portland, Oregon;* [b]*Kuzell Institute for Arthritis and Infectious Diseases, San Francisco, California*

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The release of superoxide (O_2^-) by polymorphonuclear leukocytes (PMN) is an important function that contributes to microbial death. Controversy exists as to the effect of bacterial endotoxin (lipopolysaccharide, or LPS) on the production of O_2^-. We have injected rabbits with 25 μg *Escherichia coli* LPS intravenously and studied PMN function 18 to 24 hours later. Relative to PMN from saline-injected controls, PMN from LPS-treated rabbits released markedly greater amounts of O_2^- in response to 10 ng/ml phorbol myristate acetate (PMA) as measured by nmol cytochrome C reduced in 20 minutes (40.8 ± 7.8 for LPS-treated PMN versus 10.1 ± 1.6 for control, $p < 0.01$). LPS injection, however, significantly reduced O_2^- release in response to C (complement) 5a (1.4 ± 0.6 nmole/20 minutes for LPS-treated PMN versus 5.6 ± 1.3 nmole/20 minutes for control, $p < 0.01$). O_2^- release in response to a third stimulus, n-formyl-methionyl-leucyl-phenylalanine (10^{-7} to 10^{-9} M), was not affected by LPS. O_2^- release in response to PMA was enhanced over a wide range of PMA concentrations (10 to 300 ng/ml). Kinetic studies over 30 minutes indicated that, after a brief initial latency in measurable response, LPS enhanced responsiveness to PMA at all time points observed. The reduced responsiveness to C5a corresponds to a previously reported down regulation of receptors for this ligand after intravenous LPS. The observations indicate that intravenous LPS can alter a critical function of PMN for at least 24 hours in a stimulus-specific manner.

INTRODUCTION

Studies on the effect of endotoxin (bacterial lipopolysaccharide, or LPS) on the production of superoxide by polymorphonuclear leukocytes (PMN) have yielded conflicting results. Guthrie and colleagues [1], for example, have reported that the exposure of human PMN to endotoxin *in vitro* enhances their capability to release superoxide in response to immune complexes, phorbol myristate acetate (PMA), or n-formyl-methionyl-leucyl-phenylalanine (FMLP). In contrast, Proctor [2] found that prior exposure of human PMN to LPS reduced their ability to release O_2^- in response to bacterial stimuli. Henricks and colleagues [3] used 18-hour incubations of PMN and LPS to characterize the effects of endotoxin on O_2^- release. These investigators found that LPS from *Escherichia coli* 0111B4 and from the polysaccharide-deficient mutant J5 enhanced O_2^- production when LPS itself was used as a stimulus. Using staphylococcus as a stimulus, however, LPS 0111B4 enhanced O_2^- release, while the LPS from J5 reduced it.

We have recently characterized the effects of intravenously injected endotoxin on PMN function in a rabbit model. We have previously reported that intravenously

Abbreviations: C5a: complement 5a FMLP: n-formyl-methionyl-leucyl-phenylalanine LPS: lipopolysaccharide PMA: phorbol myristate acetate PMN: polymorphonuclear leukocyte

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Address reprint requests to: James T. Rosenbaum, M.D., Oregon Health Sciences University, L329A, 3181 S.W. Sam Jackson Park Road, Portland OR 97201

injected endotoxin *(1)* inhibits the vascular permeability and PMN extravasation from blood vessels that is associated with a dermal reversed passive Arthus reaction [4]; *(2)* inhibits chemotaxis and degranulation in response to C (complement) 5a but not in response to FMLP [4,5]; and *(3)* down regulates the receptor for C5a while causing a marked increase in the number of receptors for FMLP [6].

Superoxide production plays an integral part in microbial killing by PMN and also contributes to inflammation. We have assessed the effects of intravenously injected endotoxin on the ability of rabbit PMN to release O_2^-.

METHODS

Animals

Two-kilogram New Zealand White female rabbits were purchased from Animals West (Soquel, CA). They were housed in the animal care facilities of the Medical Research Institute of San Francisco and fed standard laboratory chow.

Isolation of PMN

Rabbit PMN were isolated from acid-citrate-dextrose anticoagulated blood obtained from the central ear artery, as we have previously described [4]. Thirty-four ml of blood plus 6 to 8 ml of anticoagulant were mixed with 8 ml of 6 percent (weight/volume) Dextran T500 (Pharmacia Fine Chemicals, Piscataway, NJ) in normal saline. After sedimenting for approximately 30 minutes, the supernatant was layered over 56 percent Percoll (Sigma Chemical Co., St. Louis, MO) and centrifuged at 450 *g* for 20 minutes. The erythrocyte/PMN pellet from this centrifugation was suspended for seven minutes in 40 ml of 8.3 percent (weight/volume) ammonium chloride (pH 7.2) to produce lysis of erythrocytes. The pellet of PMN was then washed twice with phosphate buffered saline (300 mM, pH = 7.2) (GIBCO, Grand Island, New York).

Endotoxin

E. coli LPS 055:B5 was purchased from RIBI Immunochem (Hamilton, MT). It was suspended in saline prior to use and stored at −20°C. Control rabbits received sterile, pyrogen-free saline in volume comparable to that used for endotoxin injection.

Superoxide Release Assay

Rabbit PMN were suspended in a buffer of Hanks' balanced salt solution (GIBCO) plus 0.25 percent bovine serum albumin (Sigma Chemicals, St. Louis, MO) at a concentration of 5×10^6/ml. Stimuli in variable concentrations were added to the reaction mixture that included PMN and ferricytochrome C (Sigma) (0.08 mM in phosphate buffered saline, final concentration). The reaction was stopped after 20 minutes by placing the cells on ice for five minutes. Cells were removed by centrifugation at 2,000 rpm for five minutes and the reduction of ferricytochrome C was judged by the change in absorption at 550 nm [7]. The reduction of ferricytochrome C could be inhibited by including superoxide dismutase (Sigma) (20 ng/ml) in the reaction mixture. Data are expressed as the reduction of ferricytochrome C in response to stimulus minus the reduction in control reaction mixtures incubated in buffer alone. To control for day-to-day assay variability, each assay included PMN from one saline-injected rabbit and one endotoxin-injected rabbit. For kinetics experiments, cells were maintained at 37°C in a Perkin-Elmer spectrophotometer with

TABLE 1
The Effect of Intravenous LPS on PMN Superoxide Reduction

Stimulus	Source of PMN	
	LPS-Injected	Saline-Injected
C5a (1 percent) ($n = 8$)	1.4 ± 0.6	5.6 ± 1.3
PMA (10 ng/ml) ($n = 9$)	40.8 ± 7.8	10.1 ± 1.6
FMLP (10^{-7}M) ($n = 6$)	8.2 ± 3.3	9.7 ± 1.6

Data expressed as nmol cytochrome C reduced/20 minutes

continuous stirring. Absorbance readings were made once per minute. Rabbit C5a was partially purified from zymosan (ICN Biochemicals, Cleveland, OH) activated rabbit serum through a combination of sizing and ion exchange column chromatography, as we have previously described [4]. The concentration used for superoxide release was 1 percent, roughly twofold greater than the optimal concentration for chemotaxis [5]. PMA was purchased from Sigma. FMLP was purchased from Peninsula Laboratories (San Carlos, CA), dissolved in dimethyl sulfoxide, and stored frozen at −20°C.

Statistics

Data are expressed as mean ± SE and compared by the student's *t*-test.

RESULTS

Rabbits received 25 μg of LPS intravenously or a comparable volume of saline and were bled 18 to 24 hours later. As shown in Table 1, PMN from LPS-treated rabbits released significantly less O_2^- in response to 1 percent C5a than PMN from saline-injected controls ($p < 0.01$). In marked contrast, PMN from LPS-treated rabbits released fourfold more O_2^- in response to PMA (10 ng/ml) than PMN from control rabbits ($p < 0.01$). Both sets of PMN released O_2^- comparably in response to FMLP (10^{-7} M). The unstimulated release of O_2^- was also comparable for the two groups (7.2 ± 1.4 nmol/20 minutes vs. 8.0 ± 1.8 nmol/20 minutes saline-treated versus LPS-treated PMN, respectively). Superoxide release was also studied at two lower concentrations of FMLP, 10^{-8} and 10^{-9} M. For three pairs of rabbit PMN at each concentration, there was no statistical difference in O_2^- release (data not shown).

The increased capacity to release O_2^- was evident at concentrations of PMA ranging from 10 to 300 ng/ml (Fig. 1). Increasing the PMA concentration from 100 to 300 ng/ml appeared to induce minimal additional release of O_2^-, suggesting that PMN had reached a plateau in responsiveness to PMA at a similar concentration, regardless of the prior *in vivo* exposure to endotoxin (Fig. 1). In the studies summarized in Fig. 1, PMN from saline-treated rabbits were more responsive to PMA (10 ng/ml) than the control PMN, whose responses are noted in Table 1. This discrepancy reflects the daily variability of the assay. To control this variability, paired observations were made such that PMN from one endotoxin-injected rabbit and one saline-injected rabbit were each tested in the same day's assay.

As shown in Fig. 2, after a short initial latency in responsiveness, superoxide production appeared to be linear for PMN obtained after either an LPS or saline injection. The rate of production or slope differs in the two curves, but the linearity of the response is preserved.

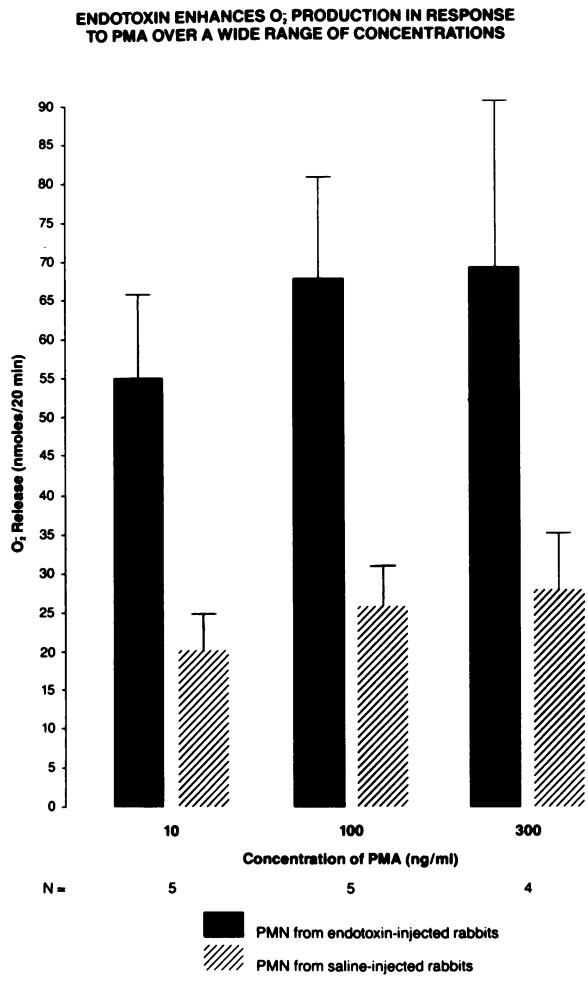

FIG 1. PMN from endotoxin-injected rabbits released greater amounts of superoxide at each concentration of PMA tested. For both PMN from endotoxin-injected rabbits and from saline-injected controls, increasing PMA from 100 to 300 ng/ml induced only a minimal increment in superoxide release.

DISCUSSION

We have previously demonstrated that intravenously injected LPS results in stimulus-specific changes in rabbit PMN [4–6]. Stimulus-specific changes have been characterized relative to receptor number and functional alterations in chemotaxis and degranulation [4–6]. Recently in clinical studies stimulus-specific changes in PMN function, including a depressed chemotactic response to C5a, have been noted in such diverse disorders as burns and inflammatory skin disease [8,9]. The present study extends our previous observations by considering the effect of intravenous LPS on the ability of PMN to generate superoxide in response to soluble stimuli.

Similar to the observations of Guthrie and colleagues [1], we find that LPS can markedly enhance the release of O_2^- by PMN. Our observations, however, are in contrast to previous studies in that we have studied a system that involves *in vivo* exposure to LPS, and we find that the enhancement of O_2^- production is highly stimulus-specific. Using PMA as a stimulus, intravenous LPS appears to enhance O_2^- release independent of the dose of PMA or of the time point studied. In contrast, intravenous LPS reduced responsiveness to C5a and did not alter responsiveness to

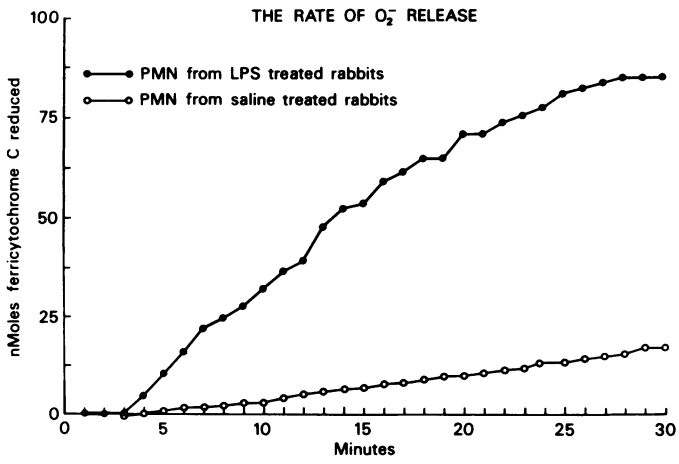

FIG. 2. PMN from LPS-treated rabbits and from controls both released superoxide in an apparently linear fashion after an initial lag period; 10 ng/ml PMA was used as a stimulus. Data are from a single experiment representative of three.

FMLP. The reduced response to C5a without comparable change in response to FMLP is also seen for chemotaxis, degranulation, and dermal vascular permeability [4,5].

A number of mechanisms might contribute to these observations. Direct binding of LPS, synthesis of soluble factors such as tumor necrosis factor, down regulation of receptors for the chemotactic ligand caused by the generation of chemotactic factors, and demargination of subpopulations of PMN could each contribute to the effects of LPS on PMN, as we have previously noted [4,5]. The alteration of PMN function after *in vivo* exposure to LPS supports the likelihood that this effect is physiologic. It may account in part for the enhanced oxidative metabolism (as measured by chemiluminescence, O_2^- release, or nitro-blue tetrazolium reduction) that has been noted in association with bacterial infection by some investigators [10–12].

Since intravenous LPS down regulates the receptor for C5a [6], the LPS-induced reduction in O_2^- release in response to this ligand may at least in part reflect a receptor-determined change. Since intravenous LPS markedly increases the number of receptors for FMLP [6] without enhancing O_2^- release in response to that stimulus, however, it appears that additional subsequent post-receptor events must be rate-limiting. Furthermore, since O_2^- release in response to PMA is enhanced by intravenous LPS, the post-receptor regulation of superoxide release may differ for PMA and FMLP. Other investigators have reported that discrepant intracellular events appear to determine PMN responses to chemotactic ligands, as opposed to responses to PMA. Pertussis toxin, for example, an inhibitor of the guanosine triphosphate binding protein, N_i, inhibits granule release in response to C5a and FMLP, but not in response to PMA [13]. In guinea pig PMN, FMLP induces the breakdown of phosphatidylinositol 4,5 biphosphate and the subsequent formation of diacylglycerol, phosphatidic acid, and free arachidonic acid. PMA fails to induce the breakdown of phosphatidylinositol 4,5 biphosphate or the release of arachidonate [14]. A recent study indicates that a phagocytosable stimulus induces O_2^- release through a different pathway, in comparison to PMA [15]. Different intracellular pathways or differential activation of regulatory substances that control the same metabolic pathway could conceivably account for discordant effects on O_2^- release in response to different stimuli. This hypothesis could account for the ability of LPS to enhance or suppress O_2^- release, depending upon the stimulus studied.

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