Stimulation of Neutrophil Oxygen-dependent Metabolism by Human Leukocytic Pyrogen

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ABSTRACT The ability of highly purified human leukocytic pyrogen (LP) to stimulate neutrophil oxygen-dependent metabolism was studied. Human peripheral blood neutrophils exposed to leukocytic pyrogen in vitro demonstrated an increase in the percentage of neutrophils reducing nitroblue tetrazolium (NBT) dye and a marked stimulation of superoxide dismutase inhibitable reduction of ferricytochrome c. LP stimulation of neutrophil oxygen-dependent metabolism was dose and time dependent. Procedures that destroyed the pyrogenicity of LP also abolished the effects on neutrophil metabolism. Neutrophil hexose monophosphate shunt activity was also stimulated by LP.

In a rabbit model, the effect of in vivo LP on neutrophil superoxide generation was also studied. There was a consistent increase in the percent and absolute number of NBT positive neutrophils. Peak stimulation of neutrophil metabolism occurred after defervescence suggesting several possible mechanisms. The observations reported here may, in part, explain the nonspecificity of the NBT test in febrile, noninfected patients and provide further understanding of neutrophil physiology during acute inflammation.

INTRODUCTION
Normal human peripheral blood neutrophils are capable of reducing nitroblue tetrazolium dye (NBT)\(^1\) in vitro. The observation that the percentage of neutrophils spontaneously reducing NBT was markedly increased during bacterial sepsis led to the original suggestion that this test would differentiate patients with fever and leukocytosis caused by bacterial infection from patients with other causes of fever (1, 2). However, numerous investigators have reported that neutrophil NBT reduction is increased in viral hepatitis (3), malaria (4), active Hodgkin's disease (5), tuberculosis (6), pulmonary embolism (7), as well as in other diseases (8). NBT dye reduction is due, at least in part, to an interaction of neutrophil generated superoxide anion (\(\text{O}_2^-\)) with the dye resulting in formation of insoluble formazan (9). Direct interaction of pyridine nucleotide-dependent diaphorases with NBT may also occur (10). Although it has been shown that perturbation of the neutrophil membrane by a variety of particulate and soluble stimuli causes \(\text{O}_2^-\) generation thereby enhancing NBT dye reduction, the stimulus for enhanced in vivo neutrophil NBT dye reduction in febrile, noninfected patients has remained obscure.

With the availability of highly purified human leukocytic pyrogen, we have recently demonstrated that this small molecular weight protein, which mediates fever, is also capable of inducing lysosomal exocytosis (11). Because virtually all neutrophil secretagogues also provoke the "respiratory burst" and \(\text{O}_2^-\) generation, we investigated the ability of leukocytic pyrogen to stimulate neutrophil oxygen-dependent metabolism. Moreover, we were interested in the possible relationship of leukocytic pyrogen to the reduction of NBT by neutrophils. The results of these studies form the basis of this report.

METHODS
Preparation of neutrophils. Heparinized venous blood was collected from healthy adult volunteers and centrifuged on Hypaque-Ficoll (Hypaque, Winthrop Laboratories, New York; Ficoll, Pharmacia Fine Chemicals Piscataway, N. J.) gradients followed by dextran sedimentation (12). Erythrocytes were lysed with hypotonic saline and the resulting leukocyte suspension washed twice in Hanks’ balanced salt solution (HBSS, National Institutes of Health media unit) containing 1.0 mM CaCl\(_2\) and 0.5 mM MgCl\(_2\). Leukocytes obtained in this manner contained 96–98% neutrophils.
Viability, based on trypan blue exclusion, exceeded 97% in all neutrophil preparations.

Preparation of leukocyte pyrogen. Leukocytic pyrogen (LP) was prepared from human mononuclear cells as described (13). Supernates containing crude LP were concentrated and placed over a rabbit antihuman LP immuno-absorbent column (14). LP was eluted from the immuno-absorbent column in citric acid buffer, chromatographed over a Sephadex G-50 column (Pharmacia Fine Chemicals) and the 15,000 mol wt LP peak isolated. Further purification was carried out using Sephadex G-15 and DEAE ion exchange chromatography as described (15). To determine the number of pyrogenic doses in preparations of LP a 2-point dose-response was employed using a rabbit bioassay. Based on the 15,000 mol wt of human LP, 1 rabbit pyrogenic dose (RPD) is ≤1 nM.

NBT dye reduction. Neutrophil NBT dye reduction was measured by a modification of the method of Park (1). Briefly, for in vivo experiments, heparinized (20 U/ml) whole blood was incubated with an equal volume of 0.2% NBT in 0.85% NaCl for 15 min at 37°C and then for 15 min at room temperature. For in vitro experiments neutrophils in HBSS (5 × 10^9/ml) or in HBSS containing varying concentrations of LP were mixed with an equal volume of 0.2% NBT and incubated as above. Duplicate stained (Wrights) smears were prepared and 200 neutrophils counted on each slide. Cells containing either stippled cytoplasmic formazan dye or more commonly, a single large cytoplasmic deposit of formazan dye were counted as positive. Results are expressed as the percentage of neutrophils reducing NBT to formazan.

Phagocytosis. The effect of LP on phagocytosis was determined by measuring uptake of 1C-radiolabeled heat-killed Staphylococcus aureus as described (16). Neutrophils suspended in HBSS plus 10% AB serum were incubated with bacteria in the presence or absence of LP (5 RPD/ml) at a ratio of 5-10 bacteria per neutrophil. After specific incubation intervals at 37°C, phagocytosis was stopped by adding iced buffer, and the suspension was centrifuged at low speed (75 g) for 10 min. Supernates were aspirated to remove non-cell associated bacteria. The cells were resuspended, and the wash procedure was repeated three times. Phagocytosis was expressed as percentage of total bacteria phagocytized (average cell associated counts per minute/total counts per minute in incubation mixture ×100).

Determination of neutrophil O2− generation. O2− production by neutrophils was measured by the capacity of O2− to reduce ferricytochrome c to ferrous cytochrome c (Neutrophil suspensions (5 × 10^9/ml in HBSS) were incubated at 37°C for 45 min in 0.1 mM ferricytochrome c, type VI (from horse heart; Sigma Chemical Co., St. Louis, Mo.), either with or without superoxide dismutase (SOD, 100 μg/ml) (Sigma Chemical Co.), and with varying concentrations of LP (final reaction volume of 2 ml). For comparison, latex particles (25/μl), (Dow Corning Corp., Midland, Mich.) were incubated with neutrophil suspensions containing the same concentrations of ferricytochrome c in the presence and absence of SOD (100 μg/ml). Control experiments were comprised of neutrophil suspensions incubated with either heat-inactivated LP (80°C for 20 min) or HBSS. For kinetic experiments, polymorphonuclear leukocyte (PMN) suspensions were first incubated at 37°C for 15 min in 0.1 mM ferricytochrome c with or without SOD. Prewarmed HBSS or LP suspended in 0.1 mM ferricytochrome c with and without SOD was then added to the reaction mixture. Additional controls consisted of buffer or LP in 0.1 mM ferricytochrome c (≥100 μg/ml SOD) in the absence of neutrophils. Aliquots (0.2 ml) were removed immediately after the addition of buffer or LP (zero time control) and then at 2, 5, 10, 20, 30, and 45 min. 0.2-ml volumes of the supernates from either the dose-response or kinetic experiments (both performed in triplicate) were added to 0.6 ml of 100 mM sodium phosphate buffer, pH 7.2 for spectrophotometric assay in 1.0 ml quartz cuvettes. The absorption spectrum of the samples was continuously measured from 540 to 560 nm (Gilford 250 spectrophotometer, Gilford Instrument Laboratories Inc., Oberlin, Ohio) and the point and amplitude of maximum absorption (usually 549 or 550 nm) was recorded. The reduced cytochrome c in the samples was then totally oxidized by the addition of a few crystals of ferricytochrome a and the absorbance remeasured at the wave length of the prior point of maximal absorption. Nanomoles of cytochrome c reduced were calculated from the maximal increase in absorbance measured using an absorption coefficient of 21.1 mM⁻¹ cm⁻¹ (reduced—oxidized) (17). Occasionally the total cytochrome c concentration was checked by reduction of the samples with dithionite. Superoxide production was calculated as the difference between parallel samples in the absence and presence of SOD. The results are expressed as nanomoles of SOD-inhibitable cytochrome c reduced/5 × 10⁶ neutrophils.

Hexose monophosphate shunt activity. Hexose monophosphate (HMP) shunt activity was measured according to a modification of the method of Goetzl and Austen (18) using the conversion of [1-14C]glucose to 14CO2. 2-ml aliquots of neutrophil suspension (5 × 10^9/ml in HBSS) were placed in duplicate or triplicate 35 mm diameter plastic Petri dishes and incubated at 37°C for 30 min. Nonadherent neutrophils were removed by washing and 0.4 μCi of [1-14C]glucose (Amersham Corp., Arlington Heights, Ill) was added to each dish followed by 1.0 ml of additional HBSS or LP in HBSS. After a 1-h incubation at 37°C, 0.1 ml of 2 N HCl was added through a small hole to each dish and resealed. The amount of 14CO2 driven off into the gaseous phase and trapped in a NaOH soaked filter paper over a 10-min period was quantitated by placing filters in 10 ml liquid scintillation cocktail (Aquasol, New England Nuclear, Boston, Mass.) and counted in a Beckman LS scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). Initial experiments with [6-14C]glucose generated ≤5% 14CO2, indicating specificity of the reaction for the HMP pathway. For protein determinations triplicate dishes containing all the reagents except [1-14C]glucose underwent identical manipulations and after the final incubation 1 ml 2 N NaOH was added to the dish and the contents aspirated. Protein content was determined by the Lowry et al. (10) method. Results are expressed as counts per minute/0.1 mg protein.

Effect of LP in vivo. A rabbit model was used to determine if LP stimulated neutrophil oxygen-dependent metabolism in vivo. Rabbits were injected with 1 RPD of LP into a marginal ear vein and temperature continuously monitored via a rectal probe (Telethermometer, model 80; Yellow Springs Instrument Co., Yellow Springs, Ohio). Heparinized venous blood samples were obtained from the contralateral ear vein collected just before LP injection and then at various time intervals. The percentage of neutrophils reducing NBT dye was then determined on duplicate samples. Control animals were given saline or heat-inactivated LP and blood samples obtained as for LP injected animals.

Statistics. Student’s t test was used to compare means and standard errors for significance.

RESULTS

NBT dye reduction in vitro. As shown in Fig. 1, 4.3±1.2% of human peripheral blood neutrophils incubated in buffer alone spontaneously reduced NBT.
When neutrophils were exposed to increasing concentrations of LP (1–4 RPD/ml), there was a progressive stimulation of neutrophil reducing capacity. In a single experiment, 83% of neutrophils exposed to a higher concentration of LP (10 RPD/ml), reduced NBT (not shown). Heating LP at 80°C for 20 min, a procedure that destroys the pyrogenicity of LP, also destroyed its ability to stimulate neutrophil NBT dye reduction.

**Ferricytochrome c reduction.** As a more quantitative measurement of superoxide generation, the ability of LP to stimulate neutrophil ferricytochrome c reduction was determined. In the absence of SOD, neutrophils in buffer reduced 1.3±0.2 nmol/5 x 10⁶ PMN/45 min. Over a wide concentration range of LP there was a progressive increase in the amount of ferricytochrome c reduced (Fig. 2). Even at only one-tenth the amount of pyrogen required to cause fever in rabbits, there was significant stimulation of neutrophil reducing capacity in vitro.

To determine if ferricytochrome c reduction was dependent on superoxide generation, SOD (100 µg/ml) was added to paired samples of each LP concentration (see Methods). As shown in Fig. 2, there was 95% ablation of the cytochrome c reduction by the LP-stimulated PMN in the presence of SOD, suggesting that the major LP stimulated neutrophil reducing substance was superoxide. Phagocytosis of latex particles also stimulated SOD-inhibitable ferricytochrome c reduction. In three separate experiments, neutrophils reduced 2.65±0.18 nmol ferricytochrome c/5 x 10⁶ cells per 45 min. These results closely agree with previous observations (20).

A series of additional controls were performed to insure that LP itself was not capable of reducing ferricytochrome c and to determine if heat inactivation of the pyrogenicity of LP would also destroy its ability to enhance neutrophil ferricytochrome c reduction (Table 1). In five separate experiments neutrophil ferricytochrome c reduction was markedly stimulated by LP (8.3±0.5 vs. 1.2±0.4 nM cytochrome c reduced/45 min with and without LP) and was inhibited to background levels by SOD. In the absence of neutrophils, LP did not produce a significant increase in ferricytochrome c reduction (P > 0.20) and the small increase which was observed was not SOD-inhibitable. These observations indicate that reduction of ferricytochrome c is not caused by the direct action of LP, but rather LP induces the liberation of O₂⁻ from neutrophils with subsequent cytochrome c reduction. As with our qualitative NBT observations, heat inactivation of LP markedly reduced its ability to stimulate neutrophil cytochrome c reduction.

![Figure 1](https://example.com/mole.png)  
**Figure 1** Dose-response of LP-induced human neutrophil NBT dye reduction in vitro. Results are the mean±SEM of four separate experiments.

![Figure 2](https://example.com/mole.png)  
**Figure 2** Dose-response of LP-stimulated human neutrophil superoxide generation. Results are the mean±SEM of three separate experiments.
TABLE I

Inability of LP or Heat-inactivated LP to Directly Cause SOD-inhibitable Ferricytochrome c Reduction

<table>
<thead>
<tr>
<th>LP (10 RPD/ml)</th>
<th>PMN (5 x 10⁶/ml)</th>
<th>SOD (100 μg/ml)</th>
<th>O₂ generated</th>
<th>nmol cytochrome c reduced/45 min</th>
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<td>Heat-inactivated</td>
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Results are the mean±SEM of five separate experiments.

The kinetics of LP stimulated neutrophil ferricytochrome c reduction are shown in Fig. 3. By 5 min neutrophils incubated with LP reduced significantly more ferricytochrome c than cells in buffer alone. The greatest rate of cytochrome c reduction occurred between 5 and 20 min. Longer incubation (30–45 min) resulted in additional ferricytochrome c reduction although at a slower rate.

HMP shunt activity. The ability of leukocytic pyrogen to stimulate neutrophil 14CO₂ production from [14C]glucose is shown in Fig. 4. Stimulation of adherent monolayers of neutrophils by 2 RPD per milliliter of LP produced a fourfold increase above buffer control in the amount of 14CO₂ generated. Higher concentrations of LP (4 and 10 RPD/ml) further augmented HMP shunt activity. When neutrophils were omitted from the reaction mixture, there was no significant generation of 14CO₂ from [1-14C]glucose. 14CO₂ generation from [6-14C]glucose was consistently <5% of that from [1-14C]glucose, implying that 14CO₂ generation was the result of HMP shunt activation. In related studies, when heat-inactivated LP was used as a stimulus there was no effect on HMP shunt activation (data not shown).

In vivo LP. In a rabbit model, the effect of in vivo LP on the percentage of peripheral blood neutrophils reducing NBT was also assessed (Fig. 5). After the intravenous injection of 1 RPD of LP, there was a typical febrile response reaching maximal temperature by 50 min with rapid defervescence over the next 1–2 h. In five separate experiments the changes in NBT positive neutrophils revealed certain trends. First, neutrophils from each animal displayed enhancement of the percentage of neutrophils spontaneously reducing NBT. In four of five animals peak response occurred after the maximal temperature elevation and in four of five animals, maximal neutrophil stimulation was observed after the animal had defervesced to within 0.3°C of basal body temperature. The absolute neutrophil counts at 0, 0.5, 1, 2, and 4 h were 1,910±510 mm³, 580±230/mm³, 4,270±1,100/mm³, 7,000±1,800/mm³, and 5,020±1,100/mm³, respectively. Leukocyte counts paralleled these changes (6,620±280/mm³, 3,950±820/mm³, 7,020±960/mm³, 10,550±1,860/mm³, and 9,620±1,020/mm³ at the same time intervals). In a single experiment neutrophils isolated from whole blood at 0, 0.5, 2, and 5 h after the intravenous injection of LP were incubated at 37°C with 0.1% NBT for 15 min. Quantitative extraction of formazan (21) revealed a ΔOD₅₉₅/2.5 x 10⁶ PMN of 0.030, 0.062, 0.173, and 0.036, respectively; the peak NBT dye reduction 2 h after intravenous pyrogen...
DISCUSSION

Multiple investigators have reported alterations in neutrophil metabolism, structure, and function during the course of febrile or inflammatory illnesses. In particular the increased ability of neutrophils to reduce nitroblue tetrazolium is often observed under these conditions. Initial reports suggested that the increase in NBT-positive neutrophils would be a useful adjunct in differentiating febrile patients with bacterial infection from those with nonbacterial inflammatory disorders. However, as a result of multiple studies showing a lack of discrimination between bacterial and nonbacterial febrile illness using the NBT test, this early optimistic view is no longer accepted. Despite the absence of specificity of the NBT test in aiding clinical evaluation of febrile patients, it has continued to be useful in the diagnosis of chronic granulomatous disease of childhood. However, a unifying mechanism underlying stimulation of neutrophil oxygen dependent metabolism in both bacterial and nonbacterial febrile inflammatory disorders has remained obscure. After a phagocytic stimulus (latex particles, bacteria), the neutrophil elaborates NBT dye reducing substances (21). Similarly, nonparticulate soluble factors including C5a (22) and staphylococcal protein A (23) are also capable of stimulating neutrophil oxidative metabolism. The results of our in vitro studies demonstrate that LP, the small molecular weight protein that mediates fever, is also capable of stimulating neutrophil superoxide generation and subsequent NBT reduction. Even at concentrations of LP below the fever-producing threshold, neutrophils elaborate a significant amount of superoxide. In addition, metabolism via the HMP shunt is activated in neutrophils exposed to LP.

The possible interpretations of our in vivo experiments are interesting. Intravenous LP has a profound effect on the absolute number of circulating peripheral blood neutrophils. We consistently observed a marked neutropenia 0.5 h after injection of LP with a rebound neutrophilia at 1–2 h. This response is reminiscent of effects produced by endotoxin or C5a administration (24). Although we have strong evidence that our LP preparation does not contain either of these contaminants (it is negative in the limulus amebocyte assay and is not chemotactic) it is possible that LP activates complement in vivo. Because C5a also stimulates the respiratory burst, such an interaction could provide an explanation for effects on neutrophil circulation kinetics and generation of superoxide. Experiments are in progress to examine this possibility.

Nevertheless, based on our observation that LP stimulates neutrophil oxygen metabolism in a serum-free system, it is not necessary to invoke an intermediate

FIGURE 4  HMP shunt activity of neutrophils stimulated by LP. HMP shunt activity is expressed as counts per minute from the conversion of [1-14C]glucose to 14CO2 by adherent neutrophils. Results are the mean±SEM of three separate experiments.
reaction. Neutrophils reducing NBT after LP may represent a population of cells already in the circulation at the time of bolus injection and hence stimulated by this interaction. Alternatively, LP could stimulate the reserve pool neutrophils either in the circulation, bone marrow, or peripheral marginal pool with subsequent mobilization. Available data does not allow better definition of this mechanism; however, studies directed at the circulation kinetics of LP and its effects on neutrophil mobilization are in progress.

The overall interaction of LP with the neutrophil appears to be quite complex. In a recent report we described the ability of LP to induce neutrophil lysosomal exocytosis (11). With the metabolic effects on peripheral blood neutrophils reported here, LP may serve as an important patho-physiologic tool for further understanding of the neutrophil during acute inflammation.

REFERENCES