

Enhancement of Lipopolysaccharide-Induced Neutrophil Oxygen Radical Production by Tumor Necrosis Factor Alpha

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Although tissues become exposed to both exogenous and endogenous cell-activating mediators during infection, there is little appreciation of the effects of subjecting cells to multiple mediators. We examined the hypothesis that the response of neutrophils to bacterial lipopolysaccharide (LPS) is significantly altered in the presence of the endogenous mediator tumor necrosis factor alpha (TNF). The data showed that human neutrophils pretreated with TNF for 10 to 30 min, displayed significantly enhanced superoxide production in response to LPS (from either *Escherichia coli* K-235 or *E. coli* 0127:B8), measured as lucigenin-dependent chemiluminescence (CL), seen as an increase in the initial peak rate as well as the total CL accumulated over the incubation period. TNF amplified the response to LPS at 1 to 100 U of TNF/10⁶ neutrophils and was able to enhance the response to a wide range of concentrations of LPS (0.01 to 1,000 ng/ml). The TNF-induced increase in the LPS response was paralleled by an increase in LPS binding to the neutrophils, which could be abrogated by an anti-CD14 monoclonal antibody. The results demonstrate that TNF significantly increases the LPS-induced release of oxygen radicals in neutrophils through the upregulation of cell surface CD14.

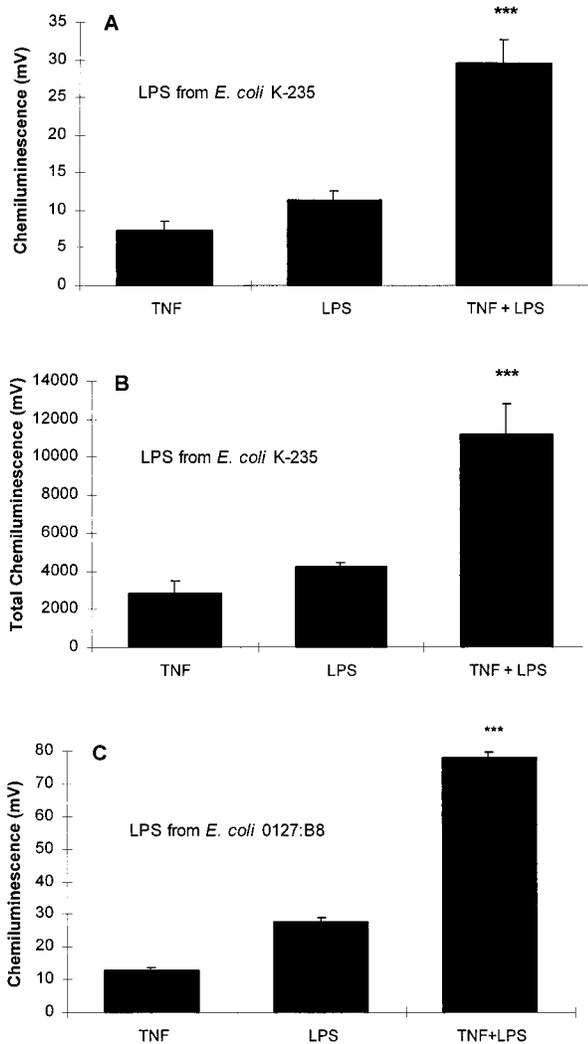
Neutrophils, while playing a key role in microbial killing in infections, may become harmful to host tissues as a result of their nonspecific stimulation by both endogenous and exogenous mediators (1, 5, 16). Bacterial lipopolysaccharide (LPS), a glycolipid from the outer leaflet of the cell wall of gram-negative bacteria, is released into body fluids during infection. Because of its marked biological activity, LPS induces severe pathological changes in host tissues (20). Tumor necrosis factor alpha (TNF) is readily produced by a range of local tissue cells and inflammatory leukocytes during bacterial infections as a result of stimulation by microbial products such as LPS (4, 11, 20). TNF has been shown to have a broad spectrum of biological activities which collectively give rise to the pathophysiological processes seen in a wide range of diseases and disorders (1, 10, 25). The cytokine has previously been shown to upregulate neutrophil responses to some surface acting agonists (5, 6, 9, 17, 21). Although LPS is known to trigger release of O[•]-₂ and H₂O₂ (2, 21), the relationship between cytokines and microbial components such as LPS in terms of the neutrophil oxidative respiratory burst has not been defined. It was therefore of interest to determine if the response of neutrophils to LPS was increased by the presence of TNF in an attempt to better understand the relationship between these two agents.

Neutrophils with >96% purity and >99% viability (by trypan blue exclusion test) were prepared by centrifugation of blood from healthy volunteers on Hypaque-Ficoll medium with a density of 1.114 (7). Superoxide production was measured by the bis-*N*-methylacridinium nitrate (lucigenin)-dependent chemiluminescence (CL) assay (14). Lucigenin was purchased from Sigma Chemical Co., St. Louis, Mo. This response is specific for superoxide and can be totally inhibited by superoxide dismutase (12, 15). Briefly, 10⁶ neutrophils in 100 μl of

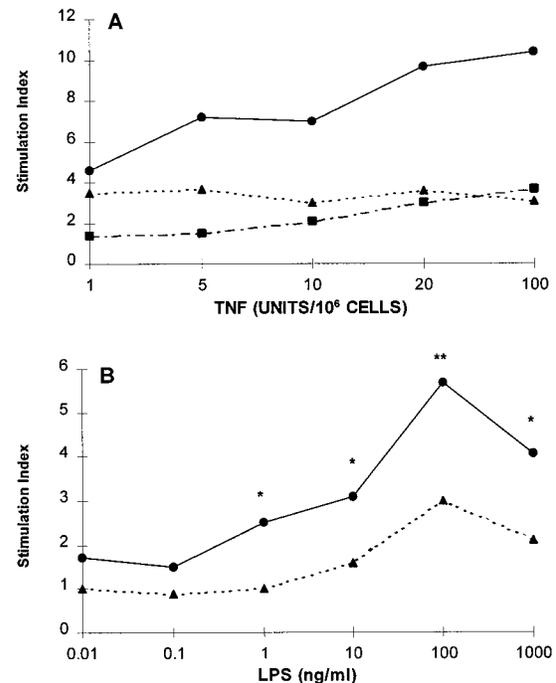
Hanks balanced salt solution, pH 7.3, were transferred into luminometer tubes, and then various reagents were added in an assay volume of 500 μl. The neutrophils were pretreated for 20 min (unless specified otherwise) with the concentrations of TNF indicated in the figure legends. Human recombinant TNF (6 × 10⁷ U/mg, 500 μg/ml, >99% pure) was produced by Genentech, Inc. (San Francisco, Calif.) and was kindly provided by G. R. Adolf (Ernst Boehringer Institute, Vienna, Austria). The endotoxin contamination was less than 0.125 endotoxin U/ml as assessed by the *Limulus* lysate assay. During the pretreatment times, the tubes were incubated at 37°C in an atmosphere of 95% air–5% CO₂ and high humidity. The neutrophils were then treated with LPS (from *Escherichia coli* K-235 [Sigma; chromatographically purified by gel filtration]) in the presence of 1% heat-inactivated (56°C, 30 min) autologous donor serum and then transferred into a luminometer (water jacketed, 37°C, model 1251 [Bioorbit Oy, Turku, Finland] with MultiUse software, version 1.08). A 500-μl volume of lucigenin was automatically added at a final concentration of 127.5 μg/ml per tube to bring the final assay volume to 1 ml. The resulting CL (in millivolts) in all tubes was measured at the same time in the luminometer chamber. The results were recorded as the peak initial rate of superoxide production and also as the total amount of superoxide produced over a fixed time period by integration of the area under the curve (in millivolts). Some data are expressed as stimulation indices. To obtain these, the means of the treatments were divided by the means of the baseline values.

Preliminary studies revealed that pretreatment of neutrophils for 20 min with 20 U of TNF per 10⁶ cells was optimal for priming of these cells for subsequent responses to other agonists (6). Examination of the lucigenin-dependent CL induced by LPS showed that TNF caused significant enhancement of this response ($P < 0.001$). The effect of TNF was seen as an increase in both the initial peak rate of CL and the total CL produced over the incubation period of 50 min (Fig. 1A and B). To ensure that the effects observed were not restricted to a specific *E. coli* serotype, the experiments were repeated with LPS from a different serotype, *E. coli* 0127:B8 (Sigma; chro-

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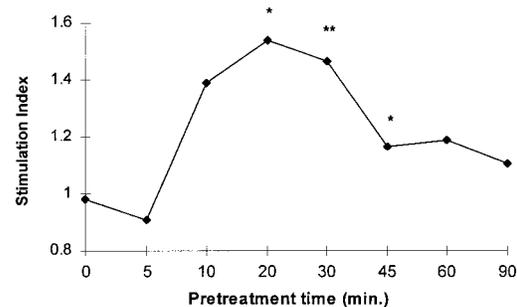


matographically purified by gel filtration). The results were similar to those obtained with serotype K-235 (Fig. 1C). Studies of the effects of different doses of TNF (1, 5, 10, 20, and 100 U/ 10^6 cells) showed that TNF pretreatment was effective in significantly enhancing the LPS-induced CL over the entire dose range (Fig. 2A). A wide range of LPS concentrations, from 0.01 to 1,000 ng/ml, was examined, and significant



priming was observed from 1 to 1,000 ng/ml, with an optimal effect at 100 ng/ml (Fig. 2B). The priming effects of TNF for the LPS-induced CL response were evident within 10 min of TNF pretreatment and maximal at 20 min. The priming effect had decreased by 45 min but was still above the baseline at 90 min (Fig. 3).

To elucidate the mechanism by which TNF alters the response to LPS, the effects of TNF on the expression of CD14



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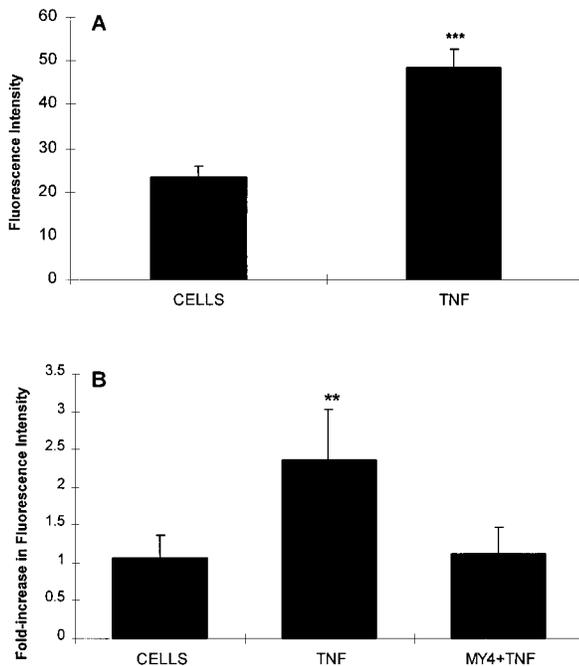


FIG. 4. (A) Effect of TNF pretreatment on surface CD14 on neutrophils. Neutrophils 10^6 were incubated with either buffer or 20 U of TNF for 20 min. At 4°C the cells were incubated with anti-CD14 and then washed and incubated with goat anti-mouse phycoerythrin. The intensity of fluorescence was determined as described in the text. The data shown are the means and standard errors of five experiments. The increase in fluorescence was 2.1-fold (***, $P < 0.001$ [Student *t* test]). (B) Effect of TNF on LPS binding. Neutrophils were stimulated as described above. The cells were washed and resuspended in serum (5%, autologous, heat inactivated for 30 min at 56°C) and incubated with FITC-labelled LPS (*E. coli* 0127:B8) at a final concentration of 16 $\mu\text{g/ml}$ for 30 min on ice. After three washes, flow cytometry was performed as described in the text. The increase was 2.2-fold and could be abrogated by incubating the cells with MY4 (dilution, 1:10) for 30 min prior to adding serum and FITC-LPS. The data shown are the means and standard errors of five experiments (**, $P < 0.01$ [analysis of variance]).

and on the surface binding of LPS were investigated. The changes in CD14 expression were examined first. Neutrophils ($10^6/100 \mu\text{l}$) were treated with either TNF at 20 U/100 μl or diluent at 37°C for 20 min, the tubes were centrifuged at 4°C, and the cells were resuspended in buffer containing murine anti-human CD14 monoclonal antibody MY4 (Coulter Corporation) at a dilution of 1:50 and left on ice for 30 min. Cells in an additional tube were incubated with a murine immunoglobulin G2b antibody in place of the anti-CD14 monoclonal antibody to serve as an isotype control. After two washes with ISOTONE II solution (Coulter Electronics), the cells were treated with a secondary antibody (affinity purified goat anti-mouse phycoerythrin diluted 1:50; Jackson Immuno-Research Laboratories, West Grove, Pa.) for 30 min on ice. The cells were washed three times at 4°C and then fixed in formaldehyde at 1:33 in ISOTONE II solution. The fluorescence intensity of the cell population was analyzed by flow cytometry on a FAC-Scan (Becton Dickinson). Ten thousand cells were counted, and the data were processed with LYSYS II software. TNF pretreatment led to a 2.1-fold increase in CD14 surface expression (Fig. 4A).

Assessment of LPS binding to neutrophils ($10^6/100 \mu\text{l}$) was done by pretreating cells with either TNF at 20 U/100 μl or diluent for 20 min at 37°C. The tubes were centrifuged at 4°C and the cells were resuspended in 5% heat-inactivated serum. Fluorescein isothiocyanate (FITC)-labelled LPS (*E. coli* sero-

type 0127:B8 [Sigma; chromatographically purified by gel filtration]) was added at a final concentration of 16 $\mu\text{g/ml}$. Unlabelled LPS at the same concentration served as a background control. After 30 min of incubation on ice, the cells were washed three times at 4°C and then fixed in formaldehyde at 1:33 in ISOTONE II solution. The fluorescence intensity of the cell population was analyzed by flow cytometry, and 10,000 cells were counted. Flow cytometry analysis showed a 2.2-fold increase in LPS binding by neutrophils pretreated with TNF compared to the buffer solution. The increase in fluorescence was prevented by incubating TNF-treated neutrophils for 30 min on ice with MY4 at a dilution of 1:10 prior to adding serum and FITC-LPS (Fig. 4B).

TNF is a cytokine with multiple biological activities. These include cytotoxicity, growth modulation, cellular differentiation, and anti-infective properties (1, 5, 8, 11, 20, 25). It has the ability to act locally (paracrine) and systemically (endocrine). While its role in infection as a mediator of the pathological effects of LPS in sepsis is well understood, little is known about how TNF modulates the interaction between neutrophils and LPS. Our data demonstrate that TNF alters neutrophil responses to LPS in vitro. Neutrophils pre-exposed to human recombinant TNF showed significantly increased oxidative respiratory activity and release of oxygen-derived reactive species in response to LPS. Thus, while TNF alone is an incomplete secretagogue and a weak stimulator of the neutrophil respiratory burst (17, 21), it is likely to substantially increase the release of oxygen radicals at foci of infection in the presence of a microbial product such as LPS. This finding may, in part, explain the skewing of the balance toward pathophysiological reactions as a result of cytokine production in the presence of microbial components. LPS, a potent stimulator of TNF production by macrophages, may hence, set up a vicious cycle of neutrophil priming for an increased release of oxygen radicals in response to LPS. In support of this concept was our finding that in the presence of TNF only 1 ng of LPS per ml was required to achieve levels of oxygen-derived radical species production by neutrophils similar to those that LPS at 100 ng/ml achieved in the absence of the cytokine (Fig. 2B). It is evident from these studies that an LPS concentration of 10 ng/ml had to be reached to stimulate neutrophils. However, an LPS concentration as low as 0.1 ng/ml has been shown to stimulate TNF production by macrophages. It is therefore likely that at initial stages of bacterial infections, the production of TNF precedes neutrophil stimulation by LPS. When neutrophils were pretreated with very low doses of LPS (0.01 to 1 ng/ml), they were still able to respond to the combined effects of subsequent TNF and LPS additions (data not presented).

The time course of the neutrophil response strongly implies a mechanism of either intracellular enzyme activation or receptor upregulation. It has been shown that the expression of the LPS receptor CD14, a 55-kDa glycoprotein on the cell surface, doubled after treatment with TNF for 20 min, suggesting mobilization of the molecule to the surface from an intracellular pool (27). While it had been suggested previously that the reservoir of CD14 in neutrophils was the specific granules, current evidence suggests that these molecules are stored primarily in the azurophilic granules (23). We confirmed that under our conditions, TNF treatment caused a significant increase in CD14 expression on neutrophils and an associated increase in LPS binding to the cells. Furthermore, this increase in binding could be abrogated by a monoclonal antibody to CD14. This provides evidence that an increase in CD14 receptor expression is the major basis for the TNF effect.

The mechanisms by which LPS induces intracellular signal-

ling are not clear. While binding of the lipoprotein binding protein-LPS complex to CD14 seems essential (27–29), the receptor is attached to the cell surface via a phosphatidylinositol anchor only. Many investigators believe that molecules anchored in this manner are unlikely to produce signals per se because of the absence of an intracellular domain, while others consider it possible (22, 24). Several investigators have postulated the existence of a membrane protein facilitating signal transduction (26). This putative LPS receptor or LPS receptor cofactor was initially described as a 73-kDa protein in murine splenocytes (18, 19, 30) and human leukocytes (13). However, this protein has since been identified as albumin (3). Nevertheless, the existence of a transmembrane molecule facilitating signalling by LPS is still a likely model to explain CD14 independent activation of cells (26). If such a molecule exists, it may also undergo changes subsequent to TNF stimulation. Irrespective of the existence of additional LPS receptors, our data demonstrated a significant increase in LPS binding by TNF-primed neutrophils. The magnitude of this increase correlated with an increase in surface CD14 and also an increase in the CL response. Based on these observations, we propose that TNF primes neutrophils for an enhanced CL response to LPS, most likely via upregulation of surface CD14, leading to increased LPS binding.

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