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

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Received 14 April 1997/Returned for modification 3 July 1997/Accepted 20 January 1998

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/10^6 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 pathological 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 H_2O_2 (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, 10^6 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 × 10^7 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% CO_2 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 10^6 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-
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

FIG. 1. (A) Effect of TNF on the LPS-induced neutrophil CL response. Neutrophils (10^6) were preincubated with 20 U of TNF for 20 min and then challenged with 100-ng/ml LPS (E. coli K-235), and CL was measured over 50 min of incubation. The values shown are the peak initial rate of CL and represent the means ± the standard error of the mean of eight experiments, each conducted in duplicate with neutrophils from eight different donors. The basal CL of 6.1 mV was deducted from each of the experimental values. Neutrophils treated with TNF and LPS showed a significantly increased response compared to responses induced by either of the agents alone (***, P < 0.001 for TNF versus TNF plus LPS and LPS versus TNF plus LPS; P < 0.015 for the sum of values for the individual TNF and LPS treatments versus cotreatment with TNF plus LPS, [analysis of variance]). (B) Data expressed as a function of the total accumulated CL generated over the incubation period. The baseline value of 2258 mV was subtracted from each column. (***, P < 0.001 for TNF versus TNF plus LPS and LPS versus TNF plus LPS; P < 0.015 for the sum of values for the individual TNF and LPS treatments versus cotreatment with TNF plus LPS [analysis of variance]). (C) Effect of LPS (E. coli 0127:B8) under the same conditions as in A. The basal CL of 13.3 mV was deducted from each of the experimental values. The increased response to stimulation with both TNF and LPS was highly significant (***, P < 0.001 [analysis of variance]).

FIG. 2. (A) Effects of varying TNF amounts on the LPS-induced CL response. Neutrophils were pretreated with the indicated concentrations of TNF or diluent for 20 min and then challenged with 1-μg/ml of LPS (E. coli K-235) or diluent for another 50 min. Neutrophils were treated with TNF only (■), LPS only (○), or TNF plus LPS (▲). Results are the means of four duplicate experiments, expressed as stimulation indices. These were obtained by dividing the means of the treatments by the means of the baseline values. At all TNF doses, the effects of the combined action of TNF and LPS were significantly different from those of either agonist alone (P values of <0.0001 to <0.05 [analysis of variance]). (B) Response of TNF-primed neutrophils to varying concentrations of LPS (E. coli K-235). The cells were pretreated with 20 U of TNF/10^6 cells (■) or diluent (○) for 20 min and then stimulated with the indicated concentrations of LPS. Values are stimulation indices. The data are the means of four experiments conducted in duplicate (●, P < 0.05; ▲, P < 0.01 [analysis of variance]).

FIG. 3. Effect of varying TNF pretreatment times on the response of neutrophils to LPS (E. coli K-235). Cells were pretreated with 20 U of TNF/10^6 cells for 20 min and then with LPS (100 ng/ml) for 50 min. The results are derived from eight experiments with different donor cells. The results are expressed as stimulation indices (●, P < 0.035; ▲, P < 0.015 [analysis of variance]).
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 treatment. Irrespective of the existence of additional LPS receptors, our data demonstrated a significant increase in LPS 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.

This work was supported by funds from the National Health and Medical Research Council of Australia. Hubertus P. A. Jersmann is a recipient of a Reginald Walker scholarship, a scholarship of the University of Adelaide for Medical Graduates.

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Editor: R. E. McCallum