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Alterations of Neutrophil Responses to Tumor Necrosis Factor Alpha and Interleukin-8 following Human Endotoxemia

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Interleukin-8 (IL-8), a neutrophil chemoattractant and activating cytokine, has been implicated as a proinflammatory mediator in gram-negative sepsis. In vitro data support the notion of IL-8 as an endothelial adherence inhibitor. To evaluate this issue, we infused six volunteers with reference endotoxin and measured plasma levels of IL-8, neutrophil tumor necrosis factor alpha (TNF-α) receptors, TNF-α-induced adherence to fibronectin, and neutrophil chemotaxis to IL-8 and other attractants. We found that, at 3 h postinfusion, IL-8 but not TNF-α plasma levels were elevated. Neutrophils had shed L-selectin (mean channel fluorescence decrease, 79 ± 9 to 49 ± 7; $P = 0.0625$) and TNF-α receptors (decrease in number of receptors per cell, 1,596 ± 340 to 574 ± 93; $P = 0.004$). Cells were chemotactically desensitized to IL-8. TNF-α-induced adherence to fibronectin was suppressed from 69% ± 5% of the phorbol myristate acetate response to 38% ± 7% ($P = 0.0154$). These findings support the notion that release of IL-8 into the vascular space may be an in vivo mechanism for suppression of neutrophil accumulation at extravascular sites. L-Selectin loss would reduce the ability of neutrophils to adhere to activated endothelial cells. The specific loss of migratory response to IL-8 would impair neutrophil delivery to areas where IL-8 was the predominant chemoattractant. Loss of TNF-α-induced adherence to fibronectin would blunt those responses, including production of oxidants, capacitated by adherence.

Gram-negative infection is often accompanied by varied manifestations of a systemic acute inflammatory response. Prominent early clinical characteristics include vascular and myocardial dysfunction which may result in circulatory failure (34). Additional early findings not attributable to hemodynamic collapse include pulmonary dysfunction (the adult respiratory distress syndrome) and a consumptive coagulopathy (5, 28, 49). The pivotal role of cytokines induced by endotoxin is supported by a range of in vitro and clinical observations (9, 12). The response to endotoxin has been conceptualized in relation to a network of epithelial, endothelial, and mononuclear cells signaling a large recruitable pool of effector cells (neutrophils) through mediators with overlapping activities (43). Cytokines of the interleukin-8 (IL-8) family appear central by inducing neutrophil migration into the interstitial space (13, 22, 25).

Neutrophil migration from the vascular space into areas of injury or contamination is initiated by neutrophil attachment via specific receptors to endothelial surface antigens. Relatively loose attachment to sialyl antigens occurs through selectins, and this is followed by β2 integrin activation and binding to endothelial counter-receptors (39, 41, 50, 51). Selectin shedding then occurs. Less well explored are subsequent events in which neutrophils adhere to various connective tissue proteins through integrins (10).

Neutrophil attachment to matrix proteins in turn activates several neutrophil responses, including phagocytosis and an oxidative response to tumor necrosis factor alpha (TNF-α) and other cytokines (17, 30). Production of oxidants allows for degradation of pro- and anti-inflammatory mediators and facilitates digestion of connective tissue matrices (7, 8). These findings are attractive mechanistic explanations for the adult respiratory distress syndrome occurring during the early phases of gram-negative bacteremia, a case supported by the histology of neutrophils in the interstitium of the lung, disruption of the interstitial matrix, and findings of IL-8 and TNF-α and neutrophil-derived enzymes and oxidants in lavage fluid from patients with adult respiratory distress syndrome (1, 11, 23, 29, 45, 53).

In recent studies of patients with serious infections and with trauma, elevated plasma levels of IL-8 have been identified (15, 18, 20). This observation, coupled with the known neutrophil-activating properties of IL-8, has led to the suggestion that IL-8 may induce the remote tissue injury seen accompanying sepsis or trauma. This notion conflicts with the in vitro finding that IL-8 in solution induces neutrophil selectin shedding and prevents neutrophil adherence to endothelial cells (16).

The current study was therefore undertaken to examine various aspects of neutrophil proinflammatory functioning and to examine possible relationships between IL-8 and observed changes in neutrophil function.

MATERIALS AND METHODS

Reference endotoxin EC-5 was obtained from the Food and Drug Administration Bureau of Biologics (21). Recombinant human TNF-α was a gift from Genentech, Inc. (South San Francisco, Calif.). *N*-Formylmethionyl leucyl-phenylalanine (FMLP) and leukotriene B_4 were obtained from Sigma Chemical Co. (St. Louis, Mo.). IL-8_{72} was obtained from Calbiochem-Behring (La Jolla, Calif.).

The protocol was reviewed and approved by the University of Cincinnati College of Medicine Institutional Review Board. Eight volunteers (five men and three women), ranging in age from 20 to 34 years, consented to participate. Volunteers were

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TABLE 1. Hemodynamic parameters in saline- and endotoxin-infused volunteers[a]

Time	Temp (°C)	Heart rate (beats/min)	Cardiac index ($L/m^2/min$)	Mean arterial pressure (mm Hg)[b]	Systemic vascular resistance index ($dynes/cm/m^2$)	QS/QT (%)[c]
Before saline infusion	36.6 ± 0.1	59 ± 4	2.9 ± 0.2	91 ± 4	1,148 ± 372	5.8 ± 0.4
Before EC-5 infusion	37.0 ± 1	62 ± 6	3.2 ± 0.3	93 ± 3	984 ± 96	8.6 ± 2
3 h Post-saline infusion	36.9 ± 0.1	66 ± 3	2.9 ± 0.2	93 ± 3	850 ± 100	9.3 ± 1.5
3 h Post-EC-5 infusion	38.1 ± 0.1	93 ± 7	5.7 ± 0.5	76 ± 2	560 ± 196	17.5 ± 4.4
Significance[d]	0.0156	0.0156	<0.01	0.0156	<0.01	<0.01

[a] The values for the saline-infused volunteers at 0 and 3 h were not different from the 0-h (preinfusion) values.
[b] 100 mm Hg = 13.3322 kPa.
[c] QS/QT, pulmonary shunt fraction.
[d] The *P* values given refer to comparisons between the pre- and postinfusion endotoxin groups. The one-tailed Wilcoxon signed rank sum test was used to compare pre-EC-5 infusion values with 3-h post-EC-5 infusion values. There were no significant differences between the pre- and post-saline infusion data.

fasted for 8 h prior to the study while receiving a normal saline infusion at 0.7 ml/kg of body weight per h. Radial arterial and pulmonary artery catheters were placed. Hemodynamic calculations were performed as described previously (44). Six volunteers, three men and three women, received 20 U of EC-5 per kg (2 ng/kg) administered over 30 s through a peripheral vein, which was flushed with 20 ml of saline. Two of these individuals and two additional volunteers were also studied after saline infusion.

Polymorphonuclear leukocytes were isolated from arterial blood collected before endotoxin or saline infusion and 3 h after infusion. Blood was drawn into EDTA and then subjected to Dextran T500 sedimentation, Ficoll-Hypaque density gradient centrifugation, and hypotonic erythrocyte lysis (47). All isolation steps were performed at 4°C with pyrogen-free reagents. All assays were performed within 2 h of blood drawing. Chemotaxis was assayed by using a modified Boyden chamber technique (14). Neutrophil adherence to fibronectin (Calbiochem) was measured by quantifying lactic dehydrogenase in plates after 0.2% Triton lysis of the cells remaining after three washes (3). Cells used for flow cytometry were prepared by using a standard whole-blood procedure (Gen Trak, Plymouth Meeting, Pa.). Cells were stained for immunofluorescence by using a phycoerythrin-labeled L-selectin antibody (Leu-8) obtained from Becton-Dickinson Immunocytometry Systems (San Jose, Calif.). Cell surface TNF-α receptors were measured by using a competition binding assay with ^{125}I-TNF-α labelled by the Iodogen method (40a). Serum levels for IL-8 and TNF-α were determined by using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, Minn.).

Data derived from preinfusion and 3-h-postinfusion in vitro cell studies were analyzed by paired one-tailed Wilcoxon signed rank sum testing. Unpaired one-tailed *t* testing was used to compare the 0- and 3-h data from volunteers receiving saline with those receiving endotoxin. Data are presented as means ± standard errors of the mean.

RESULTS

Volunteers responded to EC-5 with a mean temperature rise of 1.5°C. Hemodynamic parameters were measured 3 hours after EC-5 infusion, and changes in various cardiovascular parameters are detailed in Table 1. These data are consistent with previous reports using EC-5 (44).

Results of serum cytokine assays are provided in Fig. 1. We noted that the peak in TNF-α levels occurred approximately 30 min before that of IL-8. The peak levels obtained represent approximately 50% of those reported in volunteers receiving 4 ng of EC-5 per kg, measured with a similar ELISA (27). IL-8 plasma levels remained significantly elevated at 3 h postinfusion.

Cells harvested at 3 h postinfusion had significantly fewer TNF-α receptors than the preinfusion cells did (Table 2). There was no change in the affinity of the measured TNF-α receptor population. The ability of volunteer neutrophils to adhere to fibronectin after stimulation with either 1 ng of TNF-α per ml or 5 ng of phorbol myristate acetate (PMA) was examined. The PMA dose produced maximal adherence of control neutrophils, while the TNF dose produced 75% of maximal. Before endotoxin infusion, TNF-α-induced adherence was 69% ± 5% of the PMA values; the adherence of cells harvested 3 h after endotoxin infusion was 38% ± 7% of the PMA values ($P = 0.0154$).

Chemotactic responses of volunteer neutrophils to C5a (as zymosan-activated serum), FMLP, leukotriene B_4, and IL-8 are presented in Fig. 2. These data indicate a specific loss of migratory response to IL-8 at 10^{-9} M. Migratory responses to 10^{-8} M IL-8 were not significantly different from baseline values.

To examine the bioactivity of IL-8 identified in the patient samples, we tested the patient samples with a measured IL-8 level greater than 1,000 pg/ml as a chemoattractant source. Sera were tested with and without the addition of 4 μg of anti-IL-8 (R&D Systems) per ml. Control experiments indicated that recombinant IL-8 had a 50% effective dose of 2×10^{-10} M, and that 4 μg of antibody per ml would inhibit 75%

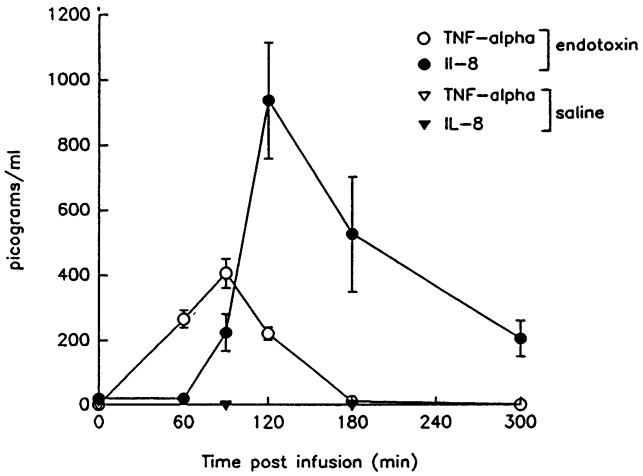

FIG. 1. Plasma cytokine levels at various time points after infusion of either 2 ng of endotoxin per kg or saline in human volunteers.

TABLE 2. Neutrophil characterization studies after endotoxin infusion[a]

Time	TNF-α receptor		L-Selectin mean channel fluorescence	Adherence fibronectin (TNF-α/PMA [%])
	Affinity (kDa)	No of receptors/cell		
Before saline infusion	134 ± 18	1,784 ± 320	82 ± 6	93 ± 10
Before EC-5 infusion	142 ± 4	1,659 ± 229	79 ± 8	69 ± 5
3 h Post-saline infusion	142 ± 15	1,596 ± 340	79 ± 9	76 ± 3
3 h Post-EC-5 infusion	142 ± 25	574 ± 93	49 ± 7	38 ± 7
Significance[a]	NS	0.0042	0.0625	0.0154

[a] A one-tailed Wilcoxon signed rank sum test was used to compare pre-EC-5 infusion values with 3-h post-EC-5 infusion values. There were no significant differences between the pre- and post-saline infusion data.

of this response. We found that the addition of anti-IL-8 to patient or control sera had no effect on chemotaxis and that chemoattractant activity in serum was destroyed by preheating at 56°C for 30 min. IL-8 is stable under these conditions (35). The chemoattractant was likely C5a, generated by interaction with the chamber.

DISCUSSION

Neutrophils possess a variety of cell surface receptors for cytokines and for serum protein-lipopolysaccharide (LPS) complexes and undergo prominent functional responses to cytokine and endotoxin stimulation (30, 54, 55). Remodeling of the neutrophil surface, through the expression of previously cryptic binding activity and shedding of receptors, provides a precise means of regulating neutrophil functions and a potential locus for suppression of tissue injury during acute inflammatory events (37, 38). IL-8 is recognized as an important in vivo mediator of neutrophil function and is a member of the chemokine family (2, 33). When present on the abluminal surface, IL-8 functions as a classical chemoattractant (22). More recently, IL-8 has been identified as an inducer of fibroblast collagen production and angiogenesis (24).

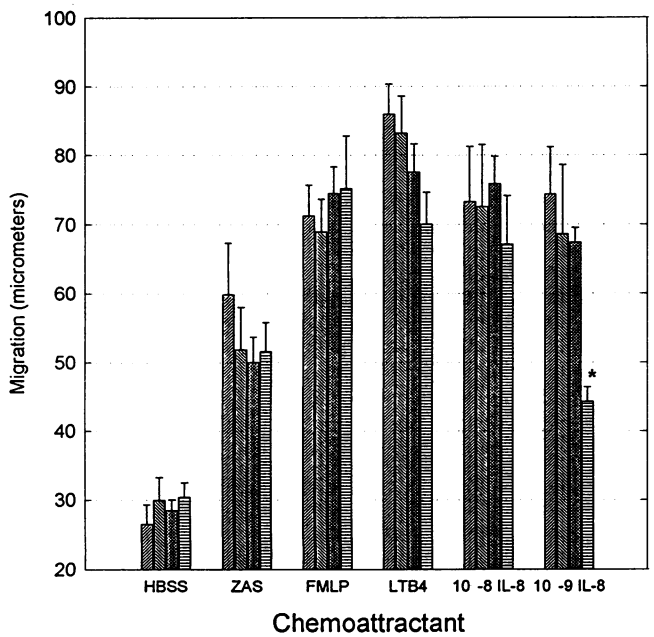

FIG. 2. Chemotactic responses of neutrophils before and 3 h after infusion of either saline or 2 ng of endotoxin per kg in normal volunteers. The chemoattractants were 25% zymosan-activated serum (ZAS), 10^{-8} M FMLP, 5 ng of leukotriene B_4 per ml, and 10^{-8} and 10^{-9} M IL-8. HBSS, Hanks balanced salt solution. Symbols: ▨, 0 h, no LPS; ▧, 3 h, no LPS; ▩, 0 h, LPS infused; ▤, 3 h, LPS infused; *, $P = 0.021$.

During septic episodes, IL-8 has been considered a proinflammatory molecule contributing to the deleterious host response (13, 18, 29). It has become apparent that IL-8 may also serve to suppress the accumulation of neutrophils at an inflammatory locus by inhibiting attachment of the cells to endothelium (16, 26). This event may be mediated in part by shedding of selectins required for the initiation of neutrophil-endothelial binding and by chemoattractant-induced F-actin polymerization (52). In an animal model, IL-8 infusion inhibited neutrophil accumulation at sites of acute inflammation (19).

The findings of this study suggest that the release of IL-8 into the vascular space may serve as an in vivo mechanism for suppression of neutrophil accumulation at extravascular sites. The loss of L-selectin documented in this study would reduce the ability of neutrophils to adhere to activated endothelial cells. The specific loss of the migratory response to IL-8 would impair neutrophil delivery to areas where IL-8 was the predominant chemoattractant. Loss of TNF-α-induced adherence to fibronectin would blunt those responses, including production of oxidants, capacitated by adherence.

The evidence supporting neutrophil exposure to IL-8 includes (i) demonstration of elevated immunoreactive IL-8 plasma levels and (ii) specific loss of neutrophil migrating response to IL-8. While we cannot rule out the participation of other mediators in the chemotactic deactivation, the pattern of polymorphonuclear leukocyte response is not recreated by neutrophil treatment by C5a, TNF-α, granulocyte colony-stimulating factor, or granulocyte macrophage colony-stimulating factor (data not presented). One recent report documented the loss of IL-8-induced chemotaxis after neutrophil exposure to endotoxins of various bacteria, including *Escherichia coli* (4). This effect required endotoxin concentrations above 100 ng/ml, concentrations far in excess of those obtained after EC-5 infusion. We found no chemotactic defect when neutrophils from normal volunteers were pretreated with EC-5 at 100 ng/ml (data not shown).

Chemotaxis has been used commonly to define agent-specific loss of cell responsiveness. Deactivation for other ligands has been related to dimunition of the specific cell surface receptor population (31). The presence of elevated plasma levels of cytokines and other neutrophil-activating factors such as C5a in patients with clinically severe gram-negative infections is uncommon, but this may be a consequence of enhanced receptor-mediated clearance (40). Under these circumstances, cell-directed consequences of intravascular cytokine production might still occur, as suggested by the

current study. Detection of intravascular trafficking of various mediators would then depend upon recognition of loss of specific cell-based responses.

We did not find IL-8 bioactivity in specimens with elevated immunoreactive IL-8. A variety of mechanisms exists to restrict the activity of IL-8 released into the vascular space. Autoantibodies to IL-8 have been identified recently, and free antibody concentrations decrease after endotoxemia (46). Receptor-mediated clearance is also likely important in removing IL-8 from the vascular space. A cytokine receptor on erythrocytes capable of binding IL-8 has been identified recently, and induction of erythrocyte-bound IL-8 after IL-2 chemotherapy has been shown (32, 48). We believe that leukocyte receptor binding of IL-8, a participant in the process of reducing plasma levels of IL-8, occurred although we did not measure IL-8 receptors on volunteer neutrophils. Such binding would explain migratory desensitization and perhaps the shedding of TNF-α receptors.

We found that postendotoxemia neutrophils were deficient in TNF-α receptors. Previous studies have shown an increase in plasma TNF-α receptors after endotoxin injection, likely due in part to neutrophil receptor shedding (42). Loss of cell surface TNF-α receptors has been shown to specifically abrogate in vitro TNF-α-induced responses, an observation supported by the current study (37).

Endotoxemia is accompanied by a prominent neutrophilia, and neutrophils appearing in the circulation after endotoxin infusion express a different antigenic pattern than preinfusion cells do (6, 36). The migratory responses of such elicited cells have not been studied after recruitment by means other than endotoxin infusion, and thus it is not possible to rule out some other phenotypic basis for the observed changes.

These data support the notion that a component of the response to endotoxemia is elevation of plasma IL-8 to an extent suppressing neutrophil responses to proinflammatory stimuli. The clinical relevance of this is supported by a recent study of patients with sepsis, 42 of 47 of whom were found to have elevated plasma IL-8 levels on admission (18). In a study of *Pseudomonas pseudomallei* infection, persistent elevation of plasma levels of IL-8 was identified (15). Whether IL-8-induced suppression of neutrophil adherence and oxidative responses to TNF-α represents a potential therapeutic strategy is unclear.

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