Two-stage response to endotoxin infusion into normal human subjects: Correlation of blood phagocyte luminescence with clinical and laboratory markers of the inflammatory, hemostatic response

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Injection of endotoxin (i.e., lipopolysaccharide [LPS] from the cell wall of Gram-negative bacteria) into healthy subjects reproduces the inflammatory changes observed during the initial phase of bacterial infection. As such, endotoxin has been used to study the immune activation and the pathophysiologic consequences of early Gram-negative infection. In human subjects, endotoxin alters capillary integrity and affects the cardiovascular system (1, 2), causes production of cytokines (3, 4), and activates the coagulation-fibrinolytic pathways (4–9). Production of tumor necrosis factor (TNF), interleukin (IL)-6, and IL-8 occurs within 2–3 hrs of endotoxin infusion (3, 10–17). No evidence, however, of complement activation has been observed after endotoxin infusion into normal human subjects (4). In all the previously cited studies, the observations were terminated at time (T) + 6–8 hrs, and/or observations were restricted to cardiovascular and rapidly responding inflammatory and hemostatic mediators.

A blood luminescence system for assessing phagocyte activity has been used in human studies of normal subjects and infected patients who were stimulated by granulocyte colony-stimulating factor (18–23). Recently, we used this system to study the phagocyte response of normal human subjects to endotoxin (24). We measured phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and myeloperoxidase (MPO)-dependent oxygen utilization. We also measured circulating (basal) and stimulated (platelet-aggregating factor [PAF]-primed) opsonin receptor (e.g., CD11b/18, CD35) dependent phagocyte functions before and after endotoxin infusion. In addition to the expected increase in phagocytic oxygenation activities immediately after endotoxin, we observed a second peak of oxidase activity at T + 24 hrs (24). This suggested a later or second-stage response to endotoxin in the human model of endotoxemia.

The present studies characterize the receptor and oxidative enzymatic responses of phagocytes in the human model of endotoxemia and correlate them with the responses of molecular markers of hemostatic and inflammatory system activation and endothelial injury. These observations establish for the first time that the compensated response of human subjects to endotoxin consists of two stages: an immediate symptomatic inflammatory stage followed by an asymptomatic stage that is characterized by a recurrence of hemostatic activity, appearance of complement activation products complexed to C-reactive protein (CRP), and evidence of endothelial cell injury.

MATERIALS AND METHODS

Subjects

The subjects were 12 (eight experimental and four control) healthy male volunteers ranging in age from 21 to 37 who worked as laboratory personnel or doctors at the University of Oklahoma Health Sciences Center. They were nonsmokers, took no prescription medications, and had normal physical examinations. Informed consent was obtained, and routine chemistry, hematology, urinalysis, and physical exams were done before entry into the study. The protocol was approved by the Institutional Review Boards of the Oklahoma Medical Research Foundation and the University of Oklahoma Health Sciences Center.

Sample Collection and Assays

The subjects reported to the Oklahoma City Veterans Administration Medical Center at 7:30 AM without having eaten (fasting) since 10:00 pm the previous evening. The studies were carried out in the Intensive Care Unit of the Veterans Administration Medical Center. After the electrocardiographic monitors were connected and intravenous catheters were established, the subjects were allowed to equilibrate for 30 mins during which time baseline vital signs and blood samples were taken. After this period, 4 ng/kg of Lot F, Difco *Escherichia coli* endotoxin was infused over a 1-min period. The subjects generally experienced chills, headache, and rigors after 1 hr followed by fever, myalgia, and nausea between 2 and 7 hrs. Recovery to normal temperature and appetite occurred between 8 to 12 hrs, at which time the subjects were discharged. No medications for relief of myalgia or fever were required. Nursing notes were used to grade clinical symptoms. Blood sam-

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ples were taken before (T-0) and at 0.5, 1, 2, 4, 8, 12, 24, and 48 hrs post-LPS injection. T-0 is defined as the time at which the first blood sample was drawn just before the bolus infusion of endotoxin. These samples were taken via a short 6-cm catheter from the cephalic vein during the first 8 hrs and by venipuncture at 12, 24, and 48 hrs. Luminescence and thrombin/antithrombin complex measurements were taken on blood samples drawn from two subjects by using both methods of blood collection, and no differences between samples were observed. The blood samples included 4 mL in 3.8% citrate buffer (pH 6.8, 1:9 v/v buffer to whole blood) for fibrinogen (25), and ELISA/radioimmunoassay determinations of thrombin/antithrombin (TAT) complex (26), TNF (27), tissue plasminogen activator (28), elastase-alpha-1-antitrypsin (elastase/α1AT) complex (29), factor VII antigen (30), factor VIIa, VIIc function (31), IL-6 (32), IL-8 (32), IL-10 (33), plasminogen activator inhibitor (34), plasma tissue factor antigen (35) and soluble fibrin (36). The concentrations of plasmin/antiplasmin (PAP) inhibitor complex and activated protein C/inhibitor complex were obtained using the plasmin inhibitor complex and TEIJIN-F tests (TEIJIN, Osaka, Japan), respectively. Soluble thrombomodulin was determined using ELISA (37). Blood (2.5 mL) also was collected in 0.1% potassium EDTA (pH 7.4, 1:9 v/v buffer to whole blood) for ELISA determination of CRP and complexes between CRP and C4d or C3d (38), and for leukocyte, platelet, and erythrocyte counts using a Coulter counter. Hematocrit, erythrocyte sedimentation rate determination, and blood smear preparation also were made from these samples. The blood smears were stained with Wright stain just after preparation to preserve the fine features of phagocyte morphology. One half milliliter blood was collected in thrombin/soybean trypsin inhibitor for fibrin degradation products (39). One milliliter was collected in heparin (10 units/mL) for endotoxin determination by using S-2423 (Coatest Endotoxin Endosafe) (whereas there was inconsistent elevation of endotoxin above one endotoxin unit per milliliter in subject 5 at 2 and 8 hrs, none of the other seven experimental or four control subjects had endotoxin levels above the one endotoxin unit per milliliter detection limit. Endotoxin, which might otherwise be detected, is assumed to have been taken up rapidly by the tissues between the T-0 and T + 0.5 hr sampling). One milliliter was collected in potassium oxalate and sodium fluoride for lactic acid determination. Plasma lactate determinations were performed on a TDX analyzer (Abbot Laboratories, Abbot Park, IL). Three milliliters of blood was clotted and the serum was analyzed for blood chemistries using automated methods (40). Finally, 0.1 mL (100 μL) of the potassium EDTA anticoagulated whole blood was used within 1 hr for blood luminescence system measurements of phagocyte function.

Phagocyte morphologic evaluations were by direct examination, and peripheral blood smears were scored immediately after collection of blood samples. They were fixed and stained within 1–2 mins. Phagocyte polar morphologic changes were classified as grade 1 if the cytoplasmic granules were polarized (directionally orientated) and grade 2 if pseudopodia plus polarization of granules were observed (41).

Clinical symptoms of nausea, vomiting, headache, rigors/chills, myalgia, and lethargy were graded on a scale of 0 to 4, with 4 being the most severe.

Differential Measurement of Phagocyte Oxidase, MPO, and Opsonin Receptor Expression

The respiratory burst metabolic activities of circulating blood phagocytes were measured by using an automated blood luminescence system and patented reagents (originally supplied by ExOxEmis, Little Rock, AR) as previously described (18, 19). Activity-specific chemiluminigenic substrates yield luminescence by different dioxygenation mechanisms and can be employed to differentially measure the products of phagocyte oxidative metabolism (42, 43). The reductive dioxygenation of lucigenin in dimethylbiacridinium balanced salt solution is NADPH oxidase-dependent, whereas the simple dioxygenation of luminol in balanced salt solution is mostly, but not exclusively, dependent on oxidase-driven MPO activity (43). The reagent tube and chemiluminigenic substrate combinations—that is, PMA_b (10 pmol/tube) tubes with dimethylbiacridinium balanced salt solution and PMA_a (5 nmol/tube) tubes with luminol in balanced salt solution—are formulated for optimum measurement of reduction and simple dioxygenation activities, respectively, and employ only 1 μL equivalent of whole blood per measurement. Different quantities of PMA are used per tube to induce different degrees of degranulation. In low concentrations, PMA (PMA_b) causes degranulation of the specific (secondary) granules and activation of NADPH oxidase, but at relatively high concentrations, PMA (PMA_a) produces extensive degranulation of both azurophilic and specific granule compartments and stimulation of both oxidase and MPO activities.

The whole blood (0.1 mL) was diluted in blood-diluting medium (9.9 mL) and loaded into the luminometer (LB953 modified; Berthold EG&G, Mainz, Germany). Basal and PMA_b-stimulated NADPH oxidase (opsonin receptor-independent) dioxygenation activities were measured automatically after luminometer injection of 0.1 mL of diluted whole blood (1 μL equivalent of whole blood) into uncoated and PMA_b-coated tubes containing 0.6 mL dimethylbiacridinium balanced salt solution, respectively. Basal and stimulated MPO (simple dioxygenation) activities were measured after injection of 0.1 mL of diluted blood into uncoated and high-dose PMA_a-coated tubes containing 0.6 mL luminol in balanced salt solution, respectively. Luminescence was measured in triplicate over a 20-min interval. The results were expressed as specific activity per phagocyte (i.e., counts per 20 mins per phagocyte). The number of phagocytes per microliter of blood was measured by routine hematology with a manual differential cell count. The total leukocyte count minus the total lymphocyte count equals the total phagocyte count.

The antigenic expression and function of blood phagocyte opsonin receptors, that is, CR1 (CD35) and CR3 (CD11b/CD18), increases in proportion to the level of exposure to inflammatory mediators such as C5a, platelet activity factor (PAF), leukotrienes (LTB_4), various cytokines and interleukins, and microbial products. Increase in phagocyte opsonin receptor expression can occur with little or no activation of respiratory burst metabolism (42). Under properly controlled assay conditions, the extent of opsonin receptor expression can be linked functionally to the initial phase of opsonin-stimulated phagocyte respiratory burst metabolism measured as luminescence (42, 44). Assessment of the circulating opsonin receptor (COR)-dependent phagocyte activity requires a nonlimiting concentration of opsonin as stimulus and a nonlimiting concentration of luminigenic substrate. The maximum opsonin receptor (MOR)-dependent activity is measured under the same testing conditions except that a priming agent (e.g., 10 pmol of PAF or 20 pmol of C5a) is included to ensure that phagocyte opsonin receptor expression is maximal.

The blood luminescence system also was employed for functional measurement of the COR and MOR activities of the blood phagocytes. As with the measurements of oxidase and MPO activities, measurements of opsonin receptor expression of phagocytes were made using a 1-uL equivalent of whole blood (see preceding description). The COR/MOR ratio provides an index of the degree of *in vivo* priming of opsonin receptor activation and has been employed to gauge the *in vivo* state of immune activation, that is, systemic inflammation (19, 21, 22, 42). The ratio approaches unity (a value of 1) with increasing *in vivo* exposure to the immunomodulating agents of inflammation. Additional assay reagents required include human complement opsonized zymosan, 2×10^9 yeast cell wall units/mL,

<0.06 endotoxin units/mL particle-free supernatant; C5a tubes, coated with 20 pmol recombinant human C5a; PAF tubes, coated with 10 pmol 1-*O*-hexadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphorylcholine; and *N*-formyl-methionyl-leucyl-phenyl-alanine tubes, coated with 100 pmol *N*-formyl-methionyl-leucyl-phenyl-alanine.

Data Analysis

Each complete data set from every luminometer run was stored to a data file and later transferred to a relational database program, Paradox for Windows (version 5.0, Borland International, Buffalo, NY). The hematology data also were added to the database. Routine statistical analyses was run on SPSS for Windows (version 8, SPSS, Chicago, IL).

RESULTS

Phagocyte Opsonin Receptor Expression in Response to Endotoxin

Figure 1 illustrates the opsonin-activated oxygenation activity of blood relative to the exposure to endotoxin. The COR and MOR activities are normalized per phagocyte concentration (Fig. 1*A*). This operation clarifies the difference in COR and MOR activities that precede and follow *in vivo* endotoxin activation. Although not statistically significant, the endotoxin effect on COR activity was seen as early as 0.5 hr post-LPS. It occurred even before the circulating phagocyte concentration reached its nadir at 1 hr. Both COR and MOR activities per phagocyte peaked at 1 to 2 hrs post-LPS and remained elevated with a slow decline during the initial 12 hrs post-LPS. The decrease in COR activity exceeded that of MOR at 24 hrs post-LPS, and the difference was even more obvious at T + 48 hrs. Figure 1*B* presents these data as the simple ratio of COR to MOR activities. The ratios were constructed for each individual subject specimen (or measurement) and not from the composite mean statistics. Ratio expression eliminates the necessity to consider activity relative to volume of blood or phagocyte concentration, because these factors are present in both the numerator and denominator of the ratio. This ratio rapidly increased to near maximum by 1.0 hr. It remained relatively constant during the first 12 hrs post-LPS and was more than three-fold higher than the pretreatment ratio. The ratio still had not completely returned to the pretreatment value at T + 48 hrs.

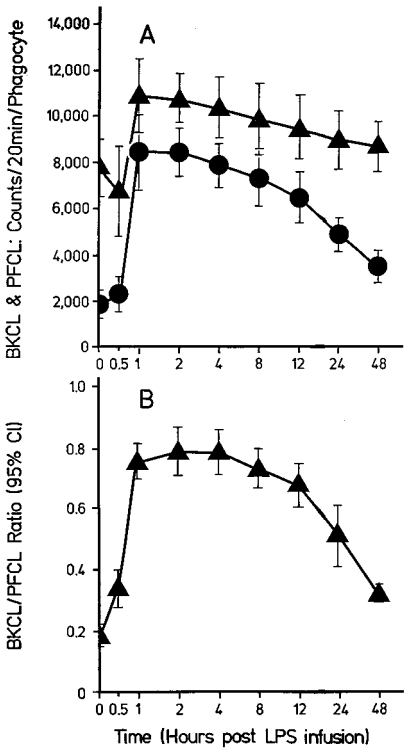

Figure 1. *A*, luminescence activity per phagocyte after stimulation with opsonized zymosan alone (*BKCL*, i.e., circulating opsonin receptor [COR] activity) and of phagocytes stimulated with opsonized zymosan plus platelet activating factor (*PFCL*, i.e., maximum opsonin receptor [MOR] activity): After a bolus infusion of endotoxin (4 ng/kg), the luminescence activity per phagocyte after exposure to opsonized zymosan *in vitro* alone (COR) began to increase at T + 0.5 hrs and peaked after increasing almost five-fold at T + 1 hr before gradually returning to baseline at T + 48 hrs (*circles*). After a bolus infusion of endotoxin (4 ng/kg), the activity per phagocyte after exposure to opsonized zymosan plus platelet activating factor (MOR) *in vitro* decreased initially at T + 0.5 hrs, peaked after increasing slightly above baseline at T + 1 hr, and remained elevated throughout the 48 hrs of the study (*triangles*). *B*, the ratio of luminescence arising from phagocytes stimulated with opsonized zymosan alone (COR) over phagocytes stimulated with opsonized zymosan plus platelet activating factor (MOR), (COR/MOR or BKCL/PFCL ratio): After a bolus infusion of endotoxin (4 ng/kg), the COR/MOR or BKCL/PFCL ratio approached unity as the endotoxin-induced opsonin-dependent receptor expression *in vivo* reached a point where the basal COR activity approached the maximum activity that can be induced *in vitro* by exposing the cells to zymosan plus platelet activating factor. Data are expressed as activity per 20 mins per phagocyte. *LPS*, lipopolysaccharide; *CI*, confidence interval.

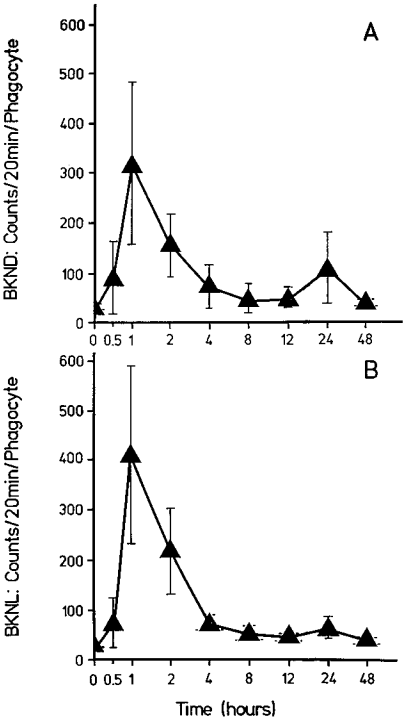

Figure 2. *A*, basal phagocyte oxidase activity expressed as lucigenin-dependent luminescence activity per phagocyte (*BKND* activity): After a bolus infusion of endotoxin (4 ng/kg), the luminescence activity per phagocyte of phagocytes incubated with the lucigenin substrate alone peaked at T + 1 hr and again at T + 24 hrs. *B*, basal phagocyte oxidase-myeloperoxidase activity expressed as luminol-dependent luminescence activity per phagocyte (*BKNL* activity): After a bolus infusion of endotoxin (4 ng/kg), the luminescence activity per phagocyte of phagocytes incubated with the luminol substrate alone peaked at T + 1 hr.

Basal Oxidase and MPO Activities in Response to Endotoxin

The COR and MOR activities are opsonin receptor functions measured as opsonin-activated phagocyte metabolism and generation of oxygenating agents. Opsonin receptor independent activities also were assessed as the PMA-stimulated oxidase and MPO activities. Figure 2*A* shows the effect of endotoxin infusion on basal oxidase activity per phagocyte. A large peak was observed at 1 hr post-LPS and a smaller secondary peak at 24 hrs post-LPS. The first peak corresponded temporally with the endotoxin-induced nadir in the circulating phagocyte count. A small increase was seen as early as 0.5 hr, which increased to a maximum at 1 hr post-LPS. Note that the peak of maximum basal oxidase activity correlated

with the large increase in COR/MOR ratio observed at this same time. The basal oxidase activity returned to the pretreatment range by 8–12 hrs post-LPS before exhibiting a second peak of activity at 24 hrs post-LPS.

Likewise, Figure 2*B* shows the effect of endotoxin infusion on basal MPO activity per phagocyte. Whereas basal oxidase activity is measured as lucigenin luminescence in the absence of an exogenous stimulus, basal MPO activity is measured as luminol luminescence in the absence of an exogenous stimulus. A relatively large peak in basal MPO activity was observed at 1 hr post-LPS, but no significant secondary peak in basal MPO activity was observed at 24 hrs post-LPS.

In summary, the circulating phagocyte response to endotoxin injection includes the following: a) a large increase in opsonin receptor expression that remained elevated at 24–48 hrs post-LPS; b) an activation of both basal oxidase and basal MPO systems that returned to normal by 8 hrs post-LPS; and c) a secondary activation of the basal oxidase system as reflected by a second peak at 24 hrs post-LPS. This second peak in basal oxidase activity occurred long after toxic symptoms had resolved and correlated with functional and antigenic decreases in factor VIIa and increases in soluble fibrin, soluble tissue factor, and soluble thrombomodulin (see subsequent discussion).

Correlation of Luminescence Measurements of Phagocyte Response to Endotoxin With Other Morphologic and Enzymatic Markers of Phagocyte Activity and Endothelial Cell Injury

Figure 3 summarizes the changes in the various variables of neutrophil function and endothelial cell injury relative to changes in COR/MOR ratio and basal oxidase responses to endotoxin. Figure 3*A* shows the relationship between COR/MOR, which reflected phagocyte opsonin receptor expression (receptor alert) and basal oxidase activity (respiratory enzyme activation). Both changed at T + 0.5 hr and peaked at T + 1 hr. The activity of oxidase, however, rapidly returned toward baseline (T + 2–4 hrs) followed by a second peak of activity (T + 24 hrs), whereas the receptor alert or receptor expression as determined by the COR/MOR ratio remained significantly elevated out to T + 24 hrs.

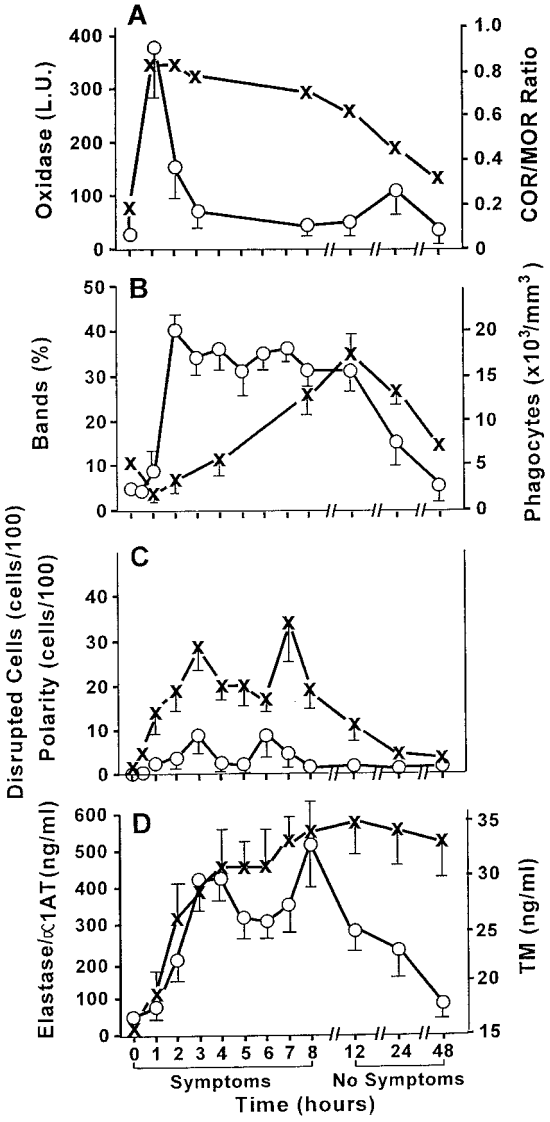

Figure 3. *A*, after a bolus infusion of endotoxin (4 ng/kg), the opsonin receptor expression (ratio of circulating opsonin receptor to maximum opsonin receptor [*COR/MOR*]) peaked at T + 1 hr and remained elevated through T + 24 hrs (*X symbols*) whereas the basal oxidase activity (BKND) (luminescence units [*L.U.*] per phagocyte) peaked twice, once at T + 1 hr and again at T + 24 hrs (*circles*). *B*, these bursts of receptor expression and respiratory enzyme activity at T + 1–2 hrs were accompanied by a sharp increase of the immature phagocyte count to 42% at T + 2 hrs (*circles*) and a more gradual increase in total phagocyte count that peaked at T + 12 hrs (*X symbols*). *C*, after a bolus infusion of endotoxin (4 ng/kg), the number of polarized (*X symbols*) and disrupted (*circles*) phagocytes peaked twice at T + 4 hrs and T + 6–8 hrs before rapidly returning to baseline. *D*, after a bolus infusion of endotoxin (4 ng/kg), the elastase-alpha-1-antitrypsin (elastase/α*1AT*) complex (*circles*) appeared and peaked twice at T + 3–4 hrs and T + 8 hrs before rapidly returning to baseline. Note that this coincides with the peak morphologic changes. In contrast, the soluble thrombomodulin (*TM*; *X symbols*) marker of endothelial cell injury was markedly elevated at T + 4 hrs and remained elevated for 24–48 hrs.

Figure 3*B* shows that this sustained receptor expression was paralleled closely by a high percentage of circulating immature neutrophils (bands), which rose to 8% at T + 1 hr at the time the total phagocyte count was at its nadir and then peaked at 42% at T + 2 hrs. This high band count occurred 1 hr after the receptor expression had peaked and before the total phagocyte count had risen above baseline. Like the opsonin-dependent receptor expression response, the percentage of immature neutrophils remained elevated out to T + 12–24 hrs, at which point the total phagocyte count reached its peak. These two panels show an early transient activation of phagocytes followed by a sustained expression of opso-

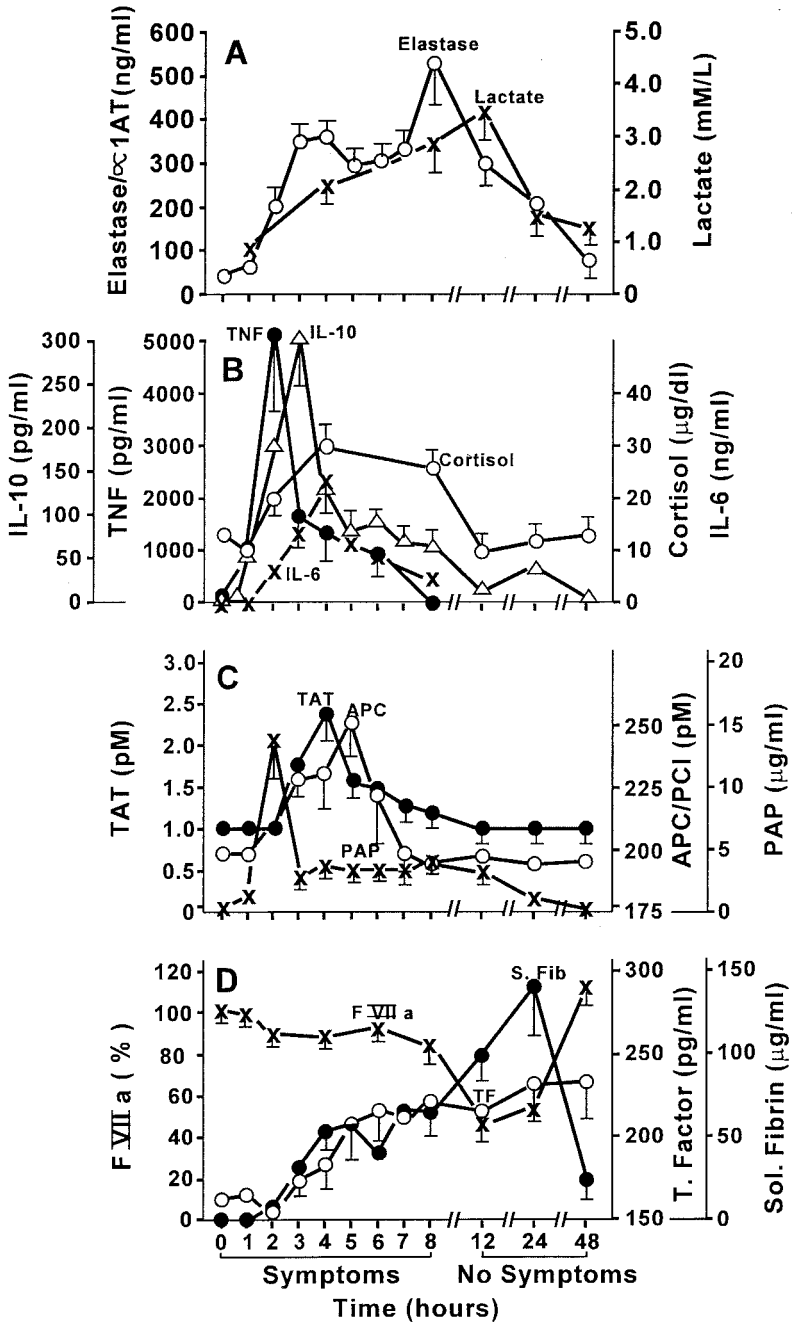

Figure 4. Qualitative correlation of phagocyte elastase production and lactate concentration with markers of the inflammatory and hemostatic system responses to endotoxin: *A*, after a bolus infusion of endotoxin (4 ng/kg), the elastase-alpha-1-antitrypsin (*Elastase/α1AT*) complexes (*circles*) appeared and peaked twice at T + 4 hr and T + 8 hr before rapidly returning to baseline. In contrast, the lactate concentration (*X symbols*), like that of the soluble thrombomodulin, remained elevated before slowly returning toward baseline at T + 48 hrs. *B*, the concentrations of inflammatory mediators tumor necrosis factor (*TNF*) (*filled circles*) and interleukin (*IL*)-6 (*X symbols*) peaked at T + 2 and 4 hrs, respectively, before rapidly returning to normal, whereas the inflammatory regulators IL-10 (*triangles*) and cortisol (*open circles*) peaked at T + 3 hrs and T + 4 hrs followed by a second peak of IL-10 at T + 7–8 hrs and a sustained elevation of cortisol out to T + 8 hrs. *C*, the concentrations of hemostatic mediator/inhibitor complexes thrombin/antithrombin (*TAT*; *filled circles*) and plasmin/antiplasmin (*PAP*; *X symbols*) peaked at T + 4 and 2 hrs, respectively, before returning toward baseline, whereas the concentration of the protein C (*PCI*) hemostatic regulator inhibitor complex (*APC*; *open circles*) peaked twice at T + 2 hrs and T + 5 hrs. *D*, the concentration of factor VIIa (*F VII a*; *X symbols*) did not reach a nadir until T + 12 hrs. This delayed response was mirrored by a reciprocal increase in the concentration of soluble tissue factor (*T. Factor*; *open circles*) and soluble fibrin monomer (*S. Fib*; *filled circles*), which also peaked at T + 12 hrs. Note that these late events occurred after the subjects had become asymptomatic and had returned to work the following day.

nin receptor (receptor alert) and elevated band and neutrophil count out to T + 24 hrs. Figure 3*C* shows changes in neutrophil morphology (polarity and cell disruption), the peak appearances of which were observed in two phases at T + 3 hrs and again at T + 6–7 hrs. The first phase of morphologic change corresponded with both the luminescence markers of neutrophil activation and appearance of immature neutrophils. The second phase occurred after the luminescence markers had returned toward baseline (T + 6–7 hrs) but within the symptomatic period. Both these phases of morphologic change corresponded to the two-phase appearance of elastase/α1AT complexes at T + 3 hrs and again at T + 7 hrs (Fig. 3*D*).

Figure 3*D* shows the response of elastase/α1AT complexes and soluble thrombomodulin. Both these markers peaked at T + 3–4 hrs, but the soluble thrombomodulin remained elevated through 24–48 hrs, whereas the elastase/α1AT complexes, after exhibiting a second peak at T + 8 hrs, returned to baseline at the same time that all of the clinical symptoms had abated (T, ≥12 hrs).

These observations raised the following questions: First, are there two stages in the response to endotoxin, and can they be better defined by examining the neutrophilic response in the context of inflammatory and hemostatic mediator responses? Second, if there are two stages, do they represent an initial inflammatory response to endotoxin (stage 1) followed by injury secondary to reperfusion (stage 2)?

Response of Mediator and Regulatory Components of the Inflammatory and Hemostatic Systems in Response to Endotoxin: Division of the Response Into Two Pathophysiologic Stages

Figure 4*A* shows the two-phase response pattern of elastase/α1AT at T + 3 and 8 hrs in relation to a sustained elevation of lactate over 12–24 hrs. Figure 4*B* summarizes the inflammatory mediator and regulator responses in which the TNF and IL-6 mediators appeared between T + 2 and 4 hrs and the IL-10 and cortisol regulators appeared between T + 4 and 8 hrs. Figure 4*C* likewise summarizes the hemostatic mediator and regulator responses in which the mediator responses (TAT) again appeared to coin-

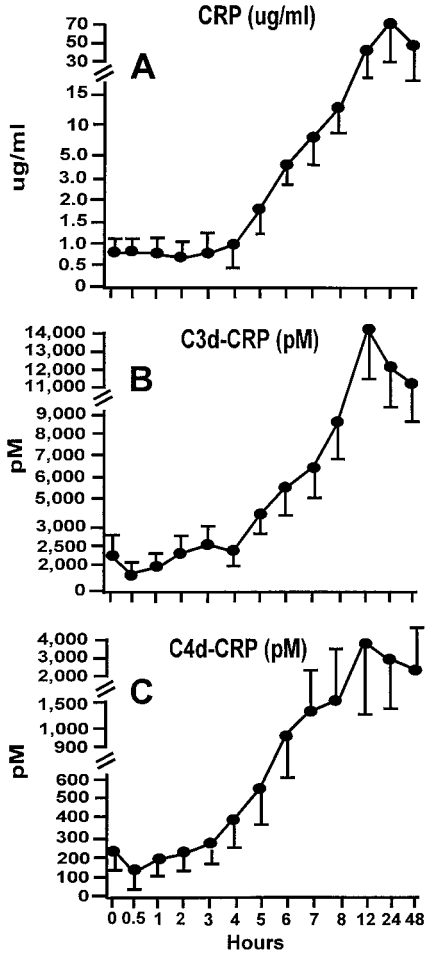

Figure 5. Correlation of C-reactive protein (*CRP*) concentration with markers of CRP/complement C3d and C4d complex responses to endotoxin. *A*, after bolus infusion of endotoxin (4 ng/kg), the CRP concentration (*circles*) increased and peaked at T + 12 hrs before gradually starting to decline. *B*, the concentration of CRP/C3d increased and peaked at 12 hrs before gradually starting to decline. *C*, the concentration of CRP/C4d increased and peaked at 12 hrs before gradually starting to decline.

cide with the regulator responses (activated protein C, PAP). All of these events except the second oxidase peak (Fig. 3*A*) and lactate responses occurred during the first 8-hr period, during which time the subjects exhibited clinical symptoms. By T + 8 hrs, all symptoms resolved and the subjects were eating and resting comfortably. Figure 4*D*, however, shows a second period of hemostatic activity at T + 12–24 hrs, as reflected by the appearance of a second and a very large peak of soluble fibrin, and a second and greater decrease in circulating factor VIIa concentration coupled with the appearance of increased concentrations of plasma tissue factor antigen. These findings, together with the second peak of oxidase activity and the sustained elevation of lactate and soluble thrombomodulin into the 12- to 24-hr period, raise the possibility of a second stage.

Response of CRP and Complement Activation Products C3d and C4d Bound to CRP

Figure 5 shows the delayed response of CRP and of CRP/C3d and CRP/C4d complexes to endotoxin. In all three cases, the responses are first evident at T + 4–5 hrs and peak at T + 12–24 hrs. Although the concentrations begin to decline after T + 24 hrs, they remain elevated far above baseline at T + 48 hrs. There is some evidence of C3d product formation at T + 3 hrs, but overall the response patterns of CRP and of the CRP/C3d and C4d complexes is delayed until the second stage of the response to endotoxin. These responses of CRP and CRP/complement product complexes coincide with the peak concentration of soluble thrombomodulin, soluble fibrin monomer, plasma tissue factor antigen, and lactate, and with the nadir of factor VIIa concentration shown in Figure 4*D*.

Control Subjects

Four control subjects were infused with saline and observed over the 48-hr period in the same manner as those subjects who were given endotoxin. In this study, none of the control subjects exhibited changes in molecular markers consistent with sustained activation of the inflammatory or hemostatic systems. Table 1 summarizes the response of white cells, neutrophils, bands, lymphocytes, and platelets and hemoglobin. This table includes both the eight subjects infused with endotoxin and the four control subjects who were catheterized but not infused with endotoxin. This table shows that the total white cell, neutrophil, band, and lymphocyte counts of the control subjects remained stable throughout the entire 48-hr period. In contrast, the white cell, neutrophil, band, and lymphocyte counts of the subjects infused with endotoxin decreased to a nadir at T + 1 hr followed by a rebound and overshoot, which peaked at T + 12 hrs. No significant change in hemoglobin or platelet counts was seen in either group. Table 2 summarizes the changes in vital signs of both control subjects and those infused with endotoxin. This table shows that the temperature, mean systemic arterial blood pressure, and heart rate remained stable throughout the entire 48-hr period, whereas the variables of the subjects infused with endotoxin exhibited changes that peaked at T + 2–4 hrs.

DISCUSSION

The neutrophil response to infusion of endotoxin into normal human subjects can be divided into membrane (opsonin-dependent receptor) and cytoplasm/organelle (enzymatic activation of oxidase and MPO) associated events, both of which can be assessed by luminimetry. This study used this technology to determine the type and magnitude of changes in phagocyte (95% to 97% neutrophils, 5% to 3% monocytes) function *in vivo* that developed after infusion of endotoxin into normal subjects. In addition, we examined these phagocyte responses relative to markers of activation of the mediator and regulatory components of both the inflammatory and hemostatic systems, as well as markers of endothelial cell injury. By using this approach we found that the human response to endotoxin occurred in two stages.

The results summarized in Figures 1–4 concerned the measurement of the membrane and cytoplasmic responses of phagocytes of eight subjects infused with endotoxin. First, the receptor response as reflected by the COR/MOR ratio was evident as early as T + 0.5 hr. This increase in ratio occurred before there was a significant decline in phagocyte count. This abrupt change in the ratio at T + 0.5 hr was a function of an increase of the COR luminescence because the MOR activity expressed per phagocyte was slightly but not significantly decreased for this T + 0.5 hr measurement (Fig. 1). This early receptor response also was associated with an early increase at T + 0.5 hr of basal oxidase and oxidase/haloperoxidase activity after endotoxin infusion. Although these early increases were not significantly different from baseline, they represented a trend leading to a significantly increased activity of both enzyme systems at T + 1 hr at a time when the phagocyte count was at its nadir. There were, however, a sufficient number of cells in circulation to give a strong signal indicating activation of both the membrane receptor and cytoplasmic respiratory enzyme components of the phagocyte/bactericidal machinery. At T + 4 hrs,

Table 1. Responses of white cells, neutrophils, bands, lymphocytes, and platelets and hemoglobin

		Hrs						
	T-0	1	2	4	8	12	24	48
Total white cell count ($\times 10^3/mm^3$)								
Experimental	5.2 ± 0.3	1.6 ± 0.16	3.5 ± 0.4	6.02 ± 0.9	13.3 ± 1.2	17.3 ± 1.8	12.6 ± 1.8	7.7 ± 0.8
Control	6.1 ± 0.08	5.9 ± 0.17	6.2 ± 0.19	7.8 ± 1.07	6.9 ± 0.4	8.8 ± 0.6	6.2 ± 0.6	5.7 ± 0.13
Neutrophil count ($\times 10^3/mm^3$)								
Experimental	3.02 ± 0.4	0.5 ± 0.1	2.06 ± 0.4	4.8 ± 0.7	11.4 ± 1.3	14.6 ± 1.7	9.2 ± 1.7	5.2 ± 0.8
Control	2.9 ± 0.15	2.8 ± 0.2	3.4 ± 0.4	3.6 ± 0.5	3.0 ± 0.6	4.6 ± 0.7	2.8 ± 0.05	2.5 ± 0.5
Band count ($\times 10^3/mm^3$)								
Experimental	0.09 ± 0.03	0.06 ± 0.03	0.2 ± 0.05	0.5 ± 0.16	1.1 ± 0.2	1.4 ± 0.4	0.6 ± 0.13	0.3 ± 0.09
Control	0.06	0.06	0.06	0.16 ± 0.02	—	0.1	0.1	0.08 ± 0.02
Lymphocyte count ($\times 10^3/mm^3$)								
Experimental	2.0 ± 0.2	1.02 ± 0.14	1.1 ± 0.19	0.6 ± 0.16	0.7 ± 0.11	1.3 ± 0.15	2.6 ± 0.2	2.2 ± 0.3
Control	2.9 ± 0.2	2.6 ± 0.3	2.5 ± 0.2	3.6 ± 0.7	3.6 ± 0.3	3.8 ± 0.4	3.2 ± 0.5	3.1 ± 0.6
Monocytes ($\times 10^3/mm^3$)								
Experimental	0.08 ± 0.01	0.01	0.14	0	0	0.03	0.01	0.17 ± 0.09
Control	0.26 ± 0.08	0.35 ± 0.06	0.2 ± 0.07	0.38 ± 0.12	0.34 ± 0.15	0.43 ± 0.02	0.15 ± 0.03	0.12 ± 0.04
Platelet ($\times 10^3/mm^3$)								
Experimental	223.9 ± 13.9	201.1 ± 19.5	187.2 ± 17.9	183.7 ± 16.7	189.5 ± 13.9	211.7 ± 16.7	204.1 ± 19.04	229.5 ± 17.01
Control	246.5 ± 33.6	291 ± 41.3	269.5 ± 38.2	245.2 ± 47.8	256.7 ± 40.6	249 ± 60.9	270 ± 43.6	250.6 ± 46.9
Fibrinogen (%)								
Experimental	100 ± 0	96 ± 13	93 ± 16	80 ± 24	95 ± 12	101 ± 20	127 ± 41	116 ± 32
Control	100 ± 0	112 ± 3	101 ± 11	102 ± 4	98 ± 7	105 ± 6	104 ± 7	95 ± 5

Experimental, n = 8; control, n = 4.

Table 2. Vital signs

		Hrs						
	T-0	1	2	4	8	12	24	48
Temperature (°F)								
Experimental	97.8 ± 0.6	98.2 ± 0.4	99.3 ± 1.0	101.5 ± 1.2	100.2 ± 0.5	99.4 ± 0.7	98.6 ± 0.4	98.5 ± 0.2
Control	98.2 ± 0.3	98.3 ± 0.3	98.7 ± 0.6	98.3 ± 0.8	98.1 ± 0.7	98.3 ± 0.8	98.4 ± 0.2	98.1 ± 0.3
Mean systemic arterial pressure (mm Hg)								
Experimental	93.6 ± 4.5	101.9 ± 11.1	102.4 ± 8.3	91.2 ± 5.4	87.1 ± 5.2	88.0 ± 7.3	92.7 ± 13.5	96.3 ± 9.3
Control	98.4 ± 3.2	99.3 ± 5.0	98.7 ± 3.0	96.1 ± 5.1	96.3 ± 5.5	94.3 ± 3.4	95.3 ± 5.2	96.1 ± 6.1
Heart rate (beats/min)								
Experimental	67 ± 12	79 ± 14	99 ± 13	114 ± 7	103 ± 7	100 ± 8	82 ± 11	78 ± 10
Control	72 ± 8	80 ± 9	85 ± 11	89 ± 12	90 ± 9	88 ± 11	88 ± 7	85 ± 10

however, the activity of both respiratory enzyme systems essentially had returned to baseline whereas the receptor expression remained high. We concluded that at T + 4 hrs, the phagocytes were on full "alert" (i.e., they showed high opsonin receptor expression) but were not "activated" (i.e., they showed no evidence of enhanced basal enzymatic activity), whereas at T + 1 hr they were on both an "alert" and "activated" status.

The second peak of basal oxidase activity at T + 24 hrs, however, occurred in the face of a declining receptor expression and was limited to the oxidase enzyme system. This was unexpected, because all inflammatory and hemostatic mediators had returned to baseline by T + 8 hrs and the subjects all appeared fully recovered and had returned to work. In contrast with the first peak of receptor and enzyme activity at T + 1 hr, which was linked to the initial inflammatory response to endotoxin (cytokines and pharmacologic mediators), this second peak occurred in the absence of proinflammatory mediators. This observation showed the following for the first time in the study of the human model of endotoxemia: a) there was a second burst of respiratory enzyme activity (postinflammatory) response in this model; and b) changes in respiratory enzyme activity of phagocytes may occur independently of activation via the opsonin receptor pathway.

Although studies of the luminescence markers of phagocyte response to endotoxin carry the advantage of being measured in real time, we wished to correlate these findings with other markers of phagocyte/neutrophil response. We observed that although the phagocyte response was dominated by peak oxidase and oxidase MPO activity that resolved by T + 4 hrs, the appearance in the circulation of polarized and disrupted neutrophils continued for an additional 4 hrs. This was accompanied by the continued appearance of elastase/α1AT complexes. This corresponded to the 8-hr symptomatic period. Other events involving the neutrophils were carried over into the succeeding 16-hr asymptomatic period out to T + 24 hrs. These included a sustained elevation of receptor expression (increased COR/MOR), a continued increase in total white cell count including immature neutrophils, and a second peak of oxidase activity and blood lactate. We concluded that these events represented a first- and second-stage response to endotoxin. We concluded that during the first

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symptomatic stage, the phagocytes expressed opsonin receptors (COR/MOR), and that they were both activated (oxidase-MPO) and engaged (morphology). Finally, we concluded that although the phagocytes continued to be mobilized from sites of production and remained on receptor alert during the second stage, they were activated and engaged only during the first symptomatic stage immediately after the infusion of endotoxin.

Studies by Suffredrini et al. (1, 3, 5), Parrillo et al. (2), and Van Deventer et al. (4) on human subjects infused with endotoxin characterized the initial inflammatory (TNF, IL-6, IL-8) and hemostatic (TAT, PAP, tissue plasminogen activator) responses out to T + 6–8 hrs. These studies, although clearly demonstrating the link between inflammatory, hemostatic, and symptomatic responses during the 6- to 8-hr interval after endotoxin, were not carried out over a length of time sufficient to demonstrate the existence of a second asymptomatic stage (T + 12–48 hrs). In addition to performing assays of cytokines and hemostatic factors normally run, we assayed for factor VIIa and soluble fibrin, which reached a nadir or peaked again at T + 12–24 hrs, indicating a second wave of procoagulant activity long after the TAT, activated protein C, and PAP molecular markers had returned to baseline. This finding is consistent with the observation that factor VIIa decreased in patients in septic shock (45). These studies also included molecular markers of endothelial injury (soluble thrombomodulin) and tissue factor expression (plasma tissue factor antigen), both of which indicated endothelial and reticuloendothelial system perturbation and injury that extended into and were amplified in the second stage. They also included molecular markers of acute phase protein/complement product complex formation which, like the concentration of soluble thrombomodulin and plasma tissue factor antigen, increased late in the first stage (T + 4–6 hrs) and peaked during the second stage. These observations support our conclusion that the human response to endotoxin occurs in two stages: the first (symptomatic) immediately after infusion of endotoxin, and the second (asymptomatic) after reperfusion of the microvasculature.

The mechanism underlying the second stage includes reperfusion of the microvasculature and the oxidative stress that attends such a reperfusion. Complement involvement in reperfusion injury has been documented by many observers, including Lehmann et al. (46), Wheeler and Ryan (47), Mannick (48), and Ward (49). Two recent studies of the effect of oxidative stress on the interaction of cultured vascular endothelium with complement and tissue factor are of particular interest. Collard et al. (50) showed that mannose-binding protein binds to and mediates complement activation and injury of endothelial cells after oxidative stress. Yan et al. (51) demonstrated that diminished levels of oxygen activate the transcription factor early growth response-1 leading to the *de novo* transcription/translation of tissue factor in monocytes. Our observations of the appearance of products arising from activation of the hemostatic and complement systems during the second stage (reperfusion) are consistent with these *in vitro* observations. The lack of evidence of thrombin production (TAT complexes) and plasmin production (PAP complexes) in this second stage, however, was unexpected and was in contrast with what was observed in the first stage. We also were surprised by the apparent disconnect between this second stage and the subjects' return to normal (asymptomatic) clinical status.

These observations emphasize that during the first stage under conditions in which the inflammatory and hemostatic responses to endotoxin are "under control" as reflected by stable platelet count and fibrinogen concentrations, there was considerable activity of many components, particularly phagocytes. These results show that there is a second stage and that during this stage, there was a sustained injury to the vascular endothelium secondary to reperfusion of the microvasculature and that this occurred in subjects who were asymptomatic. Finally, these results suggest that it should be possible in the future to assess the degree of inflammatory stress of the microvascular system in patients who are at risk before the development of an uncompensated inflammatory coagulopathy.

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