Evaluation of lipopolysaccharide-induced activation of equine neutrophils

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Objective—To evaluate lipopolysaccharide (LPS)-induced activation of equine neutrophils in blood.

Sample Population—Blood samples from 5 healthy adult Thoroughbreds.

Procedures—Neutrophil integrin (CD11/CD18) expression, size variation, degranulation, and deformability were measured with and without incubation with LPS. Time and concentration studies were done. The mechanism of endotoxin-induced neutrophil activation was investigated by inactivating complement or preincubating neutrophils with inhibitors of tumor necrosis factor-α (TNF-α) synthesis, prostaglandin-leukotriene synthesis, or platelet-activating factor.

Results—Incubation of equine neutrophils with LPS increased cell surface expression of CD11/CD18, decreased neutrophil deformability, increased and decreased neutrophil size, and induced neutrophil degranulation. The LPS-induced neutrophil activation was attenuated by addition of inhibitors of TNF-α and prostaglandin-leukotriene synthesis.

Conclusions and Clinical Relevance—Equine neutrophils are readily activated in vitro by LPS, resulting in increased expression of integrin adhesion molecules, decreased deformability, variation in neutrophil size, and degranulation. The tests used to detect activated neutrophils in this study may be useful in detecting in vivo neutrophil activation in horses with sepsis and endotoxemia. (Am J Vet Res 2002;63:811–815)

A variety of experimental studies have incriminated neutrophils as major mediators of organ injury in sepsis and endotoxemia. In these studies, neutrophils that became activated intravascularly were rigid, expressed adhesion receptors, and were primed for release of oxygen radicals but had reduced capacity to migrate into tissues. Therefore, activated neutrophils tended to lodge in small blood vessels throughout the body and release oxygen radicals and proteases, resulting in endothelial cell injury. If severe, this process can lead to altered vascular permeability and multiple organ failure.

Results of several studies indicate that neutrophils may become activated intravascularly in horses with inflammatory diseases. Plasma myeloperoxidase concentration, a neutrophil primary granule component, was reported to be high in horses with colic. Further, nonsurvivors had a greater decrease in neutrophil deformability, compared with those that survived.

The role of lipopolysaccharide (LPS) in activation of equine neutrophils has been incompletely studied. Lipopolysaccharide readily binds to equine neutrophils in the absence of plasma, but the mechanism by which activation occurs is unknown. Lipopolysaccharide was reported to be a weak stimulus for oxygen radical generation and degranulation but did prime neutrophils for agonist-induced oxygen radical release. The purpose of the study reported here was to further evaluate the capacity of LPS to activate equine neutrophils and to investigate the mechanism by which this activation occurs.

Materials and Methods

Blood samples—Blood was collected from 5 healthy 5- to 8-year-old Thoroughbreds that were part of the University of Minnesota teaching herd. Blood samples were collected from a jugular vein and placed in evacuated tubes containing sodium heparin as the anticoagulant. Total leukocyte counts were determined by use of a hemocytometer, and all counts were adjusted to 5 × 10^9/ml by addition of autologous plasma.

Experimental design—Heparinized blood was incubated with and without addition of various concentrations of Escherichia coli LPS serotype 026:B6, human recombinant tumor necrosis factor-α (TNF-α), platelet-activating factor (PAF), or human recombinant interleukin-8 (IL-8) for periods ranging from 15 minutes to 2 hours. A previous study has reported that equine neutrophils respond to human recombinant TNF-α and PAF in vitro. In other experiments, heparinized blood was incubated with inhibitors of LPS, TNF-α, prostaglandins-leukotrienes, and PAF for 15 minutes before addition of LPS. Polymyxin B (100 µg/ml) was added to inhibit LPS activity. Pentoxifylline (10 mg/ml) was added to inhibit TNF-α production. WEB 2086 (10 µM) was added as a PAF receptor antagonist. 5,8,11,14-Eicostetraynoic acid (ETYA, 100 µM) was added to inhibit prostaglandin and leukotriene synthesis. Plasma was removed from some samples by dilution of blood (1:10) with Dulbecco phosphate-buffered saline solution (PBSS) containing 1% bovine albumin. After centrifugation, the supernatant was removed and replaced with a volume of Dulbecco PBSS solution containing 1% bovine albumin equivalent to the original plasma volume. Complement was inactivated by centrifugation of blood, and the replacement of plasma with autologous plasma was heated to 56°C for 30 minutes.

A separate experiment was done to evaluate the effect of LPS on leukocyte viability. Leukocyte viability was evaluated before and after incubation with LPS (3.0 µg/ml) by use of the trypan blue exclusion technique. Tests were performed in duplicate, and the results of 2 separate experiments were analyzed. Leukocyte viability before incubation with LPS was 98.2 ± 0.1% and after 1 and 2 hours of incubation with LPS was 95.7 ± 0.7 and 94.2 ± 0.4%, respectively. Leukocyte viability after removal of plasma was 96.1 ± 1.0%.
Neutrophil CD11/CD18 expression—For determination of CD11/CD18 expression by neutrophils, 100 µl of blood was placed in a tube containing 2 ml of erythrocyte lysis buffer to which 0.5% paraformaldehyde had been added. The mixture was incubated for 10 minutes at 22 to 25 C and centrifuged at 1,000 x g for 3 minutes. The supernatant was decanted, and cells were washed and resuspended in 100 µl of Dulbecco PBS containing 2% sheep serum and 2 mM sodium azide. Neutrophil CD11/CD18 expression was determined by use of a flow cytometer as described. One of 2 antibodies that reacted against CD11/CD18 (R15.7 and MMH237) was added to leukocyte suspensions and incubated at 22 to 25 C for 30 minutes. Thereafter, cells were washed, resuspended in Dulbecco PBS containing 1% sheep serum and 2 mM sodium azide, and incubated with 20 µl of a 1:20 dilution of fluorescein-conjugated sheep anti-mouse IgG. After washing, samples were analyzed by use of a flow cytometer. Forward scatter was set at 0.0 A, side scatter was set at 473 V, amp gain was set at 1.81 A, and green fluorescence intensity (FL1) was set at 500. Cells were displayed as forward-angle versus side-angle light scatter plots, and the neutrophil population was identified. A gate was set to identify the neutrophil cluster (Fig 1). The median fluorescence intensity of the neutrophil population was determined. Positive control samples consisted of neutrophils incubated with phorbol myristate acetate (1.0 mg/ml) for 15 minutes, and negative control samples consisted of blood incubated with vehicle.

Figure 1—Scatterplots of equine neutrophils depicting the separation of cells into 3 subpopulations (R2, R3, R4) on the basis of size. Unstimulated neutrophils from a healthy horse (top). Neutrophils from the same horse after incubation with lipopolysaccharide (LPS; 1.0 mg/ml) for 2 hours (bottom). Notice the shift of neutrophils from the central gate to the smaller and larger gates.

Neutrophil size distribution—The effect of LPS on neutrophil size and granularity was evaluated by use of flow cytometry. Blood (100 µl) was incubated with and without LPS (1.0 mg/ml) for 1 or 2 hours. Thereafter, 2 ml of erythrocyte lysis buffer containing 0.5% paraformaldehyde was added and incubated for 10 minutes at 22 to 25 C. After centrifugation, the supernatant was decanted, and cells were washed and resuspended in 100 µl of Dulbecco PBS containing 2% sheep serum and 2 mM sodium azide. The leukocyte population (30,000 cells) was evaluated as forward-angle versus side-angle light scatterplots. By use of samples incubated without addition of LPS, a contour plot was used to define the neutrophil population. A rectangular gate was set to define this population (Fig 1). Two additional gates were set to detect neutrophils that were larger and smaller than the main neutrophil population. The median fluorescence intensity and percentage of neutrophils in each gate were determined. To eliminate neutrophil aggregates from the analysis, leukocytes were labeled with mouse anti-horse CD44. Fluorescent intensity of the entire neutrophil population was evaluated by use of a forward-angle light scatter versus fluorescence intensity dot plot. A small population of large cells with increased CD44 fluorescent intensity (presumably leukocyte aggregates) was observed. The size of the larger gate (R4 in Fig 1) was adjusted to exclude the neutrophils with increased fluorescence intensity.

Neutrophil degranulation—Neutrophil degranulation was measured by determining the release of acridine orange from cytoplasmic granules. Acridine orange binds to mucopolysaccharides in azurophilic (ie, primary) granules and emits red light after activation by laser light. A reduction in red fluorescence intensity of neutrophils is associated with degranulation of primary granules. Median log red fluorescence intensity of the neutrophil population was detected by use of a flow cytometer. Forward scatter was set at 0.0 A, side scatter was set at 473 V, amp gain was set at 1.81 A, and red fluorescence intensity (FL3) was set at 430.

Leukocyte deformability—Leukocyte deformability was analyzed by determining the pressure generated when blood, diluted 1:1 with Dulbecco PBS, was pumped at constant rate through polycarbonate filters containing 5-mm pores. Mean filter pore size was 4.5 ± 0.6 µm, and pore density was 4.0 ± 0.12 x 10^3/µm². The filtration device consisted of a 13-mm microanalysis filter holder connected to a constant-rate infusion pump. Filtration pressures were measured by use of a pressure transducer, and pressure readings were recorded on a polygraph recorder. Pressure curves were characterized by an initial rapid rise in pressure that plateaued within 5 seconds, followed by a secondary less-rapid pressure increase. Initial pressure increases were quantified. Results of previous studies indicate that equine erythrocyte and platelet numbers and plasma protein concentration have minimal effect on filterability through filters with 5-mm pores.

Statistical analyses—All data were determined in duplicate or triplicate, and results were reported as mean ± SD. With the exception of neutrophil deformability, data from each of 5 horses were analyzed by use of factorial ANOVA. For neutrophil deformability, data from 3 horses were analyzed. Logarithmic data were converted to linear data before analysis. Means of interest were compared by use of the Bonferroni-Dunn F-test. Results were considered significant at values of P < 0.05.

Results

CD11/CD18 expression—Incubation of blood with LPS at concentrations of 0.5, 1.0, and 5.0 µg/ml for 30 minutes resulted in a dose-dependent increase
in CD11/CD18 (Fig 2). Addition of polymyxin B to LPS before incubation with blood prevented the increase in CD11/CD18 expression. Results were similar when neutrophils were labeled with both monoclonal antibodies; therefore, in subsequent studies only R15.7 was used.

In a second study, blood was incubated with LPS (1.0 µg/ml) for 15, 30, 60, or 120 minutes (Fig 3). A significant increase in CD11/CD18 expression was observed after 30 minutes of incubation and was further increased at 60 and 120 minutes.

CD11/CD18 expression was also evaluated after incubation of blood with TNF-α, PAF, or IL-8 for 1 hour (Fig 4). Addition of TNF-α, PAF, and IL-8 induced significant increases in CD11/CD18 expression. When LPS and TNF-α were added together, no additive effect was observed.

To determine whether the effect of endotoxin on CD11/CD18 expression was a direct effect of the action of LPS on neutrophils or whether it developed secondary to secretion of inflammatory mediators, inhibitors of TNF-α, prostaglandin synthesis and leukotriene synthesis, or a PAF receptor antagonist was added to blood before addition of LPS (1.0 mg/ml; Fig 4). Incubation of blood samples with pentoxifylline, EYTA, or WEB 2086 alone had no effect on neutrophil CD11/CD18 expression. Addition of pentoxifylline and ETYA inhibited LPS-induced increase in CD11/CD18 expression. Replacement of plasma with saline (0.9% NaCl) solution also prevented LPS-induced increase in CD11/CD18 expression, whereas addition of WEB 2086 and complement depletion did not.

Neutrophil size distribution—Neutrophil size distribution was evaluated by use of flow cytometry at 1 and 2 hours after addition of LPS. Three gates were set to evaluate normal, increased, and decreased size (Fig 1). In untreated control samples that were incubated for 1 or 2 hours, neutrophils were tightly clustered, and the central gate contained 84 ± 4% of the total neutrophil population (Table 1). A progressive increase in the percentage of cells with large and small size was observed with increasing concentrations of LPS. Results for 1- and 2-hour incubation times were similar. Cells in the smaller neutrophil gate generally shifted downward, indicating that they were less granular (Fig 1). Conversely, TNF-α had little effect on neutrophil size distribution.

Neutrophil filterability—Blood was incubated with 3 concentrations of LPS for 1 hour, and filtration pressures were recorded when blood was pumped through filters with 5-mm pores. Incubation with LPS (0.5 µg/ml) resulted in a slight but significant increase in filtration pressure (Fig 5). Incubation of blood with LPS (1.0 and 5.0 mg/ml) resulted in marked increases in filtration pressure. Addition of TNF-α resulted in a significant increase in filtration pressure but addition of PAF did not.
Although the pathogenesis is complex, intravascularly activated neutrophils appear to be major mediators of the adverse systemic effects associated with endotoxemia. General characteristics of activated neutrophils include increased cell surface integrin adhesion molecule expression, decreased L-selectin expression, increased rigidity, inflammatory cytokine production, change from a spherical to elongated shape, increased size, decreased chemotaxis, enhanced oxygen radical production when activated, and degranulation. These activated neutrophils are sequestered in postcapillary venules and capillaries as a result of their rigidity and up-regulated integrin adhesion molecules. Because of decreased chemotactic capacity, intravascularly activated neutrophils remain in close contact with endothelial cells for a prolonged period. Neutrophil adhesion can result in microvascular obstruction, synthesis of inflammatory mediators, and release of toxic oxygen radicals and proteases. Release of toxic oxygen radicals and proteases directly onto the endothelial cell membrane can result in altered vascular permeability, endothelial cell dysfunction, and death. When severe, endothelial and parenchymal injury can lead to multiple organ failure.

Results of the study reported here indicate that equine neutrophils are readily activated in vitro by LPS. Evidence for activation included increased expression of CD11/CD18, decreased neutrophil deformability, both increased and decreased neutrophil size, and neutrophil degranulation. Other studies have revealed equine neutrophil degranulation, increased iodination, and priming for oxygen radical release when neutrophils are exposed to LPS. The mechanism by which LPS induces neutrophil activation appears to be complex. The effect of LPS on CD11/CD18 expression was dependent on the presence of plasma and was blocked by addition of inhibitors of prostaglandin-leukotriene and TNF-α synthesis. Unlike LPS-induced platelet activation, LPS-induced neutrophil activation was not mediated by PAF. These data indicate that LPS may act indirectly through secretion of several mediators. These mediators could be secreted by neutrophils, monocytes, or both.

Equine neutrophils are readily activated in vitro by LPS, resulting in increased expression of integrin adhesion molecules, decreased deformability, variation in neutrophil size, and degranulation. The sensitivity of equine neutrophils to endotoxin-induced activation may, at least in part, explain the sensitivity of horses to endotoxia.

### Table 1—Changes in equine neutrophil size distribution induced by incubation with lipopolysaccharide (LPS) or tumor necrosis factor-α (TNF-α) for 2 hours

<table>
<thead>
<tr>
<th>Neutrophil size</th>
<th>Negative control</th>
<th>LPS 0.5 mg/ml</th>
<th>LPS 1.0 mg/ml</th>
<th>LPS 5.0 mg/ml</th>
<th>TNF-α 0.1 mM</th>
<th>TNF-α 0.5 mM</th>
</tr>
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<tbody>
<tr>
<td>Normal (%)</td>
<td>84 ± 4</td>
<td>68 ± 8*</td>
<td>48 ± 9*</td>
<td>36 ± 4*</td>
<td>85 ± 3</td>
<td>80 ± 4</td>
</tr>
<tr>
<td>Large (%)</td>
<td>7 ± 1</td>
<td>18 ± 5*</td>
<td>25 ± 9*</td>
<td>49 ± 6*</td>
<td>7 ± 2</td>
<td>6 ± 4</td>
</tr>
<tr>
<td>Small (%)</td>
<td>6 ± 1</td>
<td>10 ± 4</td>
<td>13 ± 3*</td>
<td>11 ± 3</td>
<td>5 ± 3</td>
<td>13 ± 4*</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD.
*Significantly (P < 0.05) different from negative control value.

### Table 2—Effects of LPS on equine neutrophil degranulation as determined by measurement of the decrease in red fluorescence intensity of neutrophils stained with acridine orange

<table>
<thead>
<tr>
<th>Additive</th>
<th>Log red fluorescence intensity</th>
<th>1 h</th>
<th>2 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>163 ± 4</td>
<td>166 ± 3</td>
<td></td>
</tr>
<tr>
<td>LPS 0.5 μg/ml</td>
<td>165 ± 8</td>
<td>141 ± 5*</td>
<td>131 ± 4*</td>
</tr>
<tr>
<td>LPS 1.0 μg/ml</td>
<td>152 ± 8*</td>
<td>131 ± 4*</td>
<td></td>
</tr>
<tr>
<td>LPS 5.0 μg/ml</td>
<td>125 ± 3*</td>
<td>128 ± 3*</td>
<td></td>
</tr>
</tbody>
</table>

Values are given as mean ± SEM.
*Significantly (P < 0.05) different from control value.

Neutrophil degranulation—Neutrophil degranulation was evaluated after 1 and 2 hours of incubation with LPS. A significant decrease in red fluorescence intensity of the neutrophil population was detected at 1 hour of incubation at LPS concentrations of 1.0 and 5.0 μg/ml (Table 2). At 2 hours, a decrease in red fluorescence intensity was detected at all LPS concentrations.

Discussion

Endotoxia is thought to be involved in the systemic illness associated with gastrointestinal tract disorders and foal septicemia. When compared with other species, horses are more sensitive to the deleterious effects of LPS. The intraperitoneal lethal dose of LPS for ponies is 200 to 400 μg/kg, whereas the lethal dose for rabbits and guinea pigs is 3 and 10 mg/kg, respectively. The cause of the unique susceptibility of horses to endotoxia has not been determined.
shock and purpura.

References


