Lipopolysaccharide Enhances CD11b/CD18 Function But Inhibits Neutrophil Aggregation

By Eric B. Lyman, Scott I. Simon, Yvan P. Rochon, and Larry A. Sklar

Human neutrophils are primed in the presence of complexes of lipopolysaccharide (LPS) with its serum binding protein (LBP) in a manner dependent on CD14. Cellular consequences of priming include increased responsiveness, the upregulation of surface proteins including the adhesive integrin CD11b/CD18 (Mac-1), the increased binding of certain ligands to CD11b/CD18, and the concurrent shedding of the L-selectin homing receptor. Because expression of both CD11b/CD18 and L-selectin is obligatory for formyl peptide-stimulated neutrophil aggregation in vitro (Simon et al., Blood 82:1097, 1993), we have examined the consequences of bacterial endotoxin on the expression of neutrophil adhesive molecules. We observed that the exposure of neutrophils to LPS/LBP, while enhancing the surface numbers and adhesive function of CD11b/CD18 for latex particles, did not induce aggregation. In contrast, as the LPS/LBP concentration increased (ED50 = 30 ng/mL LPS/LBP), the ability of neutrophils to aggregate decreased in parallel with the shedding of L-selectin. Moreover, when L-selectin adhesive activity was blocked by treatment with Fab fragments of Dreg-200, aggregation was inhibited to an extent roughly proportional to the available L-selectin. Blocking of LPS/LBP with CD14-specific monoclonal antibodies suppressed L-selectin shedding and preserved formyl peptide-stimulated aggregation. Taken together, the data suggest that inhibition of neutrophil aggregation by LPS/LBP is related to the expression of L-selectin via CD14 rather than LPS inhibition of CD11b/CD18 function during cellular stimulation.

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MATERIALS AND METHODS

Neutrophil isolation. Human blood was obtained from healthy volunteers by venipuncture and collected into a sterile syringe containing 12 U heparin per milliliter of blood. Neutrophils were isolated using Mono-Poly Resolving Medium (Flow Laboratories, Irvine, Scotland) or Polymorphprep (Nycomed Pharma AS, Oslo, Norway). Blood was layered onto resolving medium and centrifuged at 400g for 28 minutes at 15°C. The granulocyte bands were collected and washed once in HEPES buffer without calcium (110 mmol/L NaCl, 10 mmol/L KCl, 10 mmol/L glucose, 1 mmol/L MgCl₂, and 30 mmol/L HEPES, pH 7.3), then pelleted at 400g for 10 minutes at 4°C. Buffer was depleted of endotoxin by affinity chromatography over a polymyxin B Sepharose column (Detox-Gel, Pierce Scientific, Rockford, IL) and then autoclaved. Neutrophils (PMN) were resuspended (10⁷/mL for receptor studies or 4 × 10⁶/mL for aggregation) in buffer containing 0.1% w/v polyvinyl pyrrolidone-human serum albumin (HSA; Armour Pharmaceutical Co, Kankakee, IL) and kept at 4°C until used. Neutrophils isolated under these conditions retain receptor levels comparable with blood, as determined by staining with fluorescent MoAbs. Isolated neutrophils retained 100% of L-selectin compared with blood. CD11b/CD18 was increased by an average of 5.1% during isolation, as compared with stimulated samples (100 ng/mL LPS/LBP).

Reagents. LPS/LBP Complexes: LPS, Salmonella minnesota RE595 (List Biochemicals, Campbell, CA) was suspended at 500 µg/mL in LPS buffer (50 mmol/L HEPES, 10 mmol/L EDTA, pH 7.5) by repeated brief sonication. Rabbit LBP was kindly provided by Drs Peter Tobias and Richard Ulevitch (Department of Immunology, Research Institute of Scripps Clinic, La Jolla, CA). Both LPS and LBP were diluted to 40 µg/mL in LPS buffer containing 0.1% human serum albumin. LPS/LBP complexes were formed by combining LPS and LBP in a ratio of 1:3, respectively, followed by 37°C incubation for 10 minutes, producing a stock solution of LPS/LBP at 10 µg/mL LPS.

Formylhexapeptide (CHO-Nle-Leu-Phe-Nle-Tyr-Lys; Bachem BioScience, Torrance, CA) stocks were dissolved in dimethyl sulfoxide at 10⁻⁵ mol/L. Daily stocks of 10⁻³ mol/L were produced in buffer containing HSA.

LPS/LBP Complexes: LPS, Salmonella minnesota RE595 (List Biochemicals, Campbell, CA) was suspended at 500 µg/mL in LPS buffer (50 mmol/L HEPES, 10 mmol/L EDTA, pH 7.5) by repeated brief sonication. Rabbit LBP was kindly provided by Drs Peter Tobias and Richard Ulevitch (Department of Immunology, Research Institute of Scripps Clinic, La Jolla, CA). Both LPS and LBP were diluted to 40 µg/mL in LPS buffer containing 0.1% human serum albumin. LPS/LBP complexes were formed by combining LPS and LBP in a ratio of 1:3, respectively, followed by 37°C incubation for 10 minutes, producing a stock solution of LPS/LBP at 10 µg/mL LPS.

Receptor analysis using flow cytometry. Neutrophils were suspended at 10⁷/mL in buffer containing 1.5 mmol/L CaCl₂ in auto-claved 12 × 75 mm cmometry tubes (Becton Dickinson). The dose response to 1 PS/LBP was determined on 200 µL of cell suspension, incubated 30 minutes at 37°C in an orbiting water bath, then chilled to 4°C. Each sample was stained with 0.2 µg/mL LDS-751. Dreg-200, and Leu-15 for 60 minutes. The kinetics of receptor expression were explored in 2-ML samples of cell suspension, treated with LPS or LPS/LBP and incubated for 60 minutes as described. At specified timepoints, 200-µL samples were removed to sample tubes on ice. Cells were stained with LDS-751, and receptors were labeled with Dreg-200 and Leu-15 as described above.

Receptor samples were analyzed at 4°C using a FACScan flow cytometer (Becton Dickinson Immunocytometry, San Jose, CA). Two thousand cellular events per sample were acquired using an LDS-751 fluorescence threshold (FL3, Detector 700). Data was analyzed using FACScan research software, as linear scale histograms of FITC fluorescence (FL1, Detector 650) and PE fluorescence (FL2, Detector 665). Specific mean channel receptor fluorescence for each sample was determined in the absence of an additional wash step by subtracting nonspecific fluorescence obtained from samples labeled with fluorescent antibody in the presence of a 50-fold excess of unlabeled antibody. The percent expression for adhesive molecules was defined as the specific-sample mean channel fluorescence (MCF) divided by specific mean fluorescence of parallel controls × 100. L-selectin expression was compared with unstimulated controls, and CD11/CD18 expression was compared with LPS/LBP (100 ng/mL) treated controls. Fluorescent FITC- and PE-conjugated microspheres standards (Flow Cytometry Standards, Research Park, NC) were measured daily as an independent control for standardization of the flow cytometer detection system.

Flow cytometric measurements of aggregation. Experimental samples (1 to 4 × 10⁶ PMN/mL) were pretreated with LPS/LBP or uncomplexed LPS as described for dose-response experiments. In bead-binding experiments, LDS-751 was withheld, because red-fluorescent, carboxylate-modified latex microspheres (CMLs) (PicoSpheres, Molecular Probes, Eugene, OR) were added (2 or 4 × 10⁷/mL). All samples were further incubated for 2 minutes at a 37°C mixing device, stirred at ~500 rpm by a 7 × 2-mm bar magnet (VWR Scientific, Media, PA). This mixing configuration has been previously established to produce optimal shear conditions for aggregation in vitro. Aggregation was stimulated by the addition of 1 µmol/L formyl peptide. LPS/LBP (100 µg/mL) was also tested as an agonist for aggregation. In neutrophil aggregation experiments, before adding stimulus, a 50-µL sample was removed to 150-µL cell buffer for receptor analysis, then constitutive aggregation was immediately measured in the remaining aggregation suspension. In CD14 specificity experiments, neutrophils were preincubated with 20 µg/mL My4, 63D3, or IgG2b for 10 minutes at 37°C, before the addition of LPS/LBP. Receptor samples were also obtained after addition of formyl peptide in CD14 specificity studies.

Two thousand events per data point were acquired from live cell suspensions at consistent starting times after stimulation, using live forward and 90° light scatter gating (bead binding) or an FL3 threshold (neutrophil aggregation). At the particle concentrations used and the low flow-rate setting (12 µL/min), acquisition required approximately 10 seconds. Aggregation was quantitated by analyzing dot plots of 90° light scatter versus FL3 (bead binding) or histograms of LDS-751 fluorescence (neutrophil aggregation). LDS-751 staining fluorescence is homogenous in intact neutrophil singlets and remains stable after stimulation. Absolute bead binding (B₀) by neutrophils was determined by equation 1.

\[ B_{0} = \frac{j}{2N_{j}} \]
Dose response of L-selectin and β2-integrin expression to LPS/LBP. Isolated neutrophils (10⁶/mL) were incubated with varying concentrations of LPS/LBP for 30 minutes at 37°C. Samples were chilled to 4°C on ice and stained with LDS-751, Dreg-200 Fab-FITC, and Leu-15-PE for flow cytometric analysis as described in Materials and Methods. The dose-dependent shedding of L-selectin (□) is shown as mean percent expression. Percent maximum expression (N) is given as the specific sample MCF divided by specific MCF of the 100 ng/mL LPS/LBP sample. Data show the mean expression ± SEM from four and three experiments, respectively.

\[ F_i = \frac{N_i}{\sum N_i} \]  
\[ R = \frac{F_i \text{(Unstimulated)} - F_i \text{(t = 15 sec)}}{15 \text{ sec}} \times 100 \]

An initial rate of aggregate formation was also computed. It describes the recruitment of singlets into aggregates during the first fifteen seconds after stimulation. The initial rate (R) was computed as the mean percent of total cells forming aggregates per second by equation 3.

Inhibiting L-selectin-dependent aggregation using Dreg-200 Fab. Neutrophil aggregation was inhibited with increasing concentrations of monovalent Dreg-200 antibody fragments. The reported kd for Dreg-200 Fab is 30 mmol/L (1.5 μg/mL). Neutrophils (4 × 10⁶/mL) were preincubated with Dreg-200 Fab-FITC (1.5 μg/mL, 3.0 μg/mL, or 20 μg/mL) for 15 minutes at 37°C before aggregation was stimulated as described above. FITC-conjugated Dreg-200 Fab was used in the qualitative comparison of L-selectin expression at different time points during the course of aggregation.

RESULTS

Effect of LPS/LBP on neutrophil L-selectin and CD11b/CD18 expression. Because adhesion and aggregation studies are performed on isolated neutrophils, conditions were first established for using LPS/LBP to alter adhesive receptor expression. The dose response and time course for the regulation of L-selectin and CD11b/CD18 by LPS/LBP complexes are shown in Figs 1 and 2. The dose-dependent depletion of L-selectin and upregulation of CD11b/CD18 are shown in Fig 1. L-selectin was shed to ~10% of control by 100 ng/mL LPS/LBP with an ED₅₀ of approximately 3 ng/mL. The percent expression of CD11b/CD18 is given in relation to the highest LPS/LBP concentration used (100 ng/mL). CD11b/CD18 upregulation occurred over a slightly wider concentration range of LPS/LBP than L-selectin (1 to 100 ng/mL). Doses of LPS/LBP above 1 μg/mL stimulated slightly higher expression of CD11b/CD18 (data not shown).

The kinetics of LPS/LBP adhesive receptor regulation are dependent upon LPS concentration. Figure 2A depicts the depletion of L-selectin from neutrophils over time. Exposure to 100 ng/mL LPS/LBP caused maximal shedding of L-selectin within 20 minutes (t½ ~ 8 minutes). At 10 ng/mL LPS/LBP, the t½ was slowed to ~17 minutes and the extent of shedding appeared slightly less than with the maximal priming dose. L-selectin shedding in response to uncomplexed LPS (10 ng/mL) did not differ significantly from
control within the first 30 minutes, but accelerated toward the end of the sample period. The kinetics of CD11b/CD18 upregulation were very similar to L-selectin shedding (Fig 2B). Additionally, between 30 and 60 minutes after exposure, neutrophils reached a maximum level of CD11b/CD18 surface expression that was reduced thereafter.

Enhanced adhesive function in primed neutrophils. The interaction between neutrophils and carboxylate modified latex beads (CMLs) was examined by flow cytometry (Fig 3). Because neutrophils are significantly larger in volume than CMLs (2 μm), they are readily discriminated on the basis of forward light scatter, with aggregates of neutrophils and beads detected by red fluorescence (FL-3) as shown in Fig 3B. Concentrations of neutrophils were maintained at ≈2 × 10⁶/mL to minimize neutrophil aggregation and to optimize CML binding.

The time courses of bead binding to neutrophils after addition of formyl peptide are shown in Fig 4. We observed that samples treated with maximal LPS/LBP concentrations exhibited enhanced bead binding over control in both stimulated and unstimulated cells. The binding increased rapidly to a maximum within the first 3 minutes after addition of formyl peptide. The presence of IB4, but not DREG-200, which are both MoAbs recognizing CD11b/CD18 and L-selectin, respectively, inhibited bead binding. The results of three bead experiments are summarized in Fig 5. In LPS/LBP-pretreated neutrophils, bead binding increased 87% upon formyl peptide stimulation, as compared with an increase of 57% in control cells. Stimulated control cells bound 39% of beads compared with stimulated LPS/LBP treated cells. Pretreatment of either population with IB4 inhibited virtually all bead binding (less than 13% of maximal). In contrast, Dreg-200 Fab, recognizing an epitope of L-selectin that is required in neutrophil aggregation, did not significantly inhibit bead binding in stimulated samples of LPS/LBP treated or control neutrophils (P = .0626 and P = .2125, respectively).

Inhibition of aggregation by LPS/LBP. The impact of LPS/LBP pretreatment on neutrophil aggregation after formyl peptide stimulation is shown in Fig 6. Under conditions of moderate to maximal LPS/LBP exposure, the ability of neutrophils to aggregate was markedly decreased. Whereas...
LPS/LBP INHIBITS NEUTROPHIL AGGREGATION

stimulated control samples recruited ~35% of cells into aggregates in response to 1 \( \mu \text{mol/L} \) formyl hexapeptide, unstimulated control or LPS/LBP-treated samples maintained ~3% of the population in aggregates under shear conditions (data not shown for LPS/LBP). There was a distinct, dose-dependent inhibition in the rate of aggregate formation for neutrophils pretreated with 1, 3, or 100 ng/mL LPS/LBP before stimulating aggregation (Fig 6, Table 1). LPS/LBP pretreatment did not alter the duration of formyl peptide-stimulated aggregation. Stirred, resting cells did not aggregate in response to LPS/LBP (100 ng/mL) within fifteen minutes after addition (data not shown), yet changes in the expression levels of L-selectin and CD11b/CD18 were consistent with the results shown in Fig 2.

**LPS/LBP effects on neutrophil aggregation through binding to CD14, but not CD11b/CD18.** Because LPS binds to both neutrophil CD14 and CD11b/CD18,12,14 we addressed the possibility that there is a direct effect of LPS on CD11b/CD18 by pretreating neutrophils with 20 \( \mu \text{g/mL} \) anti-CD14 antibodies My4 and 63D3, or with IgG2b isotype controls, then incubating the cells with 10 ng/mL LPS/LBP. Neutrophils pre-exposed to My4 and then to LPS/LBP retained a markedly greater ability to aggregate than controls pretreated with 10 ng/mL LPS/LBP alone (Fig 7). The kinetics of aggregation were comparable with untreated, stimulated controls. My4 also blocked the LPS/LBP-mediated upregulation of CD11b/CD18 and L-selectin shedding to within ~10% of untreated controls, and these samples aggregated with an identical initial rate (Table 2). Receptor expression was also measured in My4-blocked specimens and controls after aggregation as an independent measure of the functional specificity of My4 for CD14. In these samples, we observed CD11b/CD18 and L-selectin levels comparable with formyl peptide-stimulated control (data not shown). In contrast, 63D3 was less effective than My4 at blocking CD11b/CD18 and L-selectin regulation by LPS/LBP, and the initial aggregation rate was only inhibited by 63%. The IgG2b isotype control for My4 did not block L-selectin shedding or preserve aggregation as compared with 10 ng/mL LPS/LBP control, but appeared to block LPS/LBP-mediated CD11b/CD18 upregulation to a small extent. Taken together, it is

<table>
<thead>
<tr>
<th>Pretreatment Condition</th>
<th>% Maximum Expression CD11b/CD18</th>
<th>% Expression L-Selectin</th>
<th>Initial Aggregation Rate (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control stimulated</td>
<td>26.6</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>LPS/LBP 1 ng/mL</td>
<td>43.1</td>
<td>75.1</td>
<td>72.3</td>
</tr>
<tr>
<td>LPS/LBP 3 ng/mL</td>
<td>63.8</td>
<td>40.9</td>
<td>72.3</td>
</tr>
<tr>
<td>LPS/LBP 100 ng/mL</td>
<td>100.0</td>
<td>12.6</td>
<td>9.7</td>
</tr>
<tr>
<td>LPS (uncomplexed)</td>
<td>10 ng/mL</td>
<td>77.7</td>
<td>72.8</td>
</tr>
<tr>
<td>LPS 300 ng/mL</td>
<td>37.2</td>
<td>88.0</td>
<td>86.3</td>
</tr>
<tr>
<td>Control unstimulated</td>
<td>23.3</td>
<td>100.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Isolated neutrophils (4 \( \times \) 10^5/mL) were preincubated as described with moderate to optimal doses of LPS/LBP conjugate or free LPS. After pretreatment, a small aliquot of cells was removed from each, diluted to 10^5/mL, chilled, and stained for analysis of receptor expression. Receptor expression was determined as in Fig 1. The initial rate was calculated according to equation 3 (Materials and Methods), after stimulation with 1 \( \mu \text{mol/L} \) formyl peptide. Except for LBP (n = 2), the data include results from three separate experiments.
clear that the effect of LPS/LBP is mediated by binding to CD14.

Potential relationship between LPS/LBP-induced regulation of the adhesion molecules and the inhibition of aggregation. Because the effect of LPS/LBP appears to be neither on CD11b/CD18 function nor the ability of primed cells to respond to stimulus, we compared L-selectin and CD11b/CD18 expression in LPS/LBP-treated neutrophils to the initial rate of aggregation as shown in Fig 6 and summarized in Table 1. After LPS/LBP treatment, in which adhesive molecule expression was altered to varying extents, there was a subsequent inhibition of the initial aggregation rate of 28% (1 ng/mL LPS/LBP), 78% (3 ng/mL), and 90% (10 ng/mL). Pretreatment with LBP (300 ng/mL) alone had a negligible effect on CD11b/CD18 or L-selectin expression, and aggregation was comparable with control. Neutrophils pretreated with uncomplexed LPS (10 ng/mL) altered L-selectin and CD11b/CD18 to levels comparable with cells pretreated with 1 ng/mL LPS/LBP, and exhibited a corresponding decrease in initial aggregation rate compared with control. This comparison suggests a positive correlation between the decreased expression of L-selectin and the subsequent reduction in the initial rate of aggregation.

The correlation between cellular L-selectin and aggregation was further explored by blocking the adhesive epitope of L-selectin with Dreg-200 antibody fragments. Unstimulated neutrophils were pretreated using Dreg-200 Fab-FITC at varying concentrations equaling 1X, 2X, and 13.3X the reported kd for the antigen1 (Fig 8, Table 3). After stimulation, the extent of aggregation was inhibited in a dose-dependent fashion by Dreg-200. The progressive blocking of L-selectin sites also resulted in a corresponding decrease in the initial

Table 2. Requirement of CD14 for Adhesive Consequences of LPS/LBP

<table>
<thead>
<tr>
<th>Pretreatment Condition</th>
<th>% Maximal Expression CD11b/CD18</th>
<th>% Expression L-Selectin</th>
<th>Initial Aggregation Rate (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulated control (4)</td>
<td>30.4</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>My4 + LPS/LBP (4)</td>
<td>41.8</td>
<td>91.3</td>
<td>101.4</td>
</tr>
<tr>
<td>63D3 + LPS/LBP (2)</td>
<td>54.3</td>
<td>74.6</td>
<td>37.3</td>
</tr>
<tr>
<td>LPS/LBP (4)</td>
<td>96.3</td>
<td>17.6</td>
<td>14.8</td>
</tr>
<tr>
<td>IgG2B + LPS/LBP (2)</td>
<td>71.4</td>
<td>14.5</td>
<td>11.3</td>
</tr>
</tbody>
</table>

Isolated neutrophils (4 x 10^6/mL) were preincubated for 10 minutes at 37°C with 20 μg/mL My4, 63D3, or IgG2b whole antibodies. Blocked samples and isotype controls were then challenged with 10 ng/mL LPS/LBP. Before aggregation, a small aliquot of each sample was removed, diluted to 10^5 cells/mL, chilled, and labeled for receptor analysis. Receptor data are presented as described in Fig 1. Initial aggregation rates were determined and compared as in Table 1. Values in parentheses are the number of separate experiments in which each sample type was analyzed.

Table 3. Correlation of L-Selectin Levels With Neutrophil Aggregation

<table>
<thead>
<tr>
<th>Pretreatment Condition</th>
<th>% Maximum Expression CD11b/CD18</th>
<th>Predicted Unblocked L-Selectin</th>
<th>Initial Aggregation Rate (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulated control</td>
<td>30.5</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Dreg-200 Fab-FITC: 30</td>
<td>31.8</td>
<td>50.0</td>
<td>67.5</td>
</tr>
<tr>
<td>60 nmol/L (1.5 μg/mL)</td>
<td>32.1</td>
<td>33.0</td>
<td>45.8</td>
</tr>
<tr>
<td>400 nmol/L (20 μg/mL)</td>
<td>40.7</td>
<td>6.9</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Isolated neutrophils (4 x 10^6/mL) were preincubated for 15 minutes with FITC-conjugated Dreg-200 Fab. Upregulation of CD11b/CD18 was determined as in Fig 1. Estimates of available L-selectin were based on the reported kd (30 nmol/L) of Dreg-200 Fab.1 Aggregation rates represent the mean initial aggregation rate calculated as described in Table 1, compared with stimulated controls. The data include results from three experiments.
aggregation rate (Fig 8, Table 3). Inhibition of aggregation was comparable with the levels we had observed after LPS/LBP priming. Under L-selectin antibody-blocking conditions, aggregation appeared to be less sensitive to CD11b/CD18 upregulation, as was also observed with LPS/LBP treatment. Cytometric analysis of Dreg-200 Fab-FITC binding to stimulated neutrophil sinogels showed no significant loss of L-selectin over the initial 15 seconds of formyl peptide stimulation in which the aggregation rate is determined.

**DISCUSSION**

Neutrophils participate in host defense against bacteria and in the pathophysiology of sepsis. Changes in neutrophil adhesion during endotoxemia contribute to adult respiratory distress and multiple system organ failure. This study contrasts the enhanced adhesivity of neutrophils and functional responsiveness after endotoxin exposure to the diminished ability of the cells to aggregate. Several mechanisms that contribute to the results are evaluated.

**Effects of LPS on adhesive receptor regulation and aggregation.** As a framework in support of aggregation studies, we have extended previous observations that showed both L-selectin shedding in response to LPS and CD11b/CD18 upregulation by LPS/LBP. We observed the shedding of L-selectin and upregulation of CD11b/CD18 by human neutrophils in response to LPS/LBP complexes (1.0 to 10.0 ng/mL). The shedding of L-selectin within a relatively low and narrow dose range is in contrast to the somewhat broader observed response range (1.0 ng to ≈1.0 μg/mL LPS/LBP) for CD11b/CD18 upregulation. The difference in dose response and the reciprocal nature of their regulation is consistent with control of the expression levels of the two molecules through independent processes.

Pretreatment of neutrophils with LPS/LBP profoundly reduced their ability to aggregate in response to formyl peptide stimulation. The rate and extent of aggregate formation were reduced in proportion to the LPS/LBP pretreatment dose. Moreover, the rate of aggregate formation was proportional to L-selectin expression at the time of formyl peptide addition. LPS/LBP pretreatment did not greatly affect the duration of aggregation. Whereas neutrophil aggregation requires the expression of both L-selectin and CD11b/CD18, the depletion of cellular L-selectin following LPS/LBP exposure indicated that its surface numbers may limit aggregation in normal neutrophils. This conclusion is supported in part by the finding that the upregulation of CD11b/CD18 in response to LPS/LBP did not appear to compensate for L-selectin depletion. Recently, it was reported that adherence of activated neutrophils to ICAM-1 and fibrinogen is mediated by as few as 10% of the total number of CD11b/CD18 molecules expressed on their surfaces. In addition, we have observed that a MoAb that binds the CD18 subunit (Kim-127) causes extensive and persistent aggregation in stirred, otherwise unstimulated neutrophils. Therefore, native expression of CD11b/CD18 is sufficient for aggregation.

**LPS/LBP exposure and adhesive competence.** The general competence of neutrophils pretreated with LPS to adhere after activation has been verified previously. Neutrophils pretreated with LPS exhibited enhanced binding of latex beads upon subsequent stimulation with formyl peptide, whereas under certain conditions of study, LPS-pretreated neutrophils bind beads at near-control levels. The idea that the function of β2 integrins remains competent after LPS priming is supported by a number of adhesive studies. We have adapted bead-binding experiments to show that the adherence of stimulated control cells or neutrophils pretreated with LPS/LBP to CML particles depends on the function of CD11b/CD18 and not on L-selectin. Moreover, the sites on CD11b/CD18 involved in its adherence to counter structures on neutrophils and to latex microspheres are both activated by formyl peptide and are blocked by an MoAb specific to β2 integrins that inhibits neutrophil aggregation. In experiments conducted in parallel to the present study, we found that cells pretreated with LPS/LBP aggregate with untreated neutrophils through a process dependent upon β2 integrins on the population treated with LPS/LBP. Therefore, it is highly unlikely that global effects of LPS on neutrophil function or its specific effects on CD11b/CD18 disable neutrophil aggregation in single populations of cells.

**Specificity of LPS/LBP effects through CD14-dependent mechanisms.** We determined that the inhibition of aggregation by LPS depends on its binding to CD14 and not on inhibition of the adhesive function of CD11b/CD18. Under conditions in which LPS binding to CD14 was blocked by My4, the effects of LPS/LBP on aggregation, L-selectin expression, and CD11b/CD18 expression were ablated. These findings, together with the observation that LPS/LBP did not induce aggregation, suggest that the binding of LPS to CD11b/CD18 plays no significant role in aggregation at the concentrations of LPS used (3 to 10 ng/mL for near optimal activity). This cannot exclude the possibility of effects attributable to locally concentrated LPS, as in the case of neutrophil adherence to bacteria. Further, one could envision an ensemble of surface molecules where the binding of LPS by CD14 would function to concentrate LPS for recognition by other LPS binding proteins. Thus, although the binding of CD14 was required, ligation may not be sufficient to account for the observed cellular effects of LPS. Additional evidence for a role of CD14 in LPS recognition comes from studies on patients with paroxysmal nocturnal hemoglobinuria (PNH), who are deficient in phosphatidylinositol-anchored membrane proteins including CD14. Recent findings indicate that the upregulation of neutrophil CD11b/CD18 by subsaturating concentrations of LPS (1 ng/mL) in blood is inhibited. It remains to be shown whether the adhesive status of PNH neutrophils is sensitive to LPS exposure.

**Dependence of aggregation on L-selectin.** Because LPS/
L-selectin levels rise to 2 to phils to the vascular wall at inflammatory sites.4' The inhibition effects of LPS/LBP on neutrophil aggregation were reproduced by the sequential removal of L-selectin sites using Dreg-200 Fab to deplete its functional epitope. Under these conditions, the rate and extent of aggregation were reduced in proportion to the extent of blocking of cellular L-selectin. Again, the duration of aggregation was independent of available L-selectin sites. Taken together, a number of ideas suggest a role for L-selectin in neutrophil aggregation: these are (1) qualitative and quantitative studies using antibodies1,6 (Fig 8); (2) the competence of the integrins in their ability to permit stimulated adhesion to beads as well as control neutrophils6 (Figs 4 and 5); (3) the correlation between the loss of selectins from primed cells and their diminished capacity to aggregate (Fig 6, Table 1); and (4) the sensitivity of aggregation to a panel of carbohydrates appropriate to L-selectin.25

Our data support a model for neutrophil aggregation in which the surface numbers of L-selectin limit the ability of neutrophils to aggregate. The findings from Kотовури and al.7 concerning E-selectin and CD11/CD18, suggest a model in which L-selectin recognizes sialyl-lewis X displayed on activated CD11/CD18. This hypothesis is being tested.

**LPS binding and neutrophil physiology.** Recognition of LPS by neutrophils chiefly functions in the enhancement of responses to other inflammatory mediators and in the clearance of gram negative bacteria.17,20,23 We observed optimal changes in adhesive receptor expression, specifically L-selectin, after brief incubation with 10- to 1,000-fold lower doses of LPS/LBP (10 ng/mL) than previously reported for uncomplexed LPS.5,19 Given the kinetics of adsorptive modulation in response to LPS/LBP complexes12,20 (Fig 2) and the absence of aggregation under suitable shear conditions after exposure, it appears that LPS/LBP does not directly stimulate neutrophil aggregation. In contrast, the inhibition of aggregation after exposure suggests that LPS may affect a balance between aggregation and adhesion in sepsis, through the shedding of L-selectin. In septic patients, serum L-selectin levels rise to 2 to 3 times above normal.28 Soluble L-selectin at equivalent concentrations inhibits lymphocyte-endothelial adhesion.40 If shed L-selectin also inhibits neutrophil adhesion, the elevation of serum L-selectin would be predicted to reduce the processes of margination and redistribution of neutrophils to the vascular wall at inflammatory sites.43

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Lipoplyascharide enhances CD11b/CD18 function but inhibits neutrophil aggregation

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