P-selectin and ICAM-1 mediate endotoxin-induced neutrophil recruitment and injury to the lung and liver

Kamochi, M., F. Kamochi, Y. B. Kim, S. Sawh, J. M. Sanders, I. Sarembock, S. Green, J. S. Young, K. Ley, S. M. Fu, and C. E. Rose, Jr. P-selectin and ICAM-1 mediate endotoxin-induced neutrophil recruitment and injury to the lung and liver. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L310–L319, 1999.—The role of leukocyte adhesion molecules in endotoxin-induced organ injury was evaluated by administering intraperitoneal Salmonella enteritidis lipopolysaccharide (LPS) to wild-type (WT) mice, P-selectin-deficient mice, intercellular adhesion molecule (ICAM)-1-deficient mice, and P-selectin-ICAM-1 double-mutant mice. In WT mice, there was a sevenfold increase in the number of neutrophils present in the pulmonary vascular lumen fluid, and there were sevenfold more intracapillary neutrophils by electron-microscopic (EM) morphometry at 4 h after intraperitoneal LPS compared with that in control mice. Extravascular albumin accumulation increased approximately twofold in the lungs and liver of WT mice treated with LPS. In the double-mutant mice, although overall mortality after intraperitoneal LPS was not attenuated, there was a significant delay in mortality in the P-selectin-ICAM-1-deficient mutants compared with that in WT mice after intraperitoneal LPS (P < 0.01). Moreover, compared with LPS-treated WT mice, lung and liver extravascular albumin accumulation was significantly lower in LPS-treated P-selectin-ICAM-1-deficient mice, and P-selectin-ICAM-1 double-mutant mice. Lung myeloperoxidase activity, normalized per 1,000 circulating neutrophils, increased after endotoxin in WT and P-selectin-deficient mice but not in P-selectin-ICAM-1 double-mutant mice. In addition, lung and liver myeloperoxidase activity per 1,000 circulating neutrophils in endotoxin-treated ICAM-1-deficient mice and P-selectin-ICAM-1 double mutants was significantly lower compared with that in endotoxin-treated WT mice. These data suggest that P-selectin and ICAM-1 significantly contribute to lung and liver injury after systemic endotoxemia.

intercellular adhesion molecule-1; lipopolysaccharide; alumin permeability

SEPSIS IS A CATASTROPHIC ILLNESS, often resulting in profound hypotension and major injury to multiple organs, with a high incidence of mortality. The inflammatory events and organ injury occurring in septic patients are orchestrated by cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1 released in response to lipopolysaccharide (LPS) and other products of gram-negative or gram-positive bacteria (4, 31, 37). The release of these endogenous mediators leads to a number of pathophysiological reactions, including fever, neutropenia, thrombocytopenia, disseminated intravascular coagulation, neutrophil sequestration in various organs, and hemodynamic changes that ultimately may lead to lethal shock. Among these, sequestration of neutrophils in a variety of vascular beds is regarded as the critical and first step to induce leukocyte-related vascular injury in endotoxemia (20, 53).

Although the roles of leukocyte adhesion molecules have been evaluated in animal models of pneumonia induced by intratracheal administration of LPS or live bacteria (7, 13, 23, 24, 28, 34, 35), only a few studies (46, 52) have evaluated the influence of these adhesion ligands in an animal model of systemic endotoxemia.

The most catastrophic and fulminant lung injury that can occur is acute respiratory distress syndrome, a fulminant form of life-threatening pulmonary edema (3) that is most often a complication of bacteremic sepsis (26). Although neutrophil sequestration is observed in acute respiratory distress syndrome, it is the increase in vascular permeability, possibly mediated by neutrophils, that is believed to be the mechanism responsible for the flooding of the interstitial and alveolar compartments of the lung. The present experiments were conducted to evaluate the roles of P-selectin and intercellular adhesion molecule (ICAM)-1 in lung neutrophil accumulation and edema in a murine model of systemic inflammatory response syndrome. This model was chosen to simulate the fulminating bacteremia-induced lung injury that occurs in patients. Because multiorgan dysfunction frequently develops in patients with sepsis (5), the present experiments included evaluation of the changes in the liver.

MATERIALS AND METHODS

Animals

Mice genetically engineered to be deficient in the expression of ICAM-1 (40), P-selectin (7) or P-selectin-ICAM-1 (7) were obtained from the Induced Mutant Resource at Jackson Laboratory (Bar Harbor, ME). The ICAM-1 gene disruption is not a null mutation because alternatively spliced isoforms of ICAM-1 can still be produced in these mutants (22). The P-selectin mutation is a null mutation (7). All mice used in this study were backcrossed onto C57BL/6 mice for at least five generations. Wild-type (WT) C57BL/6 mice were also obtained from Jackson Laboratory. The mice used in this study were 7–8 wk old, and body weight was in the range from 17 to 30 g. All experiments were approved by the Animal Research Committee at University of Virginia (Charlottes-
euthanized animals were inflated in situ with air, rapidly. The lungs in absence of P-selectin and ICAM-1 expression. The lungs in exsanguination.

Anesthesia

Mice were deeply anesthetized with intraperitoneal ketamine (80 mg/kg) and intraperitoneal xylazine (8 mg/kg) administered no sooner than 10–15 min before euthanasia by exsanguination.

Immunohistochemistry

Initial immunoistochemical staining was performed in untreated P-selectin-ICAM-1 double mutants to confirm the absence of P-selectin and ICAM-1 expression. The lungs in euthanized animals were inflated in situ with air, rapidly removed, and frozen in liquid N2. Frozen sections were prepared and stained with rat anti-mouse P-selectin monoclonal antibody (MAB) RB40.34 (6) or rat anti-mouse ICAM-1 MAB YN1/1 (44), washed to remove unbound antibodies, and stained with biotinylated rabbit anti-rat IgG (Vector Laboratories, Burlingame, CA) followed by avidin-biotin-peroxidase complex and diaminobenzidine with a Vectastain Elite ABC Kit (Vector Laboratories). The sections were counterstained with methyl green. The P-selectin-ICAM-1 double mutation was confirmed by the absence of any demonstrable immunohistochemical staining for either P-selectin or ICAM-1 in lung tissue. Positive control immunoperoxidase staining for both P-selectin and ICAM-1 was observed in WT mice.

Immunohistochemical staining for P-selectin was also performed on WT mice 4 h after either intraperitoneal saline (n = 2) or intraperitoneal Salmonella enteritidis LPS (250 µg; n = 2). Mice were euthanized by exsanguination under deep ketamine-xylazine anesthesia, and the lungs were fix-inflated in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Paraffinized sections were mounted onto glass slides and deparaffinized, and endogenous peroxidase activity was quenched with methanol containing 0.45% H2O2. Sections were then incubated with blocking solution containing normal goat serum, rabbit anti-human P-selectin, and biotinylated goat anti-rabbit IgG, with a wash with phosphate-buffered saline between each step. Bound antibody was then detected with the Vectastain Elite ABC Kit as described above, and the sections were counterstained with hematoxylin. The positive control was lung tissue from an animal treated with TNF-α. Negative control staining was performed by substituting phosphate-buffered saline for the primary antibody.

Endotoxin Lung Injury

Mice were injected with S. enteritidis (250 µg) from the same lot utilized in a previous study by Rose et al. (37). Control mice received an equal volume (0.05 ml) of 0.9% NaCl vehicle intraperitoneally. Mice were studied in one of two protocols in which 125I-labeled human serum albumin and 51Cr-labeled red blood cells (RBCs) were administered for the measurement of organ vascular permeability or in which no radioisotopes were administered, and the organs were harvested for measurement of organ myeloperoxidase (MPO) activity.

Protocol I: Evaluation of LPS-induced increase in vascular permeability of the lung and liver. Albumin vascular permeability is an accepted index for vascular injury that occurs in the lung (7, 37) and liver (10, 51). LPS-induced organ injury in the WT and mutant mice was assessed with the double-isotope method for the evaluation of vascular leak of albumin as described by Bullard et al. (7). Fifteen minutes before intraperitoneal LPS or saline, each mouse received intravenous 125I-human serum albumin (Mallinkrodt Nuclear Medicine, Maryland Heights, MO) through a tail vein. At 225 min after intraperitoneal LPS or saline, each mouse was deeply anesthetized with intraperitoneal ketamine-xylazine, and blood was obtained from the retroorbital plexus with a heparinized capillary tube for total and differential leukocyte counts. The trachea was cannulated with a 22-gauge catheter, and the animal was ventilated with room air with a rodent ventilator at a rate of 100 breaths/min and a peak inflation pressure of 10 mmHg. A midline abdominal incision was made, and at 238 min, 51Cr-RBCs were injected into the inferior vena cava to determine intravascular volume. At 240 min, the chest was opened through a median sternotomy, and blood was withdrawn from the right ventricle to exsanguinate the animal and to obtain an equilibration blood sample. The lungs and liver were then removed and counted for 125I and 51Cr.

The following equations (7) were used to determine the extravascular albumin (EVA) accumulation for each organ: total organ albumin volume (in µl) = 125I-albumin in the organ/albumin per gram of plasma; intravascular organ volume (in µl) = (51Cr-RBCs in the organ/51Cr-RBCs per gram of blood) × (1 – hematocrit); and EVA accumulation (in µl) = total organ albumin volume – intravascular organ volume. EVA accumulation for each organ was then calculated per total organ weight.

To label mice RBCs with 51Cr, donor blood (0.5 ml) obtained from the inferior vena cava of deeply anesthetized C57BL/6 mice was mixed with 2.0 ml of sterile saline containing 50 U of heparin and 5 µCi (5 µl) of sodium chromate-51 (NEN, Wilmington, DE). After incubation at 37°C for 60 min, the RBCs were pelleted by gentle centrifugation (400 g) and washed by resuspension in sterile saline. This process was repeated three times to remove free 51Cr.

Protocol II: Assay of MPO activity of the lungs and liver. Gupta et al. and others have previously validated lung MPO activity as an index of lung neutrophil sequestration in rats (18) and rabbits (17). Gupta et al. (18) observed a significant correlation (r = 0.93; P < 0.001) between lung MPO activity (right lung) and the number of lung intracapillary neutrophils (left lung) counted by electron-microscopic (EM) morprhometry in rats treated with intraperitoneal endotoxin. Therefore, this assay was performed as an index of lung neutrophil sequestration as described previously by Gupta et al. The mice were treated identically to the permeability experiments except that radioisotopes were not administered. After ketamine-xylazine anesthesia a 15 min before euthanasia, retroorbital blood was obtained for total and differential leukocyte counts and a tracheal cannula was inserted for mechanical ventilation. The animals were euthanized by blood withdrawal from the right ventricle through a median sternotomy, and the lungs and liver were quickly removed. Each organ was minced in petri dishes containing chilled 0.5% hexadecyltrimethylammonium bromide and maximally homogenized for 90 s. The samples were then centrifuged at 30,000 g for 10 min, and the supernatant containing hemoglobin, which can interfere with the MPO assay, was discarded. The pellet was resuspended in an equal volume of hexadecyltrimethylammonium bromide and maximally homogenized for 90 s. To further disrupt the cells, the homogenate was frozen at −80°C for 20 min, thawed, and placed in an ultrasonic bath (Branson Ultrasonic, Danbury, CT) for 90 s. After a second high-speed centrifugation, 0.1 ml of the supernatant was added to a cuvette containing 2.9 ml of chilled 0.1% o-dianisidine dihydrochloride in 0.05 M sodium phosphate buffer (pH 6.0) activated by 0.007% H2O2. The enzyme reaction was allowed to proceed for 2 min, and the absorbance at 405 nm was determined with a spectrophotometer.
change in absorbance over 2 min at room temperature was measured at 460 nm (Beckman 35 spectrophotometer).

Hematology

Mice were bled from the retroorbital venous plexus with heparinized capillary tubes. Whole blood was diluted 1:40 with 2% acetic acid, and total white blood cell counts were determined with a hemacytometer. Blood smears were stained with Diff-Quik (Dade Diagnostics, Aguada, PR) for differential leukocyte counts.

Pulmonary Vascular Lavage

After induction of deep anesthesia and tracheal cannulation for mechanical ventilation, WT mice injected with either intraperitoneal saline or intraperitoneal LPS (S. enteritidis LPS, 250 µg) 4 h earlier were euthanized by blood withdrawal from the right ventricle, and polyethylene catheters (Clay Adams, Becton Dickinson) were placed in the pulmonary artery (OD 0.05 inch) and left ventricle (OD 0.062 inch) and secured with 5-0 silk ligatures. Care was taken to be sure that the tip of the pulmonary arterial catheter was beyond the pulmonic valve. To confine lavage to the right lung, the left hilar structures were then cross-clamped with a plastic tubing-shod hemostat. Pulmonary vascular lavage (PVL) was then performed according to the technique of Williams et al. (50) by perfusing the pulmonary vasculature with 20 ml of lavage solution (2% hetastarch in 0.9% sodium chloride containing 6 U/ml of heparin) at a perfusion pressure of 70 cmH2O. The lavage fluid was warmed to 37°C by being passed through a water-jacketed coil. In every lavage, application of the left hilar cross-clamp immediately stopped inflation of the lung with the ventilator breaths and abrogated the blanching that was observed in the right lung with PVL. Initially, two saline- and one LPS-treated mice underwent antegrade lavage (50), with the perfusate entering the pulmonary arterial cannula, and effluent was collected from the left ventricular cannula. However, to determine whether leukocyte recovery was related to the direction of perfusion, the remainder of mice were retrograde perfused, with the effluent collected from the pulmonary arterial cannula. After lavage, the left hilar clamp was removed, with immediate restoration of left lung inflation with each ventilator breath. The lungs were then fix-infiltrated with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer. Sections of the nonlavaged left lung were prepared for electron microscopy to determine the location of neutrophils in the lung 4 h after LPS. In addition, blocks of the lavaged right lung were submitted to evaluate the effectiveness of pulmonary vascular lavage to harvest leukocytes that were sequestered in the lung after LPS.

The number of total leukocytes and total neutrophils in the lavage fluid was determined with a hemacytometer and differential counts of Diff-Quik-stained cytocentrifuged (Shandon) leukocytes.

Lung Neutrophil EM Morphometry

The techniques for lung tissue preparation and morphometric analysis have been thoroughly previously described (18). Briefly, 1-mm blocks of lung were postfixed in 2% osmium and embedded in Epon. Sections for electron microscopy, 70–80 nm in thickness, were stained with uranyl acetate and lead citrate. The number of neutrophils within the capillaries, interstitium, and alveoli was then counted with a transmission electron microscope by an experienced microscopist totally blinded as to the treatment nature of the samples. Cells were counted from two 0.2-mm2 areas from each lung, and the number of cells is expressed per square millimeter by multiplying by 5. A previous study (19) on the ultrastructure of bone marrow in endotoxin-treated mice revealed mature granulocytes that range in diameter from 5 to 7 µm. We reasoned that visualized neutrophils in the lung would never extend beyond a 10-µm section. Therefore, we extrapolated the number of intracapillary and alveolar neutrophils from the number per square millimeter to the number per cubic millimeter by multiplying by 100, the number of 10-µm sections in 1 mm.

Statistics

Data were analyzed by general linear-model analysis with SAS software. PVL data for the percentage of neutrophils and for the total neutrophils recovered from saline- versus endotoxin-treated mice were analyzed by one-way analysis of variance (42). Multiple comparison testing between groups was performed with Tukey’s test (42). All data are presented as means ± SE, and significance was identified at the level of P < 0.05.

RESULTS

Mortality From Endotoxin

All animals survived for 30 h after intraperitoneal administration of 250 µg of LPS. However, at 36 h, 3 of 10 WT mice were dead, whereas only 1 of the double mutants had died (Fig. 1). At 48 h after LPS, 8 of 10 WT mice were dead, but only 1 of 10 double mutants had died. However, at 72 h, 9 of 10 WT mice were dead, and 8 of 10 double mutants were dead. At 80 h after endotoxin, 9 of 10 mice had died in both groups, with 1 long-term survivor in each genotype. Although overall mortality was unchanged, significant differences existed between the two survival curves by the Wilcoxon test (P < 0.01), which reflected the delay in mortality in the P-selectin-ICAM-1 double mutants.

Changes in Circulating Neutrophils After LPS Injection

In the saline-treated WT and mutant control mice, no differences were found in circulating neutrophil numbers between mice studied at 2 or 4 h after intraperitoneal saline. Therefore, the data were pooled for each genotype and are expressed as a single control value.
Total circulating neutrophil counts were significantly elevated in the saline-treated P-selectin-ICAM-1 double-mutant mice compared with those in WT mice treated with saline (P < 0.05; Fig. 2). Total neutrophil counts in saline-treated P-selectin or ICAM-1 single-mutant animals were not elevated above those in WT mice. Circulating neutrophil numbers decreased significantly at 2 and 4 h after LPS in the P-selectin-ICAM-1 double mutants (P < 0.05; Fig. 2). Circulating neutrophil numbers were significantly higher in double mutants studied 4 h after LPS compared with those in double mutants studied at 2 h after LPS administration (P < 0.05). Reduced circulating neutrophil counts were also observed in the other groups at 2 and 4 h after LPS but did not reach significance.

Neutrophil Sequestration in Lung and Liver After LPS Injection

In the subsequent studies in which EVA permeability measurements were obtained in saline- and LPS-treated mice, no significant differences in pulmonary blood volume were found either between genotypes or with LPS treatment at 4 h. Comparable pulmonary blood volumes allowed for lung MPO measurements to be corrected for the neutrophils present in the larger blood vessels in the circulating venous blood by expressing lung and liver MPO activities as activity per 1,000 circulating peripheral blood neutrophils obtained from blood sampled just before the lungs were harvested (Figs. 3 and 4). This allowed for correction for elevated circulating blood neutrophil numbers in the P-selectin-ICAM-1 mutants. In support of the index, lung MPO activity per 1,000 neutrophils was comparable among all saline-treated genotypes (Fig. 3) despite the elevation in peripheral blood neutrophils in the P-selectin-ICAM-1 mutants (Fig. 2). In WT mice, an increase in the lung MPO-to-neutrophil ratio was observed at both 2 and 4 h after LPS, which was strikingly elevated above the ratio observed in saline-treated WT mice (P < 0.05; Fig. 3). In the mutant mice, the lung MPO-to-blood neutrophil ratio was elevated above the respective genotype control only in the P-selectin mu-

Fig. 2. Circulating neutrophil (Circ Neut) numbers decreased 2 (hatched bars) and 4 (solid bars) h after LPS administration in P-selectin (P-Sel), ICAM-1 /−/− double-mutant mice. Open bars, saline control mice. Nos. above bars, no. of mice/group. Only in double mutants was there a significant difference in circulating neutrophil number between 2 and 4 h after LPS. Significant difference from:
* saline-treated mice of same genotype; † saline-treated WT mice. * Above bracket, significant difference between 2- and 4-h LPS groups, P < 0.05.

Fig. 3. Proportion of circulating neutrophils sequestered to lung as expressed by ratio of lung myeloperoxidase (MPO) to circulating neutrophils ([change in absorbance·g lung−1·min−1 divided by peripheral blood neutrophils/mm3] × 103). Open bars, saline control mice; hatched bars, 2-h LPS; solid bars, 4-h LPS. Nos. above bars, no. of mice/group. Lung MPO-to-neutrophil ratio increased in WT mice and P-selectin /−/− mutant mice after LPS. However, compared with WT mice, lung MPO-to-neutrophil ratio was significantly less in LPS-treated P-Sel /−/−, ICAM-1 /−/−, and P-Sel, ICAM-1 /−/− mutant mice. Significant difference from: * saline-treated mice of same genotype; † LPS-treated WT mice. * Above bracket, significant difference between 2- and 4-h LPS groups, P < 0.05.

Lung MPO-to-circulating neutrophil ratios were comparable among all saline-treated genotypes (Fig. 4). LPS MPO-to-circulating neutrophil ratios were significantly elevated above respective saline-treated control values in WT, P-selectin mutants, and P-selectin-ICAM-1 double mutants. Moreover, the lung MPO-to-neutrophil ratios in LPS-treated ICAM-1 and P-selectin-ICAM-1 double mutants were significantly lower in comparison to comparable periods in LPS-treated WT mice (P < 0.05).

Liver MPO-to-circulating neutrophil ratios were comparable among all saline-treated genotypes (Fig. 4). Liver MPO-to-circulating neutrophil ratios were significantly elevated above respective saline-treated control values in WT, P-selectin mutants, and P-selectin-ICAM-1 double-mutant mice at 2 (all groups) and 4 h (WT mice) after LPS (P < 0.05) but were unchanged in ICAM-1 mutants. Moreover, compared with WT mice, liver MPO-to-neutrophil ratios were significantly lower
in P-selectin mutants and P-selectin-ICAM-1 double mutants at 4 h after LPS and in ICAM-1 mutant mice at both 2 and 4 h after LPS (P < 0.05).

Increase in EVA Accumulation in the Lung and Liver After LPS Injection

In pilot studies, we found that lung EVA accumulation did not increase at 2 h after LPS. EVA measurements were therefore made only in animals at 4 h after LPS. There were no differences in lung EVA accumulation between saline-treated WT mice and any saline-treated mutant group (Fig. 5A). In WT mice, lung vascular permeability as measured by EVA accumulation increased significantly at 4 h after intraperitoneal LPS (P < 0.05). In striking contrast, lung EVA accumulation in LPS-treated P-selectin, ICAM-1, and P-selectin-ICAM-1 mutant mice was not significantly increased above that in the respective saline-treated control group for each genotype. Moreover, among all LPS-treated genotypes, only the LPS-treated P-selectin-ICAM-1 double mutants had lung EVA levels that were significantly lower than those in LPS-treated WT mice (P < 0.05).

Liver EVA levels were comparable between saline-treated WT mice and the mutant groups (Fig. 5B).

Liver EVA accumulation increased significantly 4 h after LPS in WT mice compared with that in saline-treated control mice (P < 0.05). However, liver EVA levels were not increased significantly above the respective genotype control groups in the P-selectin, ICAM-1, or P-selectin-ICAM-1 mutant mice 4 h after LPS.

Characterization of Pulmonary Vascular Leukocytes in LPS-Treated WT Mice by PVL and Morphometry

Almost twice as many leukocytes were lavaged from LPS-treated mice compared with mice treated with intraperitoneal saline (Table 1). However, Table 1 reveals that a much greater proportion of these leukocytes in LPS-treated mice were neutrophils (63% compared with 25% in saline-treated mice), which reflected a 7.5-fold increase in the number of PVL neutrophils from the right lungs of LPS-treated mice over the number obtained with saline treatment alone.

EM morphometry was performed on the nonlavaged left lung and lavaged right lung in saline- and LPS (4 h)-treated mice in which PVL was either antegrade or retrograde. Because we observed no effect regardless of the direction of perfusion on the numbers of lung neutrophils compared with that in the unlavaged left lung, the data were pooled (Table 2). We observed a sevenfold increase in the number of neutrophils within the capillary lumen of the LPS-treated mice compared with that in saline-treated mice, and the number of capillary neutrophils in the LPS-treated mice were similar between the nonlavaged left lung and the right lung after PVL. Most neutrophils were located within the vasculature, and 4 h after LPS, only an occasional neutrophil had emigrated into the alveolar space. No alveolar neutrophils were present in the saline-treated mice (Table 2).

Table 1. Right lung pulmonary vascular lavage

<table>
<thead>
<tr>
<th>Total leukocytes, 1×10⁶</th>
<th>IP Saline</th>
<th>IP LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent neutrophils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total neutrophils, 1×10⁶</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expected neutrophils, 1×10⁶</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of mice; IP, intraperitoneal; LPS, lipopolysaccharide. *Significant difference from IP saline, P < 0.05.

Liver EVA accumulation increased significantly 4 h after LPS in WT mice compared with that in saline-treated control mice (P < 0.05). However, liver EVA levels were not increased significantly above the respective genotype control groups in the P-selectin, ICAM-1, or P-selectin-ICAM-1 mutant mice 4 h after LPS.

Characterization of Pulmonary Vascular Leukocytes in LPS-Treated WT Mice by PVL and Morphometry

Almost twice as many leukocytes were lavaged from LPS-treated mice compared with mice treated with intraperitoneal saline (Table 1). However, Table 1 reveals that a much greater proportion of these leukocytes in LPS-treated mice were neutrophils (63% compared with 25% in saline-treated mice), which reflected a 7.5-fold increase in the number of PVL neutrophils from the right lungs of LPS-treated mice over the number obtained with saline treatment alone.

EM morphometry was performed on the nonlavaged left lung and lavaged right lung in saline- and LPS (4 h)-treated mice in which PVL was either antegrade or retrograde. Because we observed no effect regardless of the direction of perfusion on the numbers of lung neutrophils compared with that in the unlavaged left lung, the data were pooled (Table 2). We observed a sevenfold increase in the number of neutrophils within the capillary lumen of the LPS-treated mice compared with that in saline-treated mice, and the number of capillary neutrophils in the LPS-treated mice were similar between the nonlavaged left lung and the right lung after PVL. Most neutrophils were located within the vasculature, and 4 h after LPS, only an occasional neutrophil had emigrated into the alveolar space. No alveolar neutrophils were present in the saline-treated mice (Table 2).

Table 2. Lung neutrophil location by EM morphometry in WT mice

<table>
<thead>
<tr>
<th></th>
<th>Intracapillary</th>
<th>Intra-Alveolar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonperfused left lung, cells/mm³</td>
<td>2.250 ± 750 (n = 2)</td>
<td>15,875 ± 2,125 (n = 2)</td>
</tr>
<tr>
<td>Perfused right lung, cells/mm³</td>
<td>2.750 ± 500 (n = 2)</td>
<td>16,375 ± 875 (n = 2)</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of mice. EM, electron microscopic; WT, wild type.
Figure 6 represents EM images of representative lung sections used for EM morphometry. Neutrophils were readily found within the capillaries of the lung of the LPS-treated mouse (Fig. 6A, arrow) and were strikingly different from the thin alveolar walls of the saline-treated mouse (Fig. 6B). Capillary neutrophils were unaffected by PVL as illustrated in the right lung of an LPS-treated mouse after PVL (Fig. 6, C and D, arrows).

Lung Expression of P-Selectin and ICAM-1

In saline-treated mice, there was strong immunohistochemical staining for P-selectin by platelets within the blood vessels, and there was mild staining of the endothelium of the pulmonary arteries and veins. There was an increase in staining of P-selectin in mice studied 4 h after LPS, mainly apparent in the endothelium of pulmonary arteries and veins. Staining of platelets appeared to be comparable between endotoxin- and saline-treated mice. Last, there was no demonstrable staining of capillary endothelium in saline- or endotoxin-treated mice.

DISCUSSION

The present experiments, in a model of systemic endotoxemia chosen to mimic bacteremic sepsis in humans, demonstrate an increase in EVA accumulation in the lungs and liver of WT mice 4 h after intraperitoneal LPS. In contrast, the P-selectin-ICAM-1 double-mutant mouse was completely protected from this.
vascular albumin leak induced by LPS despite evidence of higher basal levels of circulating neutrophils (7) (Fig. 2). The protection in the LPS-treated double mutant was associated with a reduced proportion of neutrophils sequestered in the lung relative to the number of circulating blood neutrophils and with an enhanced exodus of sequestered neutrophils back into the circulation between 2 and 4 h after LPS administration. Clearly, the ratios of lung and liver MPO activity to circulating neutrophil counts were lower in LPS-treated double mutants compared with those in WT mice at both 2 and 4 h after LPS. This is consistent with the possibility that, compared with WT mice, fewer blood neutrophils were trapped in the lung microvasculature after LPS in the P-selectin-ICAM-1 double-deficient mice.

Several possible mechanisms could have contributed to abrogation in lung vascular leak in LPS-treated P-selectin-ICAM-1 mutants. In the absence of P-selectin and ICAM-1, the neutrophils may not be able to bind firmly to the endothelium, which could limit injury by either interfering with an approximation of the neutrophil membrane to the endothelial wall close enough for injury or decreasing the duration of contact between the neutrophil and the endothelium, with a rapid return of the neutrophils to the systemic circulation. We have no evidence on the first possibility, but the latter possibility is supported by the striking rebound in circulating neutrophils in the double mutants between 2 and 4 h after LPS. It should be pointed out that mechanisms other than cell adhesion molecules are important in the sequestration of leukocytes to the microvasculature, especially in the lung (15). Leukocyte stiffening induced by chemoattractants released in response to endotoxin may contribute to their early sequestration to the lung. It has been reported that leukocyte stiffening begins about 10 min after endotoxin administration in the rat (12). This mechanism may explain why the initial sequestration of neutrophils was unaltered even in P-selectin-ICAM-1 double-mutant mice. Consistent with this, Xu et al. (52) reported that neutrophil sequestration in the liver 10 h after LPS administration was significantly reduced in ICAM-1 mutant mice, whereas liver leukostasis 2 h after LPS injection was similar to that observed in WT mice.

The process of leukocyte emigration and localization to sites of inflammation is a complicated event, beginning first with the interaction with the vascular endothelium through regulated expression of adhesion ligands that result in sequential adhesive steps including 1) initial rolling of leukocytes on inflamed vascular endothelium, 2) activation of leukocytes by chemoattractants, 3) firm attachment to the blood vessel walls, and 4) transendothelial migration (1). These processes potentially involve three different classes of leukocyte adhesion ligands, including the selectins, members of the immunoglobulin superfamily, and integrins (1). Members of the selectin family, L-, E- and P-selectin, have all been shown to be capable of mediating neutrophil rolling in vitro or in vivo. Of importance to the present study, in vivo observations of postcapillary venules with intravital microscopy have identified that P-selectin has an important role in the rolling of leukocytes at sites of inflammation (27). P-selectin is rapidly expressed on the surface of platelets and endothelial cells (16), and LPS or TNF-α increases the mRNA for P-selectin in the lungs and liver of mice (39) and in bovine or murine endothelial cells (49). P-selectin may play a role in the early leukopenia after endotoxin administration (9), in leukocyte-platelet aggregation in thrombi (32), and in lung injury in rats after intravenous cobra venom factor (29). It has also been shown that the absence of P-selectin and ICAM-1 in double-mutant mice totally abolishes trauma-induced rolling of leukocytes (25), suggesting cooperation between the two adhesion ligands.

ICAM-1 and other members of the immunoglobulin superfamily expressed constitutively on the surface of endothelial cells facilitate adhesion to CD11/CD18 integrins expressed on the surface of leukocytes (1, 41). ICAM-1 is upregulated by various inflammatory mediators such as IL-1, TNF-α, endotoxin, and interferon-γ (33, 38). Blocking or loss of expression of CD11/CD18 or ICAM-1 can reduce the capability of neutrophils to adhere to the endothelium and subsequently reduces neutrophil emigration (1, 40).

In regard to the exact site of lung neutrophils 4 h after endotoxin administration, the lavage studies reveal a sevenfold increase in the number of neutrophils in the PVL fluid of LPS-treated mice compared with that in saline-treated mice. EM morphometry reveals a sevenfold increase in the number of neutrophils in pulmonary capillaries of LPS-treated mice compared with that in saline-treated mice, and a comparable number of intracapillary neutrophils were present in the lungs of LPS-treated mice even after PVL. Moreover, there was an occasional neutrophil in the alveolar space 4 h after LPS, but the major location of neutrophils is within the capillary lumen. The results of EM morphometry and PVL suggest that the major sites of lung neutrophils 4 h after intraperitoneal LPS are within the capillary lumen and within the larger pulmonary vessels from which a substantial number of neutrophils are recovered with PVL. Only a few neutrophils have emigrated to the alveolar space 4 h after intraperitoneal endotoxin.

The observations in the P-selectin-ICAM-1 double mutant in the present study differ from previous observations that studied intratracheal Streptococcus pneumoniae and intravenous cobra venom factor (7, 11). Although we observed protection of the P-selectin-ICAM-1 double mutant from lung vascular albumin leak, the P-selectin-ICAM-1 double mutant was not protected from lung albumin leak 6 h after intratracheal S. pneumoniae (7) or 30 min after intravenous cobra venom factor (11). Moreover, neutralizing antibodies to ICAM-1 inhibited the lung injury induced by cobra venom factor in WT mice (11). There are multiple explanations that should be considered in regard to the differences between the present experiments and these previous studies.
First, although the P-selectin mouse is a null mutant, the ICAM-1 mutation is not. King et al. (22) have reported alternately spliced isoforms of ICAM-1, some of which may be functional. King et al. reported that there was minimal ICAM-1 staining in the lungs of the basal ICAM-1 mutant, possibly due to type II epithelial cells. However, 24 h after intraperitoneal LPS administration to the mutants, there was an upregulation of staining of ICAM-1 in all vascular endothelium. We did not see staining of lung tissue from unstimulated double mutants with the MAb YN1, which would detect the alternately spliced isoforms of ICAM-1 (22). However, we cannot exclude the possibility of upregulation of ICAM-1 isoforms in these or previous experiments. Therefore, one possible explanation for the differences in protection between the studies is variation in the degree of ICAM-1 upregulation with the stimulus either because of the 24 h that elapsed after intratracheal S. pneumoniae or, despite the short period, the nature of the cobra venom factor stimulus.

Second, anesthetic use may have contributed to the differences. In the present study, the major period of albumin equilibration after intravenous administration of radiolabeled albumin between the vascular and extravascular spaces occurred in unanesthetized spontaneously breathing animals in the absence of anesthetic agents that might suppress alveolar ventilation or alter pulmonary or systemic cardiovascular variables. Although ketamine and xylazine anesthesia was employed in the present experiments, it was administered no sooner than 10–15 min before euthanasia. It should be pointed out that in higher doses than those employed in this study, xylazine can induce pulmonary edema in rats (2), but the earliest time interval studied was 3 h after administration, and there is no information available as to the occurrence of pulmonary edema, if any, within 15 min of administration of xylazine. It should also be mentioned that the pulmonary edema potential of xylazine was not observed when ketamine and xylazine were administered together (2), as was the case in the present experiments. In contrast, ketamine and acepromazine were administered at the onset of the previous studies before albumin administration (7, 11), and their potential effects could have occurred over the duration of albumin equilibration. This may be of significance in view of the observations that ketamine can profoundly suppress release of TNF-α by macrophages (45). In addition, in the study reporting elevated basal lung albumin permeability in the double mutant (11), the animals were euthanized with halothane. This anesthetic agent is known to influence the outcome of sepsis in mice (21) and, in particular, can potentially alter the localization of leukocytes to the lung after influenza virus infection (43) as well as inhibit superoxide production (30). It is possible that anesthetic effects, especially in the setting of elevated numbers of resident neutrophils within the pulmonary capillaries of the P-selectin-ICAM-1 double mutant, produced altered leukocyte and albumin accumulation data in the previous studies (7, 11).

An equally important difference between the present experiments and previous studies (7, 11) relates to employment of fundamentally different models of lung inflammation: pneumonia in the previous studies versus systemic inflammatory response syndrome in the present experiments. A significant amount of information exists to support quantitative and qualitative differences in inflammation between these two models in rodent species. Ulich et al. (47) were able to recover in bronchoalveolar lavage fluid (BALF) >10 million neutrophils from rats 4 h after intratracheal injection of 100 μg of Salmonella typhosa LPS, with a maximum yield of >33 million neutrophils 12 h after administration. In contrast, neutrophils do not appear in BALF until 24 h after intraperitoneal (2.5 mg/kg) (36) or intravenous (3 mg/kg) (8) LPS administration in rats, and the total number of BALF neutrophils recovered is one-tenth the maximal number recovered after intratracheal LPS. There is also evidence of qualitative differences in the chronology of cytokine upregulation in the lung depending on whether endotoxin is administered intratracheally or systemically. Ulich et al. (47) observed that intratracheal administration of S. typhosa LPS to rats induced a progressive increase in lung IL-1β mRNA, IL-10, and TNF-α 3 h after administration. Intravenous S. typhosa administration in rats resulted in peak lung expression of IL-1β at 1 h, which subsequently declined 4 h after administration. These data provide significant support for the existence of differences in mechanisms for inflammation in the lung depending on the stimulus, whether it is administered intratracheally or systemically, and the time after the stimulus when the measurements are obtained.

Last, as Ward (48) has suggested, mutant mice with targeted leukocyte adhesion ligand gene ablations may have additional unrecognized differences that have occurred either during the genetic modification or in compensation for the gene disruption over the life of the animal that renders it different in ways besides the ligand disruption. In studies of models of inflammation different from the present experiments, the response of the ICAM-1 mutant differed from effects of anti-ICAM-1 neutralizing antibody administration to WT mice. There was an absence of protection from lung injury after intratracheal LPS (24) or intravenous cobra venom factor (11) in ICAM-1 mutants, whereas administration of neutralizing antibodies to ICAM-1 in WT mice significantly inhibited lung injury with these inflammatory stimuli. However, the ICAM-1 neutralizing antibody, protective in WT mice, failed to offer any attenuation of lung injury in the ICAM-1 mutants, raising the possibility of upregulation of other inflammatory mechanisms. In support of this possibility, Eppihimer et al. (14) observed differences in constitutive and TNF-α-stimulated expression of E-selectin in various organs in ICAM-1 mutants or P-selectin-deficient mutants compared with those in WT mice. There was increased constitutive expression of E-selectin in the stomach, large intestine, and brain of the ICAM-1 mutants compared with that in the WT mice. In contrast, there was an attenuation in TNF-α stimula-
tion of brain expression of E-selectin in both the WT mice and ICAM-1 mutant mice. Moreover, compared with WT mice, there were no differences in constitutive or TNF-α-stimulated expression of P-selectin were observed between WT mice and ICAM-1 mutant mice. In the previous studies, the ICAM-1 mutant was not protected from the lung inflammation with either intratracheal S. pneumoniae (7) or intravenous cobra venom factor (11); in the present experiments, the P-selectin-ICAM-1 mutants were protected from the lung injury after intraperitoneal LPS. Nevertheless, observations with mutant mice alone need to be interpreted with these previous studies in mind and with the possibility that the mutants may be different in additional unrecognized ways besides the gene deletion.

In summary, our data reveal attenuation of lung and liver injury and delay in mortality after systemic endotoxemia in P-selectin-ICAM-1 double-mutant mice compared with that in WT mice. These results suggest that ICAM-1 and P-selectin significantly contribute to neutrophil retention and subsequent vascular damage in the lung and liver in the systemic inflammatory response syndrome induced by intraperitoneal endotoxin administration.

This work was supported in part by the American Heart Association, Virginia Affiliate (C. E. Rose); National Heart, Lung, and Blood Institute Grants HL-54136 (to K. Ley); and National Cancer Institute Grant CA-34546 (to S. M. Fu). Address for reprint requests and other correspondence: C. E. Rose, Jr., Division of Pulmonary and Critical Care Medicine, Univ. of Virginia Health Sciences Center, Charlottesville, VA 22908 (E-mail: cer@virginia.edu).

Received 28 October 1998; accepted in final form 30 March 1999.

REFERENCES


