Effects of Steroids on the Lung Accumulation of Neutrophil and Monocyte in Rabbits With Endotoxemia*

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**Study objective:** To determine the effects of steroids on the lung accumulation of polymorphonuclear leukocytes (PMNs) and monocytes in rabbits with endotoxemia.

**Design:** A prospective, randomized, controlled animal trial.

**Setting:** Surgical research laboratory, Keio University School of Medicine.

**Subjects:** Twenty-four female Japanese white rabbits.

**Interventions:** Rabbits with endotoxemia were pretreated with steroids sufficient to inhibit the production of tumor necrosis factor (TNF) and to prevent the fall of BP.

**Measurements and results:** The circulating leukocyte counts and the leukocyte accumulation in the lungs were evaluated. Endotoxin caused a rapid decrease in circulating PMNs and monocytes followed by an 8-fold greater accumulation of PMNs (p<0.001) and a 6.5-fold greater accumulation of monocytes (p<0.05). Steroids failed to inhibit this initial drop of PMNs and monocytes. However, steroids inhibited the PMN accumulation in the lung by 50% (p<0.05), without inhibiting the monocyte accumulation in the lung.

**Conclusions:** These findings suggest that the lung accumulation of PMNs is dependent on TNF or other inflammatory mediators that are inhibited by steroids while the lung accumulation of monocyte is not.

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**Key words:** endotoxemia; leukocyte; lung accumulation; methylprednisolone; monocyte; neutrophil; tumor necrosis factor

**Abbreviations:** ET group=endotoxin group; IL-1=interleukin 1; MAP=mean arterial pressure; MN=mononuclear leukocyte; CC=colloidal carbon; MP group=methylprednisolone-treated group; P(A-a)O2=alveolar-arterial PO2 difference; PMN=polymorphonuclear leukocyte; TNF=tumor necrosis factor

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Endotoxins are lipopolysaccharides located on the outer membrane of Gram-negative bacilli that can induce acute lung injury.1-4 In animal studies, polymorphonuclear leukocytes (PMNs) accumulate in the lungs at the time of endotoxin-induced acute lung injury and have an important role in its pathogenesis.5-7 Recently, monocytes and PMNs have been demonstrated to be involved in inflammatory reactions and to accumulate in the lungs at the time of endotoxemia.8,9 In view of the fact that monocytes are key cells in the production of cytokines such as tumor necrosis factor (TNF) and interleukin 1 (IL-1)10,11 that are important in the manifestations of shock and organ injury accompanying endotoxemia, circulating monocytes are likely to be involved in the development of the lung injury caused by endotoxemia.

Among the various chemical mediators that are known to mediate the organ injury during endotoxemia, TNF has been shown, when administered alone, to nearly reproduce the in vivo reactions that are seen following injection of endotoxin.12-14 Therefore, TNF appears to play a key role in the in vivo reactions during endotoxemia. Moreover, in vivo production of TNF in endotoxemia is inhibited by pretreatment with steroids,15-18 and it has also been reported that pretreatment with steroids inhibits several effects induced by endotoxemia, including acute lung injury.19-22

In the present study, rabbits pretreated with a dose of steroids sufficient to inhibit the production of TNF and to prevent the the fall of BP received an IV injection of endotoxin. This study tested the hypothesis that pretreatment with steroids inhibits the endotoxin-induced accumulation of PMNs and monocytes within the pulmonary microvasculature that is thought to be involved in the induction of acute lung injury seen in endotoxemia.

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Materials and Methods

Animals

Twenty-four female Japanese white rabbits, having an average weight of 3.1±0.2 kg, were used in this study. All animals received care according to the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research.

Experimental Protocol A

Rabbits were anesthetized by an IM injection of ketamine hydrochloride (60 to 80 mg/kg) and xylazine hydrochloride (2.0 mg/kg) and placed in the supine position. An 18-gauge flexible catheter was passed through the right carotid artery into the aorta to monitor BP and collect blood samples. This catheter was continuously flushed with 3 mL/h of heparinized saline solution. Butterfly catheters were placed in the lateral marginal veins of both ears, and these lines were connected to three-way stopcocks flushed with heparinized saline solutions. Baseline blood samples were collected from the aortic catheter for measurements of the blood cell counts and plasma TNF activity. Twelve rabbits were divided into three groups. In the endotoxin group (ET group, n = 4), 3 mL of saline solution was injected into the right marginal ear vein, and 1 h later, *Escherichia coli* endotoxin (lipopolysaccharide from *E. coli* 955-B3; Sigma, St. Louis) 50 μg per rabbit in 3 mL of sterile saline solution was injected into the left marginal ear vein. In the methylprednisolone-treated group (MP group, n = 4), 30 mg/kg of methylprednisolone sodium succinate (methylprednisolone) in 3 mL of sterile saline solution was injected into the right marginal ear vein, and 1 h later, 50 μg per rabbit of endotoxin was injected into the left marginal ear vein. In the control group (n = 4), 3 mL of saline solution was injected into the right marginal ear vein, and 1 h later, 3 mL of saline solution was injected into the left marginal ear vein. The above dose of endotoxin was chosen because preliminary studies showed that 100 or 150 μg endotoxin per rabbit administered IV caused half of the rabbits to die within 24 h, whereas only 1 of 10 rabbits given 50 μg endotoxin per rabbit died.

Blood samples were collected from the aortic catheter for blood cell counts just before endotoxin or saline solution injection and at 5, 15, 30, 60, 120, 180, and 240 min after endotoxin or saline solution injection, and for plasma TNF activity just before endotoxin or saline solution injection and at 30, 60, 90, 120, and 180 min after endotoxin or saline solution injection. The BP was recorded using the aortic catheter every 15 min throughout the study period.

Differential Blood Count in Peripheral Blood

The circulating leukocyte and erythrocyte counts in the peripheral blood were determined on the basis of the change in the electric impedance using an automatic blood cell counter (F-800T; Toa Medical Electronics Co; Tokyo, Japan). The differential cell counts were determined by visual inspection of Giemsa-stained thin-layer smear preparations under a microscope.

Bioassay of TNF Activity in Plasma

TNF activity in the plasma samples collected from the three groups studied in protocol A was assayed by measuring the cytotoxic effect of TNF on a tumorigenic murine fibroblast line L929, as previously described. Briefly, L929 cells were cultured in 96-well microtitrator plates at a density of 3.0×10⁴ cells per well in RPMI 1640 culture medium (Gibco; Grand Island, NY) containing 3% fetal calf serum (Gibco), 2 mM L-glutamine, penicillin (60 mg/L), and streptomycin (100 mg/L). After incubation for 18 h, the culture medium was removed. Either serially diluted plasma samples or recombinant human TNF-α (Genzyme; Boston) was added to the wells, followed by actinomycin D (2 μg per well, Sigma), and the cells were cultured for an additional 20 h. The culture medium was removed and the cells were stained with 0.5% crystal violet in 20% methanol for 40 min. After rinsing and drying, the uptake of crystal violet was quantitated by measuring the absorbance at 570 nm on an Easy Reader EAR-340 AT (SLT; Salzburg, Austria). Measurements of 100% cell destruction were obtained by adding 3 M guanidine hydrochloride. The cytotoxicity was calculated as follows:

% cytotoxicity = (1 - absorbance sample / absorbance control) × 100

Standard curves of the percent cytotoxicity vs the concentration of recombinant human TNF were constructed, and the TNF activity in the plasma samples was determined from this curve.

Experimental Protocol B

Twelve unanesthetized rabbits were prepared by placing a 22-gauge flexible catheter in the right central ear artery and butterfly catheters in the lateral marginal veins of both ears. These rabbits were then divided into the same three groups as in protocol A. After baseline blood sampling from the arterial line for measurement of the arterial blood gas tension before endotoxin or saline solution injection, all catheters were removed, and the animals were given free access to food and water in individual cages. Twenty-two hours after endotoxin or saline solution injection, the rabbits were anesthetized as in protocol A and placed in the supine position. A tracheotomy tube was inserted, and the animals breathed room air spontaneously. A catheter was placed in the aorta via the right carotid artery. To determine the effects of endotoxin on the phagocytic function of circulating leukocytes, colloidal carbon was infused into animals 1 h before evaluating lung and blood samples, as previously described. Colloidal carbon (51822A143 Drawing ink; Pelikan; Hanover, Germany) was filtered twice through 0.8-μm filters and diluted with an equal volume of sterile saline solution. Twenty-three hours after endotoxin or saline solution injection, 2 mL/kg body weight of this preparation was infused into the aortic line. Twenty-four hours after endotoxin or saline solution injection, blood samples were collected for measurement of the arterial blood gas tension parameters. Then the heart was stopped by injection of a saturated KCl solution into the arterial catheter, the chest and pericardial sac were opened, and a tie was placed around the base of the heart to prevent blood from draining out of the lungs. The lungs were fixed in situ by instilling 6.0% glutaraldehyde in 0.1 M sodium phosphate buffer into the tracheotomy tube at 20 cm H₂O pressure. The heart and lungs were removed and immersed in glutaraldehyde for 4 h. After fixation, the lungs were cut into small pieces.

Morphologic Calculations

Leukocyte accumulation in the pulmonary vasculature was evaluated by histologic quantification, as previously described. These studies were based on the premise that the number of leukocytes per RBC is increased in regions of the vasculature where leukocytes had accumulated. Four tissue blocks, each measuring 1×0.5×0.2 cm, were randomly selected from each rabbit lung, dehydrated, and embedded in paraffin, and 3-μm sections were placed on glass slides. The slides were stained with hematoxylin-eosin. The mononuclear leukocytes (MNs), and PMNs, and RBCs were counted at ×400 magnification in six randomly selected fields of alveolar capillaries in each section from each rabbit in the three groups. MNs that had phagocytosed colloidal carbon (MN with CC) were also counted. The ratios of PMNs, MNs, or MNs with CC per RBC were calculated to quantitate their accumulation in the lung.

Statistical Analysis

All data are expressed as the mean±SEM. To test the statistical significance of temporal changes in the values of circulating leukocyte counts, plasma TNF activity, and BP in protocol A, analyses of variance for repeated measurements were performed with application of Huynh-Feldt’s correction. In protocol B, the values for the
lungs accumulation of leukocytes and the arterial blood gas tension parameters were tested for statistical significance by the unpaired $t$ test. Statistical significance was defined as a $p$ value of $<0.05$.

RESULTS

Circulating Leukocyte Count

The values for the circulating total leukocyte count at $-1$ and 0 h were similar in each of the three treatment groups. Thereafter, in the ET group and the MP group, the total leukocyte count decreased rapidly, falling to 50% of the baseline value by 5 min after the administration of endotoxin, and remained low through the next 4 h (Fig 1, top left). In the control group, the total leukocyte count did not change throughout the observation period.

The effect of endotoxin and of pretreatment with steroids varied for each type of leukocyte. In both the ET group and the MP group, the PMN count fell to 10% or less of the baseline value within 5 min after the administration of endotoxin (Fig 1, bottom left). However, thereafter, the PMN count increased over time in the MP group, whereas it persisted at a similar low level in the ET group. The monocyte count was also markedly reduced from the baseline value at 5 min after the endotoxin administration in both the ET group and the MP group, but the degree of reduction was smaller in the MP group compared with the ET group (Fig 1, top right). Thereafter, the monocyte count remained nearly constant in each group. The lymphocyte count showed similar decreases in both the ET group and the MP group throughout the observation period (Fig 1, bottom right).

TNF Activity in Plasma

Figure 2 shows the plasma TNF activity in the three groups. After the exposure to endotoxin in the ET group, the plasma TNF level increased to 4,350±1,987 U/mL (range, 1,000 to 9,200 U/mL) with a peak at 1 h, and returned to the baseline value by 3 h. In con-
FIGURE 2. Concentration of TNF in plasma. After the exposure to endotoxin in the ET group (open circles), the TNF concentration in plasma increased to more than 4,000 U/mL with a peak at 1 h and returned to the baseline by 3 h. In the MP group (closed circles), this peak was only 470 U/mL, and no TNF was detected at any time point in the control group (open squares).

In contrast, the plasma TNF in the MP group was detected in only 2 of the 4 animals, and the levels were significantly lower than in the ET group (470±330 U/mL). In the control group, no plasma TNF was detected at any time point.

**Blood Pressure**

In the ET group, the mean arterial pressure (MAP) had decreased from a baseline value of 77±5 mm Hg to 54±3 mm Hg at 1 h after the administration of endotoxin, and remained at that level thereafter (Fig 3). In contrast, almost no fluctuation in the MAP throughout the observation period occurred in the control and the MP groups.

**Morphology**

Injection of endotoxin resulted in an eightfold increase in the accumulation of PMNs per RBC in the ET group than in the control group (Fig 4, top). The pretreatment with steroids inhibited this accumulation by 50% (Fig 4, top). Injection of endotoxin resulted in a 2.5-fold increase in the accumulation of MNs per RBC in the ET group than in the control group, and pretreatment with steroids did not alter the size of this accumulation (Fig 4, center). There was almost no PMN or MN emigration into the alveolar spaces, and no histologic evidence of interstitial edema or hemorrhage was seen in any of the groups.

The lungs in the ET group and the MP group were gray-black compared with the pinkish lungs from the control animals, indicating that colloidal carbon had accumulated within these lungs. Histologically, the colloidal carbon was found more frequently in the lungs of rabbits in the ET and MP groups than in the control group (Fig 4, bottom). Most of the colloidal carbon had been phagocytosed by MNs that were seen within the alveolar capillaries and had the morphologic characteristics of monocytes. MNs with CC were rarely seen in the control specimens. Injection of endotoxin resulted in a 6.5-fold increase in the accumulation of MNs with CC per RBC than in the control group, and the pretreatment with steroids did not prevent this increase (Fig 4, bottom). A small portion of the colloidal carbon was aggregated within the microvasculature.

Injection of endotoxin did not result in an increase of mononuclear cells not containing carbon (from 16.3±1.7 in the control group to 23.3±7.1 in the ET group; p>0.1). The pretreatment with steroids did not alter the size of this accumulation (21.0±2.0 in the MP group; p>0.1 compared with the control group).

**Arterial Blood Gas Tension**

The baseline PaO₂, PaCO₂, and alveolar-arterial Po₂ difference (P[A-a]O₂) before endotoxin or saline solution injection were similar among the three groups. At 24 h after injection, the PaO₂ decreased and the PaCO₂ and P(A-a)O₂ difference increased in all three groups, and there were no statistically significant differences among the three groups (Table 1).

**Discussion**

In patients with sepsis, endotoxin leads to the accumulation of inflammatory cells in the lungs, an early step in the development of acute lung injury due to endotoxemia. This accumulation is then followed by the progression of tissue injury, as is characterized by damage to the vascular endothelium followed by edema in the interstitial tissues and the airways. The present study investigated the changes caused by administration of a single, sublethal dose of endotoxin to rabbits. The data show that the TNF concentration in the plasma peaked at 60 min after endotoxin adminis-
tration and significant leukocyte accumulation in the lungs occurred by 24 h. However, no histologic evidence of pulmonary edema was observed, and there was no significant decrease in the gas exchange capacity of the lung. These results indicate that intravascular injection of endotoxin using this protocol induced cytokine production and accumulation of inflammatory cells in the lungs but not pulmonary edema, suggesting that changes mimicking the early stage of acute lung injury due to endotoxemia were present.

The changes in the circulating leukocyte counts caused by administration of endotoxin are most likely related to the sequestration of leukocytes in the organs. The present study shows that both the effect of endotoxin and the effect of steroids on endotoxin-induced changes varied for each leukocyte type. Endotoxin induced rapid, complete, and prolonged neutropenia within 5 min and a 7- to 8-fold increase in PMNs within the pulmonary capillaries at 24 h. In contrast, the decrease in circulating monocytes was slower (15 to 60 min) than that of PMNs, and the circulating lymphocyte numbers fell only slightly and even more slowly over 1 to 2 h. Because sequestered lymphocytes and monocytes within the pulmonary capillaries were difficult to distinguish based on purely histologic criteria, the total mononuclear cell accumulation and the number of mononuclear cells containing colloidal carbon, a phagocytic cell marker, were determined. Although this technique may underestimate the number of monocytes, particularly in the control group, as activated monocytes may be preferentially marked, the sequestered monocytes were estimated to be the colloidal carbon-containing cells.

The data show that the number of sequestered monocytes increased 6- to 7-fold 24 h after injection of endotoxin. In contrast, the number of sequestered lymphocytes, as estimated by mononuclear cells not containing carbon, was not increased. These data indicate that the mechanisms mediating endotoxin-induced sequestration differ for each leukocyte type.

Chemokines, cytokines, and adhesion molecules are thought to mediate the prolonged sequestration of leukocytes within the pulmonary capillaries. Endotoxin induces the expression of proinflammatory cytokines, including IL-1 and TNF, which in turn induce the expression of adhesion molecules and chemokines. Endotoxin also induces the production of other inflammatory mediators such as complement fragments and platelet activating factor that are potent activators of PMNs. Steroids inhibit the production of many cytokines and other mediators, as demonstrated by the inhibition of TNF production and hypotension. This study demonstrates the differential effects of steroids on the processes mediating leukocyte sequestration. Steroids did not prevent the initial sequestration of PMNs, as measured by the circulating PMN counts, but they inhibited the sequestration in
the lung at 24 h. These data support the hypothesis that the initial sequestration of PMNs is mediated through processes different than those important in maintaining the sequestered PMNs within the pulmonary capillaries. In contrast, while steroids partly inhibited the fall in the circulating monocyte counts, there was no change in the number of monocytes that accumulated within the pulmonary capillaries over 24 h, despite most complete inhibition of TNF production and the mediators that induce hypotension. These data suggest that prolonged accumulation of PMNs, presumably through adhesion to alveolar capillary endothelium, is dependent on TNF or other steroid-inhibitable mechanisms, while monocyte adhesion is not.

Variable effects of pretreatment with steroids on the adhesion of PMNs to vascular endothelium and PMN accumulation in the lungs during endotoxemia have been reported. These discrepancies are presumably due to differences in the experimental methods, including differences in the dose and timing of steroid administration and in the species examined that result in inconsistent inhibition of cytokines and other mediators. In present study, the dosage and timing of methylprednisolone was selected to be 30 mg/kg 60 min prior to the administration of endotoxin, because this dosing regimen led to inhibition of TNF production and prevention of hypotension in preliminary studies.

A number of clinical studies have been conducted to investigate the efficacy of high-dose steroids as a treatment for ARDS occurring in association with sepsis. However, these studies have not shown a beneficial effect of steroids. One explanation might be that the steroids are administered too late in the pathogenesis to prevent injury, since inflammatory mediators such as TNF or IL-1 are released soon after exposure to endotoxin. The results of the present study demonstrate that, although the steroid pretreatment inhibited the accumulation of PMNs in the lungs, it did not inhibit the lung accumulation of activated monocytes, despite administration of steroids prior to endotoxin. This finding suggests that, in clinical cases, failure to control the lung accumulation of activated monocytes may be another reason for the lack of efficacy in therapeutic trials.

In summary, pretreatment using methylprednisolone at a dosage that completely inhibited TNF production was found to significantly inhibit the endotoxin-induced accumulation of PMNs within the pulmonary capillaries, but it did not inhibit the accumulation of MNs and MNs with CC. The present experimental findings suggest that the regulation of adhesion of PMNs to endothelium of the alveolar capillaries is dependent on TNF or other inflammatory mediators that are inhibited by steroids while the regulation of monocyte adhesion is not.

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**Table 1—Arterial Blood Gas Tensions**

<table>
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<tr>
<th>Group</th>
<th>PaO₂, mm Hg</th>
<th>PaCO₂, mm Hg</th>
<th>P(A-a)O₂, mm Hg</th>
<th>p Value</th>
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<td>PRE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
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<tr>
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<td>20.6±8.2</td>
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<td>48.2±2.5</td>
<td>17.9±5.2</td>
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</tr>
</tbody>
</table>

*These data show values obtained before endotoxin or saline solution was injected (PRE) and at the end of the experiment (END) 24 h after endotoxin or saline solution was injected. Values are mean±SEM. NS=not significant.
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