Polymorphonuclear Neutrophil Contribution to Induced Tolerance to Bacterial Lipopolysaccharide

Jorge Barros-Aranda, Geert W. Schmid-Schönbein, Benjamin W. Zweifach, and John C. Mathison

The objective of this study was to investigate mechanisms by which polymorphonuclear neutrophils (PMNs) contribute to the tolerance induced by repeated lipopolysaccharide (LPS) injections. Tolerance was developed by daily intraperitoneal injections of sublethal doses of LPS for 4 days (LPS-tolerant group); controls were not pretreated (LPS-control group). Both groups were challenged with 9 mg/kg i.v. Escherichia coli LPS, a dose that resulted in 25% survival in LPS-control rats compared with 100% survival in LPS-tolerant rats. LPS injection caused an initial neutropenia in both groups. The neutropenia persisted throughout the experiment in LPS-control rats, whereas in LPS-tolerant rats the circulating PMN count increased dramatically; after 6 hours, the PMN count was 16-fold higher than that in LPS-control rats. Activation of circulating PMNs, PMN adhesion to nylon fibers, and tumor necrosis factor/cachectin activity were all increased in control rats given LPS. In contrast, LPS-tolerant rats had low activation of circulating PMNs, no trend for PMN adhesion to nylon fibers, and markedly reduced tumor necrosis factor activity. To determine whether neutropenia was associated with a trapping of PMNs in the microcirculation, we used a carbon perfusion technique 6 hours after LPS injection and examined histological sections of the myocardium. All of the arterioles and venules in both groups contained carbon; only capillaries showed evidence of obstruction. A significantly higher percentage of obstructed capillaries was observed in LPS-control rats than in LPS-tolerant rats. Obstruction of capillaries was consistently associated with trapped leukocytes. We conclude that PMN cytotoxicity induced by LPS involves microcirculatory entrapment and activation of PMNs. Repeated LPS pretreatment reduces dramatically circulating PMN activation and adhesion and is associated with an elevated circulating PMN count, a low degree of microvascular plugging, and survival after a normally lethal dose of LPS. (Circulation Research 1991;69:1196-1206)

A considerable body of evidence during the last decade suggests that polymorphonuclear neutrophils (PMNs) are a major contributor to tissue injury and organ failure in various forms of ischemia and shock. In this study we propose to examine the mechanisms by which the administration of gram-negative lipopolysaccharide (LPS) affects PMN activation, margination, and

PMN-mediated cytotoxicity. To further our understanding of PMN behavior after LPS administration, two experimental models, one with high lethality and the other with tolerance to LPS, will be investigated.

Although it has been known for more than 50 years that experimental animals can be rendered tolerant to shock by selected pretreatments, the phenomenon remains one of the most fascinating but poorly documented aspects of shock research. Tolerance to hemorrhagic, traumatic, and LPS-induced shock can be developed by repeated mild trauma,1,2 by voluntary exercise training for several weeks,3 and by administration of sublethal doses of LPS for several days.4,5

LPS has been shown to cause release of toxic O2 species from PMNs and to enhance their adhesion to the endothelium.6,7 In addition, LPS interacts with monocytes/macrophages to release a number of products that participate in the mediation of tissue

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injury, including the monokine termed tumor necrosis factor (TNF/ cachectin. Binding of TNF to PMNs enhances the adherence of PMNs to endothelial cells, inhibits migration, and stimulates oxidative respiratory burst and degranulation. LPS may also activate the alternative complement pathway to generate C5a, which in turn activates PMNs. Although the data to date clearly implicate PMNs in the systemic derangement after LPS administration, the sequence of events in which the PMNs participate has not been elucidated, and it is not clear how pretreatment with LPS can induce a tolerance against PMN-mediated tissue toxicity.

PMN kinetics were studied in LPS-induced shock by comparing a group of untreated controls with a group in which tolerance had developed by pretreatment with sublethal doses of LPS. A number of questions were addressed in the two models: After injection of LPS, what are the circulating PMN counts, the degree of PMN activation and adhesion, and the response of circulating PMNs to LPS in vitro? What is the degree of capillary obstruction as exemplified in a key organ such as the heart, and is it related to trapped leukocytes? Is there a correlation between plasma TNF levels and spontaneous PMN activation in the two groups?

Materials and Methods

Animal Preparation

Male Wistar rats (300–400 g, Charles River Laboratories, Inc., Wilmington, Mass.) were housed in a controlled environment and maintained on a standard pellet diet for at least 4 days before initiation of the experimental procedures. The rats were fasted overnight and cannulated (femoral artery and vein) under general anesthesia using pentobarbital (30 mg/kg i.m.). Heparin was administered intravenously in a dose of 10 units/ml blood volume, estimated as 8% of body weight; a second dose was administered 3 hours later. The rats were placed on a heating pad and covered with a blanket, and the body temperature was monitored rectally.

LPS-Tolerance Pretreatment

The rats were divided into a control group pretreated with saline (LPS-control group) and a group pretreated with LPS (LPS-tolerant group). Tolerance to LPS was induced by daily intraperitoneal injections of increasing doses (0.15, 0.30, 0.45, and 0.60 mg/kg body wt) of LPS (Escherichia coli 0127:B8, Difco Laboratories, Detroit, Mich.) for 4 days. The LPS was reconstituted in sterile pyrogen-free saline and filtered (pore size, 0.45 µm). LPS-control rats received daily injections of an equal volume (0.25 ml/100 g) of saline.

Experimental Protocol

After cannulation, LPS-control rats and LPS-tolerant rats were observed for 15 minutes to ensure that mean arterial pressure and heart rate had reached stable values. The rats received an intravenous LPS injection of 9 mg/kg E. coli 0127:B8 over a period of 5 minutes. Mean arterial pressure and heart rate were monitored continuously. Blood samples were taken via the femoral artery catheter at regular intervals of time for measurements of hematocrit, leukocyte count, differential white blood cell count, and the percentage of circulating PMNs activation as measured by the nitroblue tetrazolium (NBT) test (see below). In another set of rats, arterial blood samples were drawn aseptically and immediately placed on ice. The blood was centrifuged at 3,000g for 10 minutes at 4°C. Plasma was stored in sterile microcontainers at −60°C pending TNF assay measurements. Six hours after LPS injection, the catheters were sealed, and the skin incision over the femoral catheters was closed under local anesthesia using 4% lidocaine (Astra Pharmaceutical Products, Westborough, Mass.). Both groups of rats were observed over 48 hours for survival. Surviving rats were killed by inhalation of methoxyflurane (Metofane, Pitman-Moore, Washington Crossing, N.J.). All animal protocols were reviewed and approved by the University Animal Subjects Committee.

Nitroblue Tetrazolium Test on Circulating PMNs

The NBT test consists of counting the percent of circulating PMNs that showed spontaneous reduction of pale yellow NBT to blue-black formazan crystals. NBT reduction by PMNs has been found to be associated with enhanced superoxide (O2−) production. Fresh arterial blood (0.1 ml containing ~10 units/ml heparin) was immediately transferred into a clean siliconized concave microslide and mixed with an equal amount of 0.1% NBT solution. The microslide was enclosed in a Petri dish containing wet gauze to maintain humidity, incubated at 37°C in air for 30 minutes, and subsequently allowed to stand at room temperature for an additional 15 minutes. At the end of this period, the blood–NBT mixture was gently stirred. Coverslip smears were made and stained with Wright’s stain. A total of 100 PMNs were counted under ×100 oil objective magnification. PMNs that showed stippled cytoplasmic deposits of formazan or a dense clump of formazan in their cytoplasm were counted as NBT-positive cells.

Cytolytic Assay for TNF

TNF activity was measured using a cytolytic assay with 1 µg/ml actinomycin D–treated L929 cells. Each plate included TNF standard (conditioned medium from LPS-treated RAW 264.7 cells, 5 × 104 units/ml), which was calibrated using human recombinant TNF standard obtained from the National Institute for Biological Standards and Control, Heartfordshire, England.

Carbon Perfusion Technique

In this experiment, the extent of capillary plugging by PMNs was investigated using a method that consisted in the perfusion of carbon particles. This
The carbon perfusion technique was used previously by this group after hemorrhagic shock and after local myocardial ischemia. The carbon served as a contrast medium to distinguish vessels with or without flow. The extent of capillary obstruction was determined in histological sections of the heart at 6 hours after LPS administration. This organ was selected because of two important considerations: 1) It plays an important role in the pathophysiology of shock. 2) Its capillaries are distributed outside the myofibrils so that the histological analysis is relatively easy to perform. Three groups of rats were used for this purpose: LPS-control rats, LPS-tolerant rats, and rats neither pretreated nor challenged with LPS (no-LPS rats) as a baseline.

The carbon perfusion technique was carried out in two steps: 1) Blood cells and circulating fibrinogen were washed out by infusion of modified Ringer’s solution to prevent coagulation in the presence of the contrast medium and to induce cardiac arrest within 3–5 minutes. This initial step was performed immediately after general anesthesia. Modified Ringer’s solution was then infused at 38°C through a catheter in the right carotid artery (PE-90 polyethylene tubing) placed as close as possible to the heart. The reservoir pressure was maintained at 180 mm Hg to obtain a pressure of 120 mm Hg at the end of the catheter. Exchange of the blood requires between 6 and 8 minutes and was terminated when the fluid draining from the jugular vein contained only traces of red blood cells. 2) Infusion of contrast medium was used to identify perfused vessels. The blood washout was followed immediately without a shift in pressure by infusion of a carbon suspension (17 black drawing ink A in Plasma-Lyte A, ratio 1:2, Pelikan, RFA) at 38°C through the carotid artery at 120 mm Hg for 15 minutes. An equivalent volume was withdrawn from the jugular vein.

Tissue Preparation

After the carbon infusion, the chest was opened, the vessels that enter and leave the heart were tied and severed, and the heart was immersed in EIG solution (87% ethanol, 10% formaldehyde, and 3% glacial acetic acid) and fixed overnight. The fixed heart was cut transversely into 1.5-mm-wide slices and then rinsed in cacodylate buffer (pH 7.4, 350 mosm). Tissue from the right (six to eight specimens) and left (eight to 10 specimens) ventricles was trimmed to 0.5×1.5×4 mm, postfixed in 1% OsO₄ for 1 hour, dehydrated in graded ethanol, and embedded in araldite resin.

Tissue specimens were oriented so that the microvessels would predominantly yield cross sections. Sections of 1 μm thickness were made and stained with 1% toluidine blue and viewed through a light microscope with an oil immersion objective (×100, 1.32 n.a.) and a ×10 eyepiece, which permits recognition of capillaries, endothelial cells, carbon particles, platelets, erythrocytes, and leukocytes.

Histological Analysis

The fraction of arterioles, capillaries, and venules in which flow was present (i.e., vessels with carbon) and the average frequency of leukocytes in capillaries were determined. Whenever carbon was observed in a blood vessel, the entire vessel was considered perfused (Figure 1, top panels). When no carbon was observed in the vessel, the blood vessel was considered nonperfused (Figure 1, bottom panels).

The total number of capillaries, the number of capillaries without carbon, and the number of capillaries with a leukocyte were recorded. An obstructed capillary was identified unequivocally only when the endothelial cell surrounding an open lumen could be recognized. The criteria for identification of a leukocyte were that the leukocyte nucleus and cytoplasm had to be recognizable together with the surrounding endothelial cell (Figure 1, bottom panels).

This extensive histological procedure was carried out on a cohort of three rats for each group. In each rat, ~5,000 capillaries in the right ventricle and ~7,500 capillaries in the left ventricle were investigated.

In Vitro Activation of PMNs With LPS

Selected fresh blood samples were mixed in vitro with LPS to determine whether the level of spontaneous in vivo PMN activation could be further enhanced by LPS in vitro. Arterial blood samples (0.1 ml), drawn before and 1 and 6 hours after systemic LPS injection (9 mg/kg body wt E. coli), were mixed with LPS (E. coli, at 75 μg/ml blood). The mixture of blood and LPS was incubated at 37°C for 15 minutes and subjected to the NBT test.

In Vitro Activation of PMNs With Plasma After LPS Injection

Plasma (50 μl) from LPS-control rats (n=12) and LPS-tolerant rats (n=12) obtained 6 hours after LPS injection was mixed in vitro with 100 μl heparinized arterial blood (20 units/ml) collected from donor rats. The mixture was incubated at 37°C for 15 minutes and then subjected to the NBT test. Increments in the percentage of NBT-positive PMNs in donor blood due to the addition of plasma from the two groups were determined.

In Vitro PMN Adhesion

PMN adherence to nylon fibers in whole blood was determined at 4 and 6 hours after LPS injection in both groups according to the fiber filtration method. Aliquots (0.8 ml) of heparinized whole blood (10 units/ml) at 37°C were placed with a pipette onto nylon fiber columns (leukopak, Travenol Laboratories, Deerfield, Ill.) and allowed to pass by gravity. Standard columns contained 30-mg nylon fibers packed into 1-ml tuberculin syringes from the 0- to the 0.22-ml mark (13 mm in length). Percent PMN adherence was computed as
Neutrophil Contribution to LPS Tolerance

Statistical Analysis
The data are presented as mean±SD. Statistical comparisons between groups were carried out on the basis of the mean of individual sets of variables (Student’s t test). A value of p<0.05 was considered significant.

Results

Survival Rate
The survival rate after 48 hours was 25% in the LPS-control group (n=20) compared with 100% in the LPS-tolerant group (n=20).

Blood Pressure and Heart Rate
After LPS administration, a decrease in mean arterial pressure was observed in both groups. There was a return of mean arterial pressure toward normal in the LPS-tolerant group. In contrast, in the LPS-control group, mean arterial pressure remained low throughout the experimental protocol (Figure 2). Heart rate was significantly higher in LPS-control rats than in LPS-tolerant rats before and over 4 hours after LPS injection (Figure 2).

Blood Counts
Rats subjected to the LPS-tolerance regimen showed a reduction in hematocrit of ~12% compared with LPS-control rats (p<0.0005) (Figure 3).
Injection of LPS resulted in a rapid increase in hematocrit in both groups, which was significantly higher in LPS-control rats than in LPS-tolerant rats ($p<0.005$) (Figure 3). Subsequently, there was a gradual decrease in the hematocrit in both groups with a slow return to preshock values in the LPS-control group. In contrast, the hematocrit in LPS-tolerant rats returned to preshock values after ~3 hours.

After the injection of LPS, monocyte/lymphocyte counts were increased in both groups. This phase was followed by a progressive fall in the circulating monocyte/lymphocyte counts throughout the experimental period (Figure 4). However, the response of the circulating PMNs differed for the two groups (Figure 4): LPS produced an initial disappearance of circulating PMNs in both groups. However, in contrast to the LPS-control group, in which circulating PMN levels remained low throughout the experiment, the LPS-tolerant group showed a conspicuous increase in the circulating counts. At 6 hours after LPS injection, the PMN counts in the LPS-tolerant rats were 16-fold higher than those in LPS-control rats ($p<0.0005$) (Figure 4).

**Degree of Circulating PMN Activation**

There were no differences between the two groups in the percentages of circulating NBT-positive PMNs before LPS injection and during the following hour (Figure 5). The percentage of circulating NBT-positive PMNs in LPS-control rats was increased dramatically and reached peak levels of $33.8\pm19.2\%$ at ~4 hours after LPS injection. In LPS-tolerant rats, the percentage of NBT-positive circulating PMNs remained low throughout the experiment. The differences in the mean values during this period between the two groups were significant ($p<0.005$) (Figure 5).

When the findings are expressed as the absolute number of NBT-positive PMNs, the differences between the LPS-control and LPS-tolerant rats were less pronounced (Figure 5). The basis for this difference was the following: LPS-control rats showed low circulating PMN counts but high percentages of NBT-positive PMNs, whereas in LPS-tolerant rats, the response pattern was inverted (high circulating PMN counts with low percentages of NBT-positive cells).
TNF Activity in Plasma

LPS injection of 9 mg/kg E. coli produced a rapid but transient increase in blood levels of TNF in LPS-control rats. Injection of the same dose of LPS into LPS-tolerant rats resulted in markedly reduced or undetectable TNF activity (Figure 6).

There was a significantly higher percentage of capillaries in the right and left ventricles of the LPS-control group that contained trapped leukocytes (Figure 7B) than in the LPS-tolerant group (p<0.05). A strong positive correlation was found between the fraction of total capillaries without carbon (obstructed) and the fraction of total capillaries with trapped PMNs (Figure 8), suggesting that the obstruction of capillaries primarily involves the PMNs.

In Vitro PMN Activation With LPS

A significantly higher PMN activation (NBT test) was detected after incubation with LPS in vitro in the LPS-tolerant rats when compared with activation in the LPS-control group (Table 1). This trend could be seen in blood samples that were taken before LPS injection and 6 hours later (p<0.05). No significant additional changes in NBT-positive PMNs could be obtained in blood samples that were taken from the two groups of rats 1 hour after LPS injection and then exposed to LPS in vitro.

In Vitro Studies on PMN Activation With Plasma After LPS Injection

Table 2 shows the percentage of NBT-positive PMNs in the blood of an untreated donor rat after in vitro mixing with plasma of LPS-control rats or LPS-tolerant rats. The data show clearly that the plasma of the LPS-tolerant rats induces much less activation in the donor PMNs than the plasma from LPS-control rats.

In Vitro PMN Adherence

Before LPS injection, there was almost no adhesion of PMNs to nylon fibers in both groups. Administration of LPS (9 mg/kg i.v. E. coli) to LPS-tolerant rats did not cause an increase in PMN adherence to
nylon fiber columns. In contrast, in LPS-control rats, there was a significantly greater increase in the fraction of the PMNs that adhered to nylon fibers (Figure 9). When the density of the nylon fiber column is increased to 40 mg nylon fibers/13 mm syringe length, the percentage of PMN adherence increases in both groups: 49.8±14.9% in LPS-control rats ($n=3$) and 8.1±2.3% in LPS-tolerant rats ($n=3$) ($p<0.005$). The latter measurements were made on blood taken 6 hours after LPS injection.

**Discussion**

**Induced Tolerance to LPS**

A state of tolerance to LPS can be induced by LPS concentrations that are 1,000-fold less than those required to induce TNF production. The phenomenon has been characterized by a decrease in LPS-induced TNF mRNA without any change in TNF mRNA half-life. The current study sheds additional light on the mechanisms of LPS tolerance from several angles. Circulating PMNs in LPS-tolerant rats display fundamentally different properties compared with those in LPS-control rats; they are present in the circulation in large numbers but are nonactivated and nonadhesive. Even several hours after LPS administration, the LPS-tolerant rats show no evidence of an activator for circulating PMNs, in sharp contrast to the LPS-control rats.

The LPS-tolerance regimen leads to a reduction in hematocrit. Two mechanisms have been suggested. Repeated LPS administration (several days) produces an anemia in rats by decreasing red blood cell synthesis and by reducing the life span of circulating red blood cells. The reduced adhesion of PMNs to the endothelium in tolerant animals may be associated with a modified endothelial permeability.

**Circulating PMNs**

The number of PMNs in the active circulation after LPS injection represents a balance between the cells that fall out of the circulation through postcapillary margination, physical entrapment in the capillaries, and the rate at which cells are introduced into the circulation from sites of formation (bone marrow) or by demargination. Since LPS has the capacity to stimulate PMNs immediately after its administration there is an almost complete removal of the circulating PMNs that appears to be due to microvascular trapping of PMNs in line with the increase in their membrane adhesion energy. However, after the initial neutropenia (a phenomenon characteristic also for hemorrhagic shock), new PMNs may enter the circulation. The administration of LPS has been found to increase the acute release of granulocyte/macrophage–colony stimulating factor from stromal cells in culture. This factor serves to increase the differentiation and release of bone marrow PMNs.

A substantial difference in the number of circulating PMNs between the two groups becomes apparent shortly after the initial neutropenia. Such differences are striking at 2 hours after LPS injection and become increasingly prominent thereafter, presumably a reflection of the high degree of PMN trapping...
in the microcirculation in the LPS-control rats. This possibility is further supported by the observation that in LPS-control rats the PMNs exhibit a high degree of adherence to nylon fibers, a phenomenon that was found to be paralleled by adhesion to endothelial cells, whereas in LPS-tolerant rats the circulating PMNs exhibited an extraordinarily low level of adhesion. PMN-endothelial interactions involve bidirectional recognition processes. The expression of endothelial leukocyte adhesion molecule 1 and intercellular adhesion molecule 1 located on the endothelial surface enhances PMN adhesion to vascular endothelium. TNF is a potent stimulus for the expression of these adhesion molecules. Since TNF activity was essentially null after LPS injection in tolerant animals, it is possible that the absence in TNF induction of expression of endothelial leukocyte adhesion molecule 1 and intercellular adhesion molecule 1 has contributed to the reduced accumulation of PMNs in the capillary beds. It was concluded that PMN-endothelial adhesive interactions after LPS administration are less expressive in LPS-tolerant rats than in control rats.

Evidence for PMN Activation

A particularly important observation in this study is the low level of spontaneous activation of PMNs in the LPS-tolerant rats. PMN activation appears to be a critical issue in multiorgan failure and survival. In a previous report on an acute form of hemorrhagic shock, we were able to show that survival was strongly linked to the degree of spontaneous PMN activation (NBT test) preceding the trapping of these cells in the microcirculation. Animals with high initial PMN activation have a higher degree of PMN trapping and obstruction of capillaries, whereas those with low activation have a high probability for survival. The current study reinforces the importance of PMN activation in LPS-induced shock.

Injected LPS binds to the plasma membrane of PMNs, producing a direct and potent activation of these cells. Concurrent with the direct activation of PMNs by LPS, an indirect equally important activation has been shown to develop in LPS-control rats via TNF induction as well as by interaction with plasma factors that facilitate the formation of other PMN activators. The latter phenomena occur to a lesser degree in LPS-tolerant rats, possibly because of the markedly reduced, almost undetectable, TNF activity and/or because of the low response to C5a in this tolerant group after LPS injection. Maximum levels of TNF activity in controls were reached ~90 minutes after LPS administration. Grisham et al observed a maximum myeloperoxidase activity 90 minutes after LPS administration, which suggests PMN activation in vivo. However, the NBT test at this stage indicated very low percentages of activated circulating PMNs. An important distinction in this regard derives from the fact that the circulating PMNs sampled shortly after the initial period of neutropenia could not be activated by LPS under in vitro conditions (Table 1).

TABLE 1. Increment in the Percentage of Nitroblue Tetrazolium–Positive Polymorphonuclear Neutrophils After Lipopolysaccharide Addition In Vitro

<table>
<thead>
<tr>
<th>Time</th>
<th>NBT-positive PMNs in blood</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>LPS-control (n=15)</td>
</tr>
<tr>
<td></td>
<td>Unstimulated</td>
</tr>
<tr>
<td></td>
<td>Mean±SD (mm⁻³) (%)</td>
</tr>
<tr>
<td>Before LPS injection</td>
<td>3.9±2.8 65</td>
</tr>
<tr>
<td>1 Hour after LPS injection†</td>
<td>1.8±1.7 17</td>
</tr>
<tr>
<td>6 Hours after LPS injection†</td>
<td>19.6±21.8 135</td>
</tr>
</tbody>
</table>

NBT, nitroblue tetrazolium; PMNs, polymorphonuclear neutrophils; LPS-control, rats pretreated with saline and challenged with lipopolysaccharide (LPS) injection; LPS-tolerant, rats pretreated and challenged with LPS injection; n, number of rats; Unstimulated, unstimulated in vitro; Stimulated, stimulated in vitro with LPS injection (75 µg/ml blood of 0127:B8 E. coli at 37°C for 15 minutes); Δ, increment in NBT-positive PMNs when LPS is added in vitro.

†p<0.05 LPS-control vs. LPS-tolerant.
†9 mg/kg i.v. LPS injection (0127:B8 E. coli).

TABLE 2. Nitroblue Tetrazolium Activation of Donor Polymorphonuclear Neutrophils by Plasma of Rats 6 Hours After Lipopolysaccharide Injection

<table>
<thead>
<tr>
<th></th>
<th>LPS-control plasma (n=12)</th>
<th>LPS-tolerant plasma (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ NBT-positive PMNs (%)</td>
<td>19.9±14.4</td>
<td>1.0±1.5*</td>
</tr>
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</table>

Values are mean±SD. LPS-control, rats pretreated with saline and challenged with lipopolysaccharide (LPS) injection; LPS-tolerant, rats pretreated and challenged with LPS; n, number of rats; NBT, nitroblue tetrazolium; PMNs, polymorphonuclear neutrophils; Δ NBT-positive PMNs, increment in percentage of NBT-positive PMNs in donor blood due to the addition of plasma from the two groups.

*p<0.0005 LPS-tolerant vs. LPS-control plasma.
In LPS-tolerant rats, there was a progressive increase in the number of circulating PMNs after the initial neutropenia (at 30 minutes in Figure 4), whereas in LPS-control rats, the numbers of PMNs remained low throughout the period of observation. Concurrently, there was a conspicuous increase in the degree of circulating PMN activation in LPS-control rats, whereas activation remained low in LPS-tolerant rats. In the case of LPS-control rats, the peak level of NBT-positive PMNs in the circulation was reached at 4 hours after LPS injection at the time when TNF activity had fallen to 5% of its maximum value. Such differences in the PMN counts and degree of circulating PMN activation between the two groups, and in the times required to reach peak TNF activity and the maximum percentage of circulating NBT-positive PMNs, may be due to the presence of PMNs that are freshly released from the bone marrow or from the marginalizing pool. In the case of LPS-control rats, a significant fraction of the recruited PMNs may already be in an activated state, suggesting the interdiction of other plasma activators, produced at different steps in the cascade of events. Parenchymal damage to the intestine during the shock state may be another source of PMN activators, such as intestinal LPS, entering from the gut flora. The intestines were consistently hemorrhagic and edematous in controls at autopsy. In contrast, in the LPS-tolerant rats the intestines had a virtually normal appearance at the time of the study. Greater numbers of PMNs are margined and trapped in the microcirculation in LPS-control rats, as was shown in the histological studies, leading to lower numbers of circulating PMNs.

At 6 hours after the injection of LPS into LPS-tolerant rats, although there was a 16-fold higher level of circulating PMNs, the percentage of circulating PMN activation was eightfold below that in LPS-control rats. No additional PMN activation is seen in LPS-control rats when a blood sample was mixed in vitro with LPS; apparently the majority of activatable circulating PMNs are already in an activated state (Table 1). Finally, plasma from the control group of rats contains a potent activator of PMNs drawn from donor rats (Table 2). These separate observations suggest that plasma activators for PMNs persist in LPS-control rats for at least 6 hours after LPS injection. The situation is completely different in LPS-tolerant rats. The data in the latter are consistent with the hypothesis that plasma activators do not appear in this group since a high percentage of circulating PMNs can be activated under in vitro conditions by LPS, whereas their plasma does not activate PMNs of donor animals.

**TNF Activity**

TNF has been proposed as a primary mediator of LPS-induced injury, which initiates the cascade of events leading to activation and capillary trapping of PMNs and culminates in death. TNF administration to experimental animals has produced lethal shock. Pretreatment of baboons with monoclonal anti-TNF antibodies protects against LPS-induced shock. In the present study, TNF levels in the LPS-tolerant group remained low after LPS challenge. In this context, it is also of interest to note that pretreatment with a methylyanthine derivative (pentoxifylline, Trental), which has been shown not only to reduce the degree of circulating PMN activation but also to inhibit the appearance of TNF in plasma after LPS injection, led to a significant improvement in survival.

**PMNs in the Microcirculation During Shock Induced by LPS**

In the procedure in which carbon particles were infused as markers, the heart was first arrested with the infusion of a modified Ringer's solution (30 meq K⁺). The perfused carbon particles were then able to reach those capillaries that were not obstructed, irrespective of the hemodynamic changes associated with the experimental protocol. The type of capillary obstruction by PMNs was similar to the phenomenon observed after hemorrhagic shock or after local tissue ischemia in the heart. The fact that obstruction occurs exclusively at the capillary level without involvement of arterioles or venules suggests that leukocyte capillary plugging is an important contributory factor to the fatal outcome after LPS administration. Although PMNs also adhere to postcapillary venules, few of these cells remain attached under the conditions of the current reperfusion protocol. Thus, only those PMNs that have been trapped in capillar-
ies persist in the microcirculation after LPS administration. After entrapment, PMNs have the capacity to induce endothelial cell and tissue injury. Since the trapping is a capillary event, no organ is completely spared, and multiple organ failure can develop, depending on the extent of capillary obstruction and on the degree of cell activation. Both of these events are much less prominent in LPS-tolerant rats.

The distribution of nonperfused capillaries was seemingly random; perfused and nonperfused capillaries are often side by side. In none of the obstructed capillaries was there evidence of aggregates of erythrocytes or a swelling of the capillary endothelium to the point of lumen closure (Figure 1). Platelet plugs were likewise not observed in capillaries, a feature that is in accord with observations in the acutely ischemic heart and in the heart after hemorrhagic shock. The extent of capillary obstruction in the heart during the shock state induced by LPS in control rats appears to be similar in magnitude to that seen in the heart after irreversible hemorrhagic shock.

Summary

Injection of normally lethal doses of LPS in tolerant rats produces a situation that is completely different from that occurring in control rats; in line with low levels of plasma activators, only a small fraction of the circulating PMNs are in an activated state. The markedly low TNF activity, the paucity of PMN adherence, and the low degree of circulating PMN activation in the LPS-tolerant group indicate that survival is associated with almost undetectable PMN capillary trapping and high circulating PMN counts.

References

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KEY WORDS • nitroblue tetrazolium test • endotoxic shock • lipopolysaccharide tolerance • leukocyte capillary plugging • tumor necrosis factor
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