Role of Nitric Oxide in the Failure of Neutrophil Migration in Sepsis

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The cecal ligation and puncture (CLP) model was used to investigate whether failure of neutrophil migration occurs in sepsis and whether it correlates with disease outcome. It was observed that the severity of sepsis correlates with the number of punctures in the cecum: mice with 2 punctures (sublethal [SL]-CLP) developed mild peritonitis (100% survived), whereas mice with 12 punctures (lethal [L]-CLP) developed severe peritonitis and bacteremia that evolved to sepsis (none survived). The production of tumor necrosis factor-α, interleukin-1β, and interleukin-10 was higher in L-CLP than in SL-CLP mice. The impairment of neutrophil migration to the peritoneum and to the cecum wall was observed only in L-CLP mice. This phenomenon was shown to be mediated by nitric oxide, because aminoguanidine prevented the failure of neutrophil migration and improved the survival of L-CLP animals. In conclusion, impairment of neutrophil migration is a crucial event in the worsening of sepsis, and nitric oxide seems to be responsible for the phenomenon.

Bacterial invasion of body tissues often leads to organ failure, septic shock, and death despite aggressive surgical intervention, adequate antibiotic therapy, and intensive life support. It has become clear that bacteria and their byproducts (endotoxin and exotoxin) do not injure host tissues directly but act through a variety of inflammatory mediators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and nitric oxide (NO), mainly produced by inflammatory cells. The release of these inflammatory mediators is an essential event in the development of endotoxemic and septic shock [1].

Previous studies have demonstrated that intravenous (iv) administration of endotoxin decreases the migration of neutrophils into the inflammatory sites [2–5]. It seems that impairment of neutrophil migration is mediated by the inflammatory cytokines TNF-α, IL-8, and macrophage-derived neutrophil chemotactic factor (MNCF), which are released by the host cells after stimulation by endotoxin or other bacterial byproducts. Intravenous administration of these cytokines reduced the neutrophil migration induced by different inflammatory stimuli in various animal species [6–9].

Recently, we demonstrated that pretreatment of mice with the NO synthase (NOS) antagonist Nω-monomethyl-L-arginine prevented the TNF-α-, IL-8-, or MNCF-induced neutrophil migration failure toward thioglycolate-challenged peritoneal cavities of mice [10]. These data suggest that NO mediates the impairment of neutrophil migration caused by iv injection of these cytokines. In this context, in vitro and in vivo evidence shows that NO inhibits neutrophil adhesion and that NOS inhibitors increase neutrophil-endothelium adhesion [11–15] as well as migration of these cells to extravascular tissues [13, 15].

Despite early evidence showing that failure of neutrophil migration is observed in endotoxemia [4, 5], it has not yet been investigated whether this process is also observed in septic shock. In an infectious process, the neutrophil migration is extremely important for control of bacterial growth by preventing bacterial dissemination. On the other hand, in endotoxemic shock, neutrophil migration and its activation cause only tissue damage because of the absence of an infectious focus. Thus, in the present study we investigated whether the failure of neutrophil migration occurs during septic shock and the importance of this event to the evolution of sepsis. The cecal ligation and puncture (CLP) model was selected for the investigation because it more closely resembles the course of human sepsis [16, 17]. Because the production of NO is increased during sepsis [18], we also investigated whether it is a mediator involved in the failure of neutrophil migration.

Materials and Methods

Induction of sepsis. For all experiments, male C57BL/6 mice (weight, 18–20 g) were used. The animals were housed in cages in temperature-controlled rooms and received water and food ad libitum. Sepsis was induced through CLP as described elsewhere [16, 19]. Briefly, mice were anesthetized with 2.5% tribromoethanol (diluted in 0.9% saline), a 1-cm midline incision was made on the anterior abdomen, and the cecum was exposed and ligated below the ileocecal junction, without causing bowel obstruction. The cecum was punctured 2, 6, or 12 times with a 21G1/2 × 8–gauge needle, and the cecum was squeezed to observe cecum contents.
expressed through the punctures. Sham-operated animals underwent identical laparotomy but did not undergo cecum puncture and served as controls. The cecum was placed back in the abdomen, and the peritoneal wall and skin incisions were closed. All animals received 1 mL of saline subcutaneously (sc) immediately after the surgery. We chose 2 experimental groups: CLPs with 2 and 12 punctures in the cecum, which were called sublethal CLP (SL-CLP) and lethal CLP (L-CLP), respectively. Induction of peritonitis with 6 punctures was not used further, because we were not interested in a 50% mortality.

All mice undergoing L-CLP developed early clinical signs of sepsis, including lethargy, piloerection, and tachypnea. They were analyzed for the following: survival rate, assessed daily for 5 days; the effect of NO inhibitors on neutrophil migration, survival rate, and cytokine levels; and the effect of NO scavengers on survival rate.

**Bacteremia and number of bacteria in peritoneal cavities.** At given time points (4 and 24 h after CLP), mice were killed, and the peritoneal cavities were washed with sterile saline. For peritoneal lavage, the skin of the abdomen was cut open in the midline after thorough disinfection and without injury to the muscle. Sterile PBS buffer (2 mL) was injected into and aspirated out of the peritoneal cavities. Aliquots of serial log dilutions of these peritoneal lavage fluids were plated on Mueller-Hinton agar dishes (Difco Laboratories, Detroit); colony-forming units were counted after overnight incubation at 37°C, and the results were expressed as the number of colony-forming units per peritoneal cavity. Blood was collected at the same time points under sterile conditions. Ten microliters of blood was diluted 10-fold in brain-heart infusion medium, and the density of the medium was analyzed after 24-48 h at 37°C. The results were graded from – to +++.

**Leukogram.** Animals were anesthetized in an ether chamber, and a sample of blood (diluted in 15% EDTA K3) was collected from the caudal vein at determined time points (2, 4, 8, and 12 h). Total counts were made in a cell counter (Coulter A T Series Analyser; Coulter Corp., Miami), and differential counts were made on slides stained by the May-Grünwald-Giemsa method [20]. The results are expressed as the number of cells per milliliter.

**Neutrophil migration into the peritoneal cavities and to cecum walls.** Neutrophil migration was evaluated at 4 and 24 h after injection. At these time points, the animals were killed in an ether chamber, and the cells present in the peritoneal cavity were collected by injecting 3 mL of PBS containing 1 mM EDTA. Total counts were made in a cell counter (Coulter), and differential cell counts were made on cyt centrifuge slides (Cytospin 3; Shandon Southern Products, Astmoore, UK) stained by the May-Grünwald-Giemsa method. The results are expressed as the number of neutrophils per cavity.

**Cytokine measurements.** The concentrations of TNF-α, IL-1β, and IL-10 in the serum and peritoneal exudates were determined by using a double-ligand ELISA. Briefly, each well of flat-bottomed 96-well microtiter plates was coated with 100 μL of antibody specific to one of the above cytokines at a dilution of 2 μg/mL (TNF-α and IL-1β) or 1 μg/mL (IL-10) in coating buffer and incubated overnight at 4°C. Plates were then washed, and nonspecific binding was blocked for 120 min at 37°C with 1% bovine serum. Samples (nondiluted or diluted 1:2) and standards were loaded onto plates. Recombinant murine (rm) TNF-α, IL-1β, and IL-10 standard curves were used to calculate the cytokine concentrations. The plates were thoroughly washed, and the appropriate biotinylated polyclonal or monoclonal anti-cytokine antibody was added. After 1 h, the plates were washed, avidin-peroxidase (diluted 1:5000) was added to each well for 15 min, and each plate was thoroughly washed again. Next, substrate (0.4 mg of o-phenylenediamine and 0.4 μL of H2O2 in 1 mL of substrate buffer) was added, the reaction stopped with H2SO4 (1 N), and the optical density was measured on an ELISA plate scanner (Spectra Max 250; Molecular Devices, Menlo Park, CA) at 490 nm. The results were expressed as picograms of TNF-α, IL-10, or IL-1β per milliliter of the supernatant or serum, comparing the optical density in the samples with standard curves.

**Histopathology.** To evaluate the neutrophil migration to the

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Table 1. Bacteria in serum and peritoneal fluid of mice undergoing cecal ligation and puncture (CLP), either sham-operated, sublethal (2 punctures; SL-CLP), or lethal (12 punctures; L-CLP).

<table>
<thead>
<tr>
<th>Time point, h after surgery</th>
<th>No. with bacteremia/total (grade)</th>
<th>Bacteria in peritoneal fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Shαm</td>
<td>SL-CLP</td>
</tr>
<tr>
<td>4</td>
<td>0/3 (–)</td>
<td>0/3 (–)</td>
</tr>
<tr>
<td>12</td>
<td>0/5 (–)</td>
<td>1/3 (+)</td>
</tr>
<tr>
<td>24</td>
<td>0/5 (–)</td>
<td>2/4 (+)</td>
</tr>
</tbody>
</table>

NOTE. Bacteremia was graded, ranging from none (–) to ++++. No. of bacteria present in peritoneal cavity is expressed as mean ± SE cfu × 107 per cavity; results are representative of 2 different experiments (n = 3).
Assay for NOS activity. NOS activity was determined in the hearts, lungs, and livers of animals undergoing CLP. Animals were killed at 4 and 12 h after surgery, and these organs were collected and frozen at −70°C until processed for NOS activity, as described elsewhere [21, 22]. Briefly, tissue was homogenized, followed by centrifugation at 100,000 g for 10 min at 4°C. Supernatants were assayed for NOS activity by measuring the conversion of L-[14C]-arginine to [14C]-citrulline as described elsewhere, except that the incubation was carried out at room temperature for 60 min. The protein content of the supernatant was determined by the Coomassie blue binding according to the manufacturer’s recommendations (Coomassie Blue Reagent; Pierce Chemical, Rockford, IL). NOS activity was expressed as picomols of citrulline per milligram of protein per hour.

Measurement of nitrate concentration in urine of sham-operated and CLP animals. The sham-operated, SL-CLP, and L-CLP mice were hydrated orally (1 mL) 2 h after surgery, and 3–4 h after surgery, urine was collected from each animal. The samples were stored in a freezer at −20°C until testing. The nitrate concentration was determined by enzymatically reducing nitrate to nitrite with nitrate reductase, as described elsewhere [23]; the total amount of nitrite was then determined by means of the Griess method. Results are reported as micromolar NO3−. Briefly, 40 μL of urine sample (diluted 1:8) was incubated with an equal volume of the Griess reagent at room temperature. The absorbance at 550 nm was determined with a Titertek Multiskan apparatus (Flow Laboratories, McLean, VA). The NO3− concentration was determined by use of a standard curve of 1–200 μM of NaNO3.

Treatment of CLP mice with the NOS inhibitors aminoguanidine and NO-nitro-L-arginine and with the NO scavenger d-penicillamine. Mice were treated sc with aminoguanidine (10, 30, or 90 mg/kg) 30 min before CLP surgery, and the survival rate, neutrophil migration, and cytokine levels were determined. CLP mice also underwent 3 different schedules of sc aminoguanidine treatment (30 mg/kg) to observe the survival rate: 30 min before surgery, 30 min before and 12 h after surgery, or 12 h after surgery. In other sets of experiments, SL-CLP mice were treated 2 h after surgery with aminoguanidine (90 mg/kg), and the survival rate was determined. Mice were also treated sc with NO-nitro-L-arginine (30 mg/kg) 30 min before CLP surgery, and the survival rate and neutrophil migration were determined. In another set of experiments, 30 min before surgery, CLP mice were pretreated with iv administration of d-penicillamine (20 mg/kg), and the survival rate was analyzed.

Drugs, reagents, and antibodies. The following materials were obtained from the sources indicated. rmIL-1β (lot 63/668; specific activity, 100,000 IU/0.1-μg amuple), rmTNF-α (lot 99/532; specific activity, 200,000 IU/1-μg amuple), purified anti-mouse IL-1β, purified anti-mouse TNF-α, biotinated anti-mouse rmTNF-α (lot 250697), and biotinated anti-mouse IL-1β (lot 250997) were gifts of S. Poole (National Institute for Biological Standards and Control, London). rmIL-10 (417-ML), anti-mouse IL-10 monoclonal antibody (MAB417), and biotinated anti-mouse IL-10 (BAF417) were purchased from R&D Laboratories (Minneapolis). d-penicillamine and NO-nitro-L-arginine were purchased from Sigma (St. Louis) and aminoguanidine from Research Biochemicals International (Natick, MA). All other reagents were purchased from Sigma.

Table 2. Leukograms of normal mice and mice undergoing cecal ligation and puncture (CLP), either sham-operated, sublethal (2 punctures; SL-CLP), or lethal (12 punctures; L-CLP).

<table>
<thead>
<tr>
<th>Group, cell type</th>
<th>Normal</th>
<th>SL-CLP</th>
<th>L-CLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group, cell type</td>
<td>2 h</td>
<td>4 h</td>
<td>8 h</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Normal</th>
<th>Total</th>
<th>Neutrophils</th>
<th>Eosinophils</th>
<th>Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>17.31 ± 2.32</td>
<td>20.53 ± 8.02</td>
<td>13.41 ± 3.31</td>
<td>8.62 ± 4.04</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>4.29 ± 0.54</td>
<td>6.91 ± 3.09</td>
<td>5.12 ± 0.72</td>
<td>5.25 ± 2.95</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.26 ± 0.11</td>
<td>0.32 ± 0.19</td>
<td>0.14 ± 0.07</td>
<td>&lt; 0 ± 0</td>
</tr>
<tr>
<td>Macrophages</td>
<td>12.11 ± 0.34</td>
<td>13.29 ± 4.89</td>
<td>8.15 ± 2.93</td>
<td>3.37 ± 1.12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sham-operated</th>
<th>Total</th>
<th>Neutrophils</th>
<th>Eosinophils</th>
<th>Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>16.02 ± 4.20</td>
<td>6.80 ± 1.54</td>
<td>8.46 ± 1.16</td>
<td>7.17 ± 2.19</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>7.62 ± 2.01</td>
<td>3.49 ± 0.96</td>
<td>5.83 ± 0.89</td>
<td>2.94 ± 0.73</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.12 ± 0.07</td>
<td>0.07 ± 0.00</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Macrophages</td>
<td>8.29 ± 3.41</td>
<td>3.32 ± 0.55</td>
<td>2.62 ± 0.41</td>
<td>4.22 ± 1.46</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SL-CLP</th>
<th>Total</th>
<th>Neutrophils</th>
<th>Eosinophils</th>
<th>Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>15.12 ± 1.51</td>
<td>12.04 ± 3.74</td>
<td>19.40 ± 9.96</td>
<td>12.12 ± 2.88</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>9.12 ± 1.65</td>
<td>6.11 ± 2.10</td>
<td>7.42 ± 2.31</td>
<td>6.43 ± 1.07</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.17 ± 0.17</td>
<td>0.07 ± 0.07</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Macrophages</td>
<td>5.82 ± 0.56</td>
<td>6.22 ± 1.65</td>
<td>11.98 ± 8.26</td>
<td>5.69 ± 1.82</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>L-CLP</th>
<th>Total</th>
<th>Neutrophils</th>
<th>Eosinophils</th>
<th>Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>27.5 ± 3.57</td>
<td>17.7 ± 5.44</td>
<td>20.05 ± 8.04</td>
<td>18.20 ± 7.36</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>15.95 ± 2.74</td>
<td>8.80 ± 3.29</td>
<td>8.19 ± 3.16</td>
<td>6.31 ± 3.64</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.22 ± 0.22</td>
<td>0.13 ± 0.08</td>
<td>0.14 ± 0.10</td>
<td>&lt; 0 ± 0</td>
</tr>
<tr>
<td>Macrophages</td>
<td>11.32 ± 0.74</td>
<td>8.77 ± 2.12</td>
<td>11.72 ± 5.43</td>
<td>11.06 ± 3.73</td>
</tr>
</tbody>
</table>

NOTE. Samples of peripheral blood were taken 2, 4, 8, and 12 h after CLP surgery, and total and differential cell counts were made. Each value is mean ± SE × 106 cells/mL, and results represent data from 2 different experiments (n = 3).

a P < .05 compared with sham-operated group (analysis of variance, followed by Bonferroni’s test).
Results

Mortality following CLP. Figure 1 illustrates the survival curves of mice undergoing CLP with 2, 6, or 12 cecum punctures. Similar to the sham-operated animals, all mice with 2 punctures survived for 5 days after surgery, whereas animals that received 6 or 12 punctures showed 40% or 100% mortality, respectively, at day 2 after surgery. These rates did not change until day 5 after surgery. In the following experiments, the investigated parameters were determined in sham-operated and CLP animals with 2 or 12 punctures, which were designated SL-CLP and L-CLP, respectively.

Bacteremia and peritoneal bacterial infection. Table 1 shows the degree of bacterial infection in blood of the animals in the various experimental groups. The sham-operated animals did not exhibit bacteremia at 4, 12, or 24 h after surgery. In the SL-CLP group, the animals also did not exhibit bacteremia 4 h after surgery and exhibited a mild degree in a few animals at 12 h (1/3) and 24 h (2/4) after surgery. However, all animals in the L-CLP group exhibited a high degree of bacteremia at all times investigated. Furthermore, the number of bacteria present in the peritoneal cavities of L-CLP mice was high compared with that observed in SL-CLP mice: 80-fold at 4 h and 3200-fold at 12 h after surgery.

Leukogram. Table 2 shows the leukogram (total number of leukocytes, neutrophils, eosinophils, and mononuclear cells) of normal, sham-operated, SL-CLP, and L-CLP mice at 2, 4, 8, and 12 h after surgery. Neutrophilia was observed at 2 h after surgery in the L-CLP group. The numbers of other cell types were similar in all experimental groups at investigated times.

Impairment of neutrophil migration. Neutrophil migration into peritoneal cavities of sham-operated, SL-CLP, and L-CLP mice was analyzed at 4 and 24 h after surgery. Significant neutrophil migration, compared with that in sham-operated controls, was observed in SL-CLP mice at both 4 and 24 h after surgery. The migration observed at 24 h after surgery was higher than that observed at 4 h (figure 2). A massive neutrophil migration to the cecum wall tissue in SL-CLP animals compared with sham-operated animals was observed at 4 h after the surgery (figure 3B). Interestingly, in the L-CLP animals, despite the high degree of infection (table 1), the neutrophil migration toward the peritoneal cavity was not statistically different from that observed in the sham-operated animals at either 4 or 24 h after surgery (figure 2). In this group, reduction of neutrophil migration toward the cecum wall tissue was also observed (figure 3C). This reduced migration was not due to neutropenia, because no reduction in neutrophil counts was observed in the blood at 2, 4, 8, or 24 h after surgery in the L-CLP group.

Statistical analysis. The data (except survival curves) are reported as mean ± SE and are representative of 2 or 3 different experiments. The means between different treatments were compared by analysis of variance. If significance was determined, individual comparisons were subsequently tested with Bonferroni’s $t$ test for unpaired values. Statistical significance was set at $P < .05$. Survival rates were expressed as percentages, and a log rank test ($\chi^2$ test) was used to test for differences in survival curves.
Peritoneal fluid and serum cytokine levels following CLP. Figure 4 shows the concentrations of TNF-α, IL-1β, and IL-10 in the peritoneal fluids and serum at 4 and 24 h after surgery in sham-operated, SL-CLP, and L-CLP groups. The TNF-α concentration increased significantly in the exudates and serum of L-CLP animals compared with that in sham-operated animals at 4 and 24 h after surgery. In SL-CLP animals, the concentration of this cytokine also increased significantly in exudate at 4 h after surgery over that in sham-operated mice. The IL-1β concentration increased significantly in the exudate and serum of L-CLP and SL-CLP mice at 4 and 24 h after the surgery, compared with that in sham-operated animals. The IL-10 concentration increased significantly in exudates and serum of L-CLP animals only.

Effect of aminoguanidine on the survival rate and neutrophil migration in CLP mice. After establishing the failure of neutrophil migration toward the infectious site in L-CLP mice, we investigated whether aminoguanidine, a selective inhibitor of inducible (i) NOS, prevents the failure of the neutrophil migration and thus increases the survival rate of the animals in the L-CLP group. As seen in figure 5A, pretreatment of L-CLP animals with aminoguanidine, at sc doses of 10, 30, and 90 mg/kg, 30 min before the surgery prevented the failure of neutrophil migration observed in the L-CLP animals. The degree of prevention was similar with the 3 doses of aminoguanidine used. For comparison, figure 5A also shows the neutrophil migration observed in sham-operated and SL-CLP animals. Figure 5B shows the survival rates of sham-operated, SL-CLP, and L-CLP animals treated with aminoguanidine at doses of 10 and 30 and 90 mg/kg administered 30 min before the surgery. The survival rates of L-CLP animals pretreated with 10 and 30 mg/kg aminoguanidine were 60% and 80%, respectively. On the other hand, the L-CLP animals pretreated with 90 mg/kg showed 100% mortality, the same as that observed in nontreated
L-CLP animals. Furthermore, aminoguanidine administered at a dose of 30 mg/kg 12 h after surgery did not protect the L-CLP animals from lethality. Moreover, the administration of aminoguanidine at 30 mg/kg 30 min before and 12 h after surgery protected only 40% of the L-CLP animals from lethality (figure 6A). The SL-CLP animals posttreated 2 h after surgery with aminoguanidine at 90 mg/kg showed 80% mortality, compared with 100% survival of nontreated SL-CLP animals (figure 6B).

Pretreatment of the L-CLP animals with iv administration of D-penicillamine (20 mg/kg) 30 min before the surgery protected mice from lethality (L-CLP mice, 100% mortality; D-penicillamine-pretreated L-CLP mice, 20% mortality; n = 5).

Effect of L-arginine on the survival rate and neutrophil migration in CLP mice. The pretreatment of L-CLP mice with L-arginine, an inhibitor of the constitutive isoform of NOS, at a dose of 30 mg/kg sc, 30 min before the surgery, did not prevent the failure of neutrophil migration to the peritoneal cavity (sham-operated, 1.2 ± 0.2 × 10⁶ neutrophils/cavity; SL-CLP, 5.0 ± 0.8 × 10⁶ neutrophils; L-CLP, 1.6 ± 0.08 × 10⁶ neutrophils; L-CLP receiving L-arginine, 0.92 ± 0.22 × 10⁶ neutrophils/cavity; L-CLP, 100%; L-CLP receiving L-arginine, 100%) observed in L-CLP animals.

Bacterial counts in the peritoneal cavities of L-CLP animals pretreated with aminoguanidine. Next, we investigated whether the dose of 90 mg/kg aminoguanidine did not prevent the mortality of animals, even though the high dose of the drug is able to inhibit the production of NO for a long time, avoiding the bacterial killing by migrated neutrophils. For this, the mice were pretreated with aminoguanidine at 30 and 90 mg/kg 30 min before the surgery, and bacteria in the peritoneal cavity were counted 4 h after the surgery. Mice pretreated with 90 mg/kg had 10-fold more bacteria in the peritoneal cavity than did mice that received 30 mg/kg aminoguanidine (sham-operated, 0; SL-CLP, 0.03 ± 10⁷ cfu/cavity; L-CLP, 7.64 ± 3.01 × 10⁷; L-CLP receiving aminoguanidine 90 mg/kg, 3.46 ± 1.82 × 10⁷; L-CLP receiving aminoguanidine 30 mg/kg, 0.25 ± 0.5 × 10⁷).

Effect of aminoguanidine pretreatment of L-CLP animals on the production of TNF-α, IL-1β, and IL-10. We then investigated whether the protective effect of aminoguanidine was due to inhibition of cytokine production. Figure 7 shows that sc pretreatment of the L-CLP animals with aminoguanidine (30 mg/kg) 30 min before surgery did not affect TNF-α, IL-1β, or IL-10 levels in either peritoneal exudates or serum.

Evaluation of nitrate concentration in the urine of CLP mice. L-CLP mice had increased levels of NOx 4 h after surgery compared with those in sham-operated and SL-CLP animals (sham-operated, 203 ± 9.62 μM; SL-CLP, 244 ± 52 μM; L-CLP, 522 ± 30 μM).

Figure 5. Effect of aminoguanidine pretreatment of mice undergoing lethal cecal ligation and puncture (12 punctures; L-CLP) on neutrophil migration to the peritoneal cavity (A) and on survival (B). L-CLP mice were pretreated with saline or 10, 30, or 90 mg/kg aminoguanidine, subcutaneously, 30 min before CLP surgery, and neutrophil migration was determined 4 h after. The survival rate was determined daily for 5 days. Neutrophil migration was also determined in sham-operated mice and mice undergoing sublethal CLP (2 punctures; SL-CLP). In A, results are expressed as mean ± SE neutrophils per cavity and are representative of 3 different experiments (n = 5). *P < .05 compared with sham-operated animals; **P < .05 compared with SL-CLP mice; ***P < .05 compared with L-CLP mice without aminoguanidine treatment (analysis of variance, followed by Bonferroni’s test). In B, results are expressed as % survival and are representative of 2 different experiments (n = 5). L-CLP groups treated with saline or with 90 mg/kg aminoguanidine were significantly different from the sham-operated group. The L-CLP group treated with 30 mg/kg aminoguanidine was significantly different from L-CLP mice without aminoguanidine treatment (P < .05; Mantel-Cox log rank test).
Discussion

In the present study, we investigated the failure of neutrophil migration toward the infectious site in sepsis and the importance of this phenomenon to the evolution of the disease. We chose the CLP sepsis model [16] because it closely resembles the course of human sepsis [17]. The severity of sepsis correlated with the number of cecum punctures: mice with 2 punctures (SL-CLP) developed peritonitis with 90%-100% survival, whereas animals with 12 punctures (L-CLP) developed early clinical signs of sepsis, including lethargy, piloerection, and tachypnea, which evolved to septic shock and 100% mortality. Animals with 6 punctures showed the clinical signs of sepsis but with lower intensity, and the survival rate of the group was 60%. Furthermore, the number of bacteria in the peritoneal cavities of L-CLP mice was already elevated at 4 h after surgery and increased progressively at 12 and 24 h after. Also, the infection was not restricted to the peritoneal cavity but spread throughout the body, because all animals had intense bacteremia at sampling times. However, the bacterial count in peritoneal cavities of SL-CLP animals was 80-fold less than that in L-CLP mice at 4 h after surgery, decreasing to undetectable at 24 h after surgery. In association with the survival curves, these data suggest that SL-CLP mice were able to resolve the peritonitis successfully, whereas in L-CLP mice it evolved to sepsis.

The incapacity of the L-CLP animals to restrict the infection in the peritoneal cavity may be due to the impairment of neutrophil migration to the infection focus in these animals. It was observed in this group that, despite the substantial number of bacteria present in peritoneal cavities, the number of neutrophils that migrated to this site was similar to that observed in the sham-operated animals. On the other hand, in SL-CLP mice, in which an impairment of neutrophil migration was not observed, the bacterial infection was restricted to the peritoneal cavities. In these animals, the number of neutrophils that migrated to the peritoneal cavities was significantly higher than in sham-operated mice at 4 and 24 h after surgery (figure 2). Failure of neutrophil migration to the cecum wall tissue of L-CLP animals was also observed (figure 3). The failure of neutrophil migration was not due to neutropenia, because L-CLP mice had levels of circulating neutrophils higher than those observed in sham-operated or SL-CLP mice at 2 h after surgery (table 2). This phenomenon was also not due to hypotension, because the mean arterial pressure of L-CLP mice at 4 h after surgery was similar to that observed in SL-CLP mice (SL-CLP, 85–90 mm Hg; L-CLP, 83–90 mm Hg). Impairment of neutrophil migration has already been described in endotoxemic shock [2, 4, 24] and other diseases in which the risk of secondary infection is high, such as AIDS and diabetes mellitus [25, 26].

It has been suggested that the failure of neutrophil migration observed in endotoxemic animals may be due to the systemic release of such cytokines as TNF-α, IL-8, and MNCF [9], because the iv administration of these cytokines in normal animals mimicked the failure of neutrophil migration observed in en-
Figure 7. Effect of aminoguanidine treatment of mice undergoing lethal cecal ligation and puncture (12 punctures; L-CLP) on tumor necrosis factor (TNF-α), interleukin (IL)-1β, and IL-10 levels in peritoneal exudates and serum. L-CLP animals were treated with aminoguanidine, 30 mg/kg subcutaneously, 30 min before surgery, and TNF-α (A, B), IL-1β (C, D), and IL-10 (E) were measured in peritoneal exudates and serum (except IL-10, which was determined only in exudates) 4 h after surgery. Results are expressed as mean ± SE and are representative of 3 different experiments (n = 4). *P < .05 compared with sham-operated animals (analysis of variance, following by Bonferroni’s test).
sepsis is a product of iNOS, we treated the L-CLP mice with CLP animals (figure 6). To confirm that the NO involved in mortality, compared with 100% survival of the nontreated SL-CLP animals, they had 100% mortality. This latter result reinforced the importance of NO for the microbicidal activity of the neutrophils, as discussed above.

The fact that the iNOS activity in the organs and the nitrate in the urine increased in the L-CLP animals confirmed that NO has been produced during the CLP sepsis. Several other authors also demonstrated the induction of NO production in sepsis [18]. Of note, NO mediates not only the failure of neutrophil migration in sepsis but also the cardiovascular collapse and organ lesions [18, 33]. We did not investigate the mechanism by which NO reduced the ability of neutrophil migration in L-CLP mice, but probably it inhibited neutrophil adhesion in the endothelium. This suggestion is supported by in vivo and in vitro evidence showing that NO inhibitors increase the adhesion of the neutrophils to the endothelial cells [11–15]. Moreover, neutrophils from iNOS −/− mice adhere more avidly to an E-selectin-coated coverslip after lipopolysaccharide treatment than do neutrophils from wild-type control animals [34]. The fact that the production of the cytokines in the L-CLP animals was not affected by their treatment with aminoguanidine (figure 7) suggests that NO is the final mediator involved in the inhibition of neutrophil migration during septic shock. This fact also suggests that, although IL-10 has an antiadhesive effect [29], it is not involved in the reduction of neutrophil migration in CLP. Thus, instead of inducing NO production, it inhibits this mediator [35].

In conclusion, we demonstrated that, in CLP sepsis, the failure of neutrophil migration is an important event in disease pathogenesis and that NO is involved in this phenomenon.

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