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Inhibition of Leukocyte Rolling by Nitric Oxide during Sepsis Leads to Reduced Migration of Active Microbicidal Neutrophils

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We developed two models of sepsis with different degrees of severity, sublethal and lethal sepsis, induced by cecal ligation and puncture. Lethal sepsis induced by cecal ligation and puncture (L-CLP) resulted in failure of neutrophil migration to the infection site and high mortality. Treatment of septic animals with aminoguanidine (AG), a nitric oxide (NO) synthase inhibitor, precluded the failure of neutrophil migration and protected the animals from death. However, cytokine-induced NO synthase (iNOS)-deficient ($iNOS^{-/-}$) mice subjected to L-CLP did not present neutrophil migration failure, but 100% lethality occurred. $iNOS^{-/-}$ mice subjected to sublethal sepsis induced by cecal ligation and puncture (SL-CLP) also suffered high mortality despite the occurrence of neutrophil migration. This apparent paradox could be explained by the lack of microbicidal activity in neutrophils of $iNOS^{-/-}$ mice present at the infection site due to their inability to produce NO. Notably, SL- and L-CLP $iNOS^{-/-}$ mice showed high bacterial numbers in exudates. The inhibition of neutrophil migration by NO is due to inhibition of a neutrophil/endothelium adhesion mechanism, since a reduction in leukocyte rolling, adhesion, and emigration was observed in L-CLP wild-type mice. These responses were prevented by AG treatment and were not observed in the $iNOS^{-/-}$ L-CLP group. There was no significant change in L-selectin expression in neutrophils from L-CLP mice. Thus, it seems that the decrease in leukocyte rolling is due to a defect in the expression of adhesion molecules on endothelial surfaces mediated by iNOS-derived NO. In conclusion, the results indicate that despite the importance of NO in neutrophil microbicidal activity, its generation in severe sepsis reduces neutrophil migration by inhibiting leukocyte rolling and their firm adhesion to the endothelium, in effect impairing the migration of leukocytes and consequently their fundamental role in host cell defense mechanisms.

Sepsis and septic shock represent an intense systemic inflammatory response syndrome (SIRS) with multiple physiological and immunological abnormalities, which is commonly caused by bacterial infection (1, 9). Once the host fails to restrict the invading pathogens to a localized area of tissue, an overwhelming systemic inflammatory response may occur (8). Therefore, the host's response toward the pathogens must be under strict regulation because the consequences of uncontrolled inflammation can be more fatal than the original inciting pathogens (8, 35).

The evolution of SIRS may have resulted from an imbalance in the endogenous production of cytokines. The production of proinflammatory cytokines at the infection site is important to the recruitment and activation of leukocytes, which mediate local host defenses (3, 14, 16). On the other hand, high levels of the same proinflammatory cytokines in the circulation result in SIRS with multiorgan dysfunction syndrome, culminating in an increase in the morbidity and mortality of individuals (7, 34, 52).

Some of the deleterious and beneficial effects of cytokines have been ascribed to the release of nitric oxide (NO), the production of which is catalyzed by cytokine-induced nitric oxide synthase (iNOS) in leukocytes (7, 13). NO by itself, and the products yielded by its interaction with other reactive oxygen intermediates, plays a crucial role in the microbicidal activity of leukocytes against a great number of pathogens, including gram-positive and gram-negative bacteria (18, 30, 33). However, the overproduction of NO in circulation has been implicated in several disorders observed in sepsis, such as vascular relaxation associated with hypotension (54, 48), refractoriness to vasopressor catecholamines (6, 46), and organ lesions (17, 38). Indeed, iNOS inhibitors prevent the decrease in systemic vascular resistance and unresponsiveness to catecholamines induced by experimental endotoxemia (29) and in patients with septic shock (42). Furthermore, iNOS-deficient ($iNOS^{-/-}$) mice subjected to cecal ligation and puncture (CLP) showed improved microvascular catecholamine responsiveness and survival compared with wild mice (28).

Early studies from our laboratory demonstrated that failure of neutrophil migration to the inflammatory site is observed in severe sepsis induced by endotoxemia and by CLP (5, 44). The outcome for severely septic animals correlated with failure of neutrophil migration to the infection site (5). Investigating the mechanism involved in this phenomenon, we observed that inhibitors of nitric oxide synthase (NOS), such as aminoguanidine (AG) and N^G-mono-methyl-L-arginine, protected animals from impairment of neutrophil migration, suggesting that NO mediates this phenomenon (5, 50). Accordingly, NO reduces leukocyte-endothelial cell adhesion (25, 26, 27). In the CLP-sepsis model, treatment of mice with AG at doses of up to

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30 mg kg^{-1} were shown to protect animals from lethality. On the contrary, the administration of 90 mg kg^{-1}, despite preventing the failure of neutrophil migration, did not abolish the lethal effect. It was suggested that a high dose of a selective iNOS inhibitor abrogates the microbicidal activity for an extended period of time, allowing enough bacterial replication to cause death (5).

In the present study, we used $iNOS^{-/-}$ mice and aminoguanidine-treated wild-type mice undergoing CLP to clarify this paradoxical effect of NO in sepsis, in which NO plays a key role in the microbicidal activity of the neutrophil present at the infection site but inhibits the migration of this cell type towards its target. Also, we investigated the NO mechanisms involved in the failure of neutrophil migration towards the infection focus, examining whether NO reduces neutrophil-endothelium adhesion mechanisms.

MATERIALS AND METHODS

Mice. C57BL/6 (wild-type) mice from the Animal Facility of the Faculty of Medicine of Ribeirão Preto, University of São Paulo, and C57BL/6 $iNOS^{-/-}$ mice purchased from Jackson Laboratories (Bar Harbor, Maine), both comprised of males weighing between 18 and 22 g, were used in this study. The animals were housed in cages in temperature-controlled rooms and received water and food ad libitum. The $iNOS^{-/-}$ mice were housed in a sterile laminar flow cabinet until experiments were conducted.

All experiments were conducted in accordance with the ethical guidelines of the School of Medicine of Ribeirao Preto, University of São Paulo, São Paulo, Brazil.

Animal model. Sepsis was induced through CLP as described elsewhere (4, 53). Briefly, mice were anesthetized with tribromoethanol (250 mg kg^{-1}, diluted in saline), a 1-cm midline perturbance 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 two times with a 24-gauge needle or 14 times with a 21-gauge needle, and the cecum was squeezed to allow cecum contents to be expressed through the punctures. Sham-operated animals underwent identical laparotomy but without cecum puncture and served as controls. The cecum was placed back in the abdomen, and the peritoneal wall and skin incision were closed. All animals received 1 ml of saline subcutaneously immediately after the surgery.

The sublethal-CLP (SL-CLP) and lethal-CLP (L-CLP) groups consisted of animals with 2 and 14 punctures in the cecum, respectively. All mice subjected to L-CLP developed early clinical signs of sepsis, including lethargy, piloerection, and tachypnea. Some L-CLP animals were treated with the iNOS inhibitor AG at 30 mg kg^{-1} subcutaneously 30 min before the CLP surgery. The SL-CLP and L-CLP animals were examined for survival rate (assessed daily for 5 days), neutrophil migration to the peritoneal cavity, number of bacteria in exudates from the peritoneal cavity, cytokine levels in the peritoneal exudates, leukocyte rolling, adhesion, and migration to the mesentery, and expression of CD62L and CD18 in peripheral blood neutrophils.

Neutrophil migration in the peritoneal cavity. Neutrophil migration was assessed 4 h after CLP. The animals were killed, and the cells present in the peritoneal cavity were harvested by introducing 3 ml of phosphate-buffered saline (PBS) containing 1 mM EDTA. Total counts were performed with a cell counter (Coulter A^C T series analyzer; Coulter Corp., Miami, Fla.), and differential cell counts were carried out on cytocentrifuge slides (Cytospin 3; Shandon Southern Products, Astmoore, United Kingdom) stained by the May-Grünwald-Giemsa (Rosenfeld) method. The results are expressed as the number of neutrophils per cavity.

Number of bacteria in peritoneal cavity and in cecum luminal content. At given times (4 and 24 h after CLP), animals were killed, and the peritoneal cavity was washed with sterile saline. For peritoneal lavage, the skin of the abdomen was opened at the midline after thorough disinfection and without injury to the muscle. Sterile PBS buffer (3 ml) was injected into and aspirated out of the peritoneal cavity. Aliquots of serial log dilutions of the peritoneal lavage fluid were plated on Mueller-Hinton agar dishes (Difco Laboratories, Detroit, Mich.). CFU were counted after overnight incubation at 37°C, and the results were expressed as the number of CFU $cavity^{-1}$.

To determine the number of bacteria in the cecum luminal content of $iNOS^{-/-}$ and wild-type mice, the cecum wall was opened and the cecum luminal content was collected. The content was weighed, and a 10% suspension was prepared in sterile saline. Aliquots of serial log dilutions of the suspensions were plated on Mueller-Hinton agar dishes (Difco Laboratories, Detroit, Mich.). CFU were counted after overnight incubation at 37°C, and the results were expressed as the number of CFU (mg of cecum luminal content)$^{-1}$. All procedures were done under sterile conditions.

Cytokine measurements. The concentrations of tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), and IL-10 in the peritoneal exudates were determined by using a double-ligand enzyme-linked immunosorbent assay (ELISA). Briefly, flat-bottomed 96-well microtiter plates were coated with 100 μl per well of antibody specific to one of the above cytokines at a dilution of 2 μg ml^{-1} (TNF-α and IL-1β) or 1 μg ml^{-1} (IL-10) of coating buffer and incubated overnight at 4°C. After, the plates were washed, and nonspecific binding was blocked for 120 min at 37°C with 1% bovine serum. Samples (nondiluted and diluted 1:2) and standards were loaded in plates. Recombinant murine 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 anticytokine antibodies were added. The plates were washed 1 h later, avidin-peroxidase (diluted 1:5,000) was added to each well for 15 min, and each plate was thoroughly washed again. After, substrate (0.4 mg of *o*-phenylenediamine [OPD] plus 0.4 μl of H_2O_2 for 1 ml of substrate buffer) was added, the reaction was stopped with H_2SO_4 (1 M), and the optical density was measured on an ELISA plate scanner (Spectra Max 250; Molecular Devices) at 490 nm. The results were expressed as picograms of TNF-α, IL-10, and IL-1β per milliliter of supernatant or serum, comparing the optical density in the samples with standard curves.

RNA extraction and RT-PCR analysis. RNase-free plasticware and solutions were used throughout the procedure. Peritoneal macrophages (4×10^6 cells) obtained from $iNOS^{-/-}$ and wild-type mice were homogenized in 1.0 ml of Trizol reagent (Gibco-BRL-Life Technologies, Grand Island, N.Y.), and the extraction of total RNA was performed as described elsewhere (49). Afterward, 1 μg of total RNA was reverse transcribed using Superscript II reverse transcriptase (Gibco-BRL-Life Technologies). The cDNA was then used for specific amplification of iNOS and β-actin mRNAs using *Taq* DNA polymerase (5 U μl^{-1}). The primers (sense and antisense), shown from 5′ to 3′, as well as the number of cycles and expected sizes of the PCR products used in our reverse transcription (RT)-PCR, were as follows: sense, CATGGCTTGCCCCTCTGA TGGTGCCAYTCG, and antisense, GCAGCATCCCCTCTGATGGTGCCAT CG, 35 cycles and 754 bp; β-actin: sense, TGGAATCCTGTGGCATCCATGA AAC, and antisense, TAAAACGCAGCTCAGTAACAGTCCG, 25 cycles and 349 bp (21). After an initial incubation at 95°C for 3 min, temperature cycling was started with each cycle as follows: 95°C for 1 min, 54°C for 1 min, and 72°C for 2 min (21).

Detection of PCR products. The 4 μl of the final reaction mix with 3 μl of 5× TBE (Tris-borate-EDTA) was run at 120 V for 45 min on a 6% polyacrylamide gel. Thereafter, the gel was stained with silver.

Measurement of nitrite (NO_2^-) concentration in supernatant from macrophages obtained from iNOS-deficient mice and wild-type mice. Wild-type and $iNOS^{-/-}$ mice were sacrificed, and their peritoneal cells were harvested. Cells were seeded (2×10^5 cells) in 96-well plates and incubated for 1 h at 37°C in a 5% CO_2 atmosphere. Thereafter, the plates were washed, and the adherent macrophages were incubated with medium with and without lipopolysaccharide (LPS) (200 ng ml^{-1}) plus recombinant murine gamma interferon (rmIFN-γ) (200 IU ml^{-1}) for 12, 24, and 48 h for further analyses of NO_2^- in the supernatants. The samples were stored at −20°C until time of dosage. The nitrite concentration in the supernatants was determined using the Griess method (23). Briefly, 50 μl of supernatant samples was incubated with an equal volume of the Griess reagent at room temperature. The absorbance was measured on a plate scanner (Spectra Max 250; Molecular Devices, Menlo Park, Calif.) at 540 nm. The NO_2^- concentration was determined using a standard curve for 1 to 200 μM $NaNO_2$.

Intravital microscopy of leukocytes to assess rolling, adhesion, and migration to the mesentery. The leukocyte parameters were examined as previously described (2, 20, 43). Briefly, mice were anesthetized with an intraperitoneal injection of 40 mg of sodium pentobarbital kg^{-1}. The mesenteric tissue was exteriorized for microscopic examination in situ. This was performed through a longitudinal incision of the skin and abdominal muscle on the right side of the body and then exposure of the mesentery. The preparation was not affected by respiratory movements of the animals, and its microcirculatory characteristics remained basically invariant throughout the course of the experiment. The animals were maintained on a special board thermostatically controlled at 37°C, with a transparent platform on which the tissue to be transilluminated was

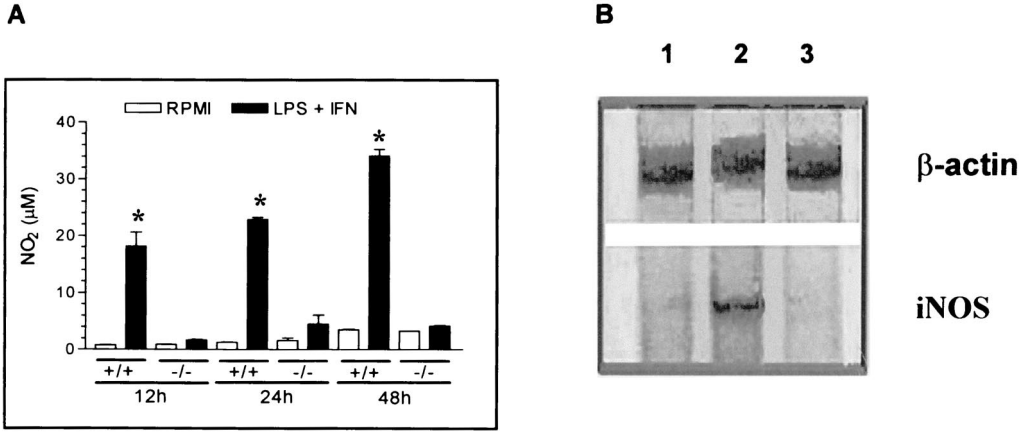

FIG. 1. Lack of NO_2^- production and absence of detection of iNOS mRNA in iNOS$^{-/-}$ mice. (A) Concentration of NO_2^- in the supernatant of macrophages stimulated with RPMI medium and LPS (200 ng ml^{-1}) plus IFN-γ (200 IU ml^{-1}) and incubated for 12, 24, and 48 h. The results were expressed as means ± SEM of quadruplicates. *, $P < 0.05$ compared with RPMI group (control) (analysis of variance, followed by Bonferroni's test). (B) iNOS mRNA products of 754 bp were obtained after amplification using total RNA from peritoneal macrophages. Lane 1, nonstimulated macrophages from wild-type mice; lanes 2 and 3, macrophages from wild-type and iNOS$^{-/-}$ mice, respectively, stimulated with LPS (200 ng ml^{-1}) plus IFN-γ (200 IU ml^{-1}) for 6 h. The same result was repeated two times, and 10 wild-type and iNOS$^{-/-}$ animals were used to obtain peritoneal macrophages in each experiment.

placed. The preparation was kept moist and warm by irrigating the tissue with warmed (37°C) Ringer Locke's solution, pH 7.2 to 7.4, containing 1% gelatin.

A 500-line television camera was incorporated onto a triocular Zeiss microscope to facilitate observation of the enlarged image (×3,400) on the video screen. Images were recorded on a video recorder with a ×40 long-distance objective with a 0.65 numerical aperture. An image-splitting micrometer was adjusted to the phototube of the microscope as described by Baez (2). The image splitter sheared the optical image into two separate images and displaced one with respect to the other. By rotating the image splitter in the phototube, the shearing was maintained at right angles to the axis of the vessel. The displacement of one image from the other allowed measurement of the vessel diameter. Vessels selected for study were third-order venules, defined according to their branch-order location within the microvascular network. These vessels corresponded to postcapillary venules, with a diameter of 12 to 18 μm.

The interaction of leukocytes with the luminal surface of the venular endothelium was studied in a segment of the vessel. Rolling leukocytes (rollers) were defined as those white blood cells that moved at a lower velocity than erythrocytes in the same stream. The number of rolling leukocytes was determined at 10-min intervals. These leukocytes moved at a sufficiently slow pace to be individually visible and were counted as they rolled past a 100-μm length of venule (20). Rolling was assessed 3 h after CLP surgery. A leukocyte was considered to be adherent to the venular endothelium if it remained stationary for >30 s (22). The number of adherent cells (stickers) was expressed as the number per 100-μm length of venule. Leukocyte adhesion was investigated 3 h after CLP surgery. To assess leukocyte transmigration, the number of cells that accumulated in a 2,000-μm^2 standard area of connective tissue adjacent to a postcapillary venule was determined. Cells were counted in the recorded image, using five different fields for each animal to avoid variability due to sampling. Data were then averaged for each animal. Leukocyte emigration was determined 3 h after CLP surgery.

Flow cytometric analyses. Cell surface antigen immunostaining was performed as previously described (55). Leukocytes from peripheral blood of both wild-type and iNOS$^{-/-}$ mice subjected to CLP and from wild-type mice subjected to L-CLP and treated with AG (30 mg kg^{-1}, 30 min before surgery) were blocked with 10% normal goat serum for 20 min at 4°C and then stained with monoclonal antibody for 30 min at 4°C (anti-CD62L and anti-CD18). Cells stained with the appropriate isotype-matched fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-labeled monoclonal antibody immunoglobulin G (IgG) but of irrelevant specificity (Pharmingen, Mississauga, Canada) were used as negative controls. After being stained, the cells were fixed with 2% paraformaldehyde and analyzed with a FACSort flow cytometer (Becton Dickinson, San Jose, Calif.). Control experiments with purified peripheral blood neutrophils and monocytes were analyzed by flow cytometer in order to determine the appropriate gate of neutrophils.

Drugs, reagents, and antibodies. The following materials were obtained from the sources indicated: rmIL-1β (lot 63/668, specific activity of 100,000 IU per 0.1-μg ampoule), rmTNF-α (lot 99/532, specific activity of 200,000 IU per 0.1-μg ampoule), anti-mouse IL-1β purified antibody, anti-mouse TNF-α purified antibody, biotinylated anti-mouse rmTNF-α antibody (lot 250697), and biotinylated anti-mouse IL-1β antibody (lot 250997) were gifts from S. Poole (National Institute for Biological Standards and Control, London, United Kingdom); rmIL-10 (417-ML), anti-mouse IL-10 monoclonal antibody MAB417, and biotinylated anti-mouse IL-10 antibody BAF417 were purchased from R&D; aminoguanidine was obtained from RBI; and all other reagents were purchased from Sigma.

Statistical analysis. The data (except for the survival curves) are reported as the means ± standard errors of the means (SEM) of values obtained from three 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. Some results were analyzed by the unpaired Student t test. Statistical significance was set at a P of <0.05. The survival rate was expressed as the percentage of live animals, and a log rank test (χ^2 test) was used to determine differences in survival curves.

RESULTS

Absence of NO production in iNOS$^{-/-}$ mice. Macrophages obtained from iNOS$^{-/-}$ and wild-type mice were assayed for NO_2^- production and iNOS mRNA expression. It was observed that, unlike with macrophages obtained from wild-type mice, the macrophages obtained from iNOS$^{-/-}$ mice stimulated with LPS (200 ng ml^{-1}) plus IFN-γ (200 IU ml^{-1}) were unable to produce NO_2^- (Fig. 1A). Panel B of the same figure shows that macrophages obtained from iNOS$^{-/-}$ mice 6 h after stimulation were negative for iNOS mRNA (lane 3) compared with stimulated macrophages obtained from wild-type mice (lane 2). Nonstimulated macrophages of wild-type mice expressed almost undetectable mRNA for iNOS (lane 1).

Neutrophil migration to peritoneal cavity and survival rate of wild-type and iNOS$^{-/-}$ mice subjected to CLP. Neutrophil migration into the peritoneal cavity in sham-operated and SL- and L-CLP mice was determined at 4 h after surgery. Com-

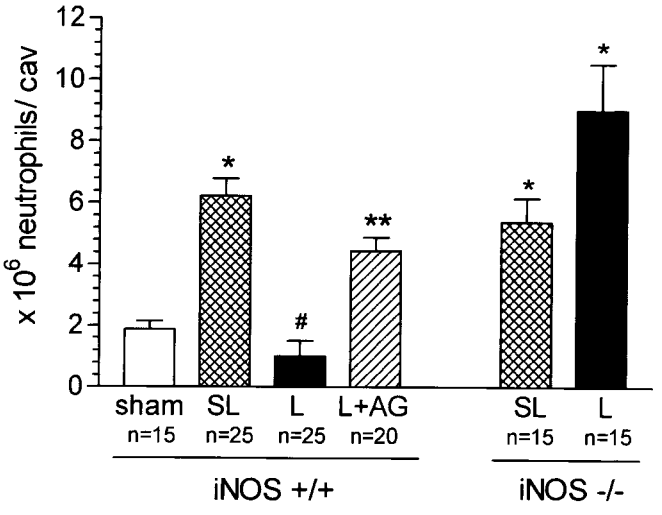

FIG. 2. Neutrophil migration into the peritoneal cavity in wild-type and iNOS$^{-/-}$ mice subjected to SL- and L-CLP. Assessment of neutrophil migration into the peritoneal cavity was performed 4 h after surgery. Wild-type mice subjected to L-CLP were treated 30 min before the surgery with saline and 30 mg of AG kg^{-1} subcutaneously. Results are expressed as mean numbers of neutrophils per cavity ± SEM. *, $P < 0.05$ compared with sham-operated animals; #, $P < 0.05$ compared with wild-type SL-CLP group; **, $P < 0.05$ compared with wild-type L-CLP group (analysis of variance, followed by Bonferroni's test).

pared to sham-operated wild-type mice, significant neutrophil migration into the peritoneal cavity was observed in SL-CLP wild-type and iNOS$^{-/-}$ mice. L-CLP wild-type mice presented with impaired neutrophil migration into the peritoneal cavity, which was prevented by AG treatment of these animals. The L-CLP iNOS$^{-/-}$ mice also did not present with neutrophil migration failure (Fig. 2).

Upon investigation of the mortality of these animals, it was shown that although wild-type and iNOS$^{-/-}$ mice subjected to SL-CLP presented similar neutrophil migration into the peritoneal cavity, the iNOS$^{-/-}$ animals suffered 60% mortality up to 5 days after surgery, whereas the wild-type mice showed 100% survival. With regard to L-CLP mice, both wild-type and iNOS$^{-/-}$ animals showed 100% mortality, despite the fact that the iNOS$^{-/-}$ mice did not show failed neutrophil migration. Among the iNOS$^{-/-}$ mice, there was 100% lethality on the first day after CLP surgery. On the other hand, wild-type mice that had undergone L-CLP and received 30 mg of AG kg^{-1} 30 min prior to surgery showed only 20% mortality (Fig. 3).

Bacterial counts in the peritoneal cavity and in the cecum luminal content of wild-type and iNOS$^{-/-}$ animals after CLP. Next, we investigated the number of bacteria in the peritoneal cavity of iNOS$^{-/-}$ and wild-type mice that underwent SL-CLP and L-CLP surgery. It was found that 4 h after surgery, iNOS$^{-/-}$ SL-CLP mice already presented significantly higher numbers of bacteria in the peritoneal cavity than were observed in wild-type mice. At 24 h postsurgery, the number of bacteria in the peritoneal cavity increased significantly (compared with 4 h) in SL-CLP iNOS$^{-/-}$ mice and was extremely higher than that observed in SL-CLP wild-type mice, which controlled the infection (no detection of bacteria in the peritoneal cavity). In the L-CLP group, a large number of bacteria were observed in the peritoneal cavity of both wild-type and iNOS$^{-/-}$ mice at 4 h after surgery (Fig. 4). The number of bacteria at 24 h in L-CLP mice was not determined because of the death of the majority of the mice (60 to 80%). It is important to note that before the surgery, the number of bacteria in the cecum luminal content of iNOS$^{-/-}$ mice was similar to that observed in the wild-type mice (iNOS$^{-/-}$, 1.5×10^3, versus wild-type, 1.05×10^3 CFU per mg of cecum luminal content).

Cytokine levels in peritoneal exudates following CLP. Figure 5 shows the concentrations of TNF-α, IL-1β, and IL-10 in the washed peritoneal exudate 4 h after surgery in the sham-operated and SL- and L-CLP groups for both wild-type and iNOS$^{-/-}$ mice. The concentrations of TNF-α, IL-1β, and IL-10 in wild-type and iNOS$^{-/-}$ SL-CLP mice were not statistically different from those observed in sham-operated animals. However, the concentrations of these cytokines increased sig-

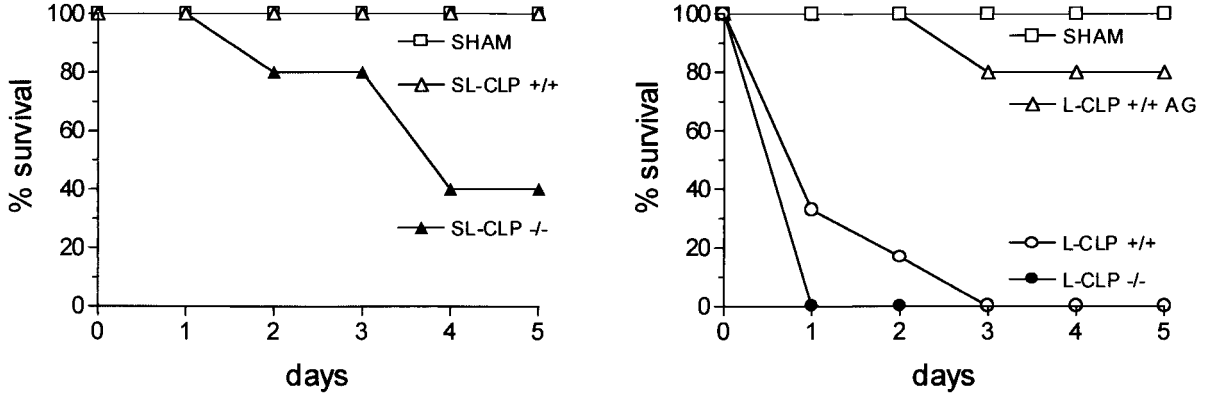

FIG. 3. Survival of wild-type and iNOS$^{-/-}$ mice after SL- and L-CLP. (A) The survival rates of sham-operated ($n = 25$), wild-type ($n = 15$), and iNOS$^{-/-}$ SL-CLP ($n = 15$) mice were determined daily up to 5 days after surgery. The iNOS$^{-/-}$ SL-CLP group was significantly different from sham-operated and wild-type SL-CLP mice. $P < 0.05$, Mantel-Cox log rank test. (B) The survival rates of wild-type mice treated subcutaneously with saline ($n = 30$) and AG (30 mg kg^{-1}, 30 min prior) ($n = 15$) and iNOS$^{-/-}$ mice ($n = 15$), both subjected to L-CLP surgery, were determined daily up to 5 days after surgery. The wild-type and iNOS$^{-/-}$ L-CLP mice were significantly different from sham-operated and wild-type L-CLP mice treated with AG. $P < 0.05$, Mantel-Cox log rank test. Results are expressed as percent survival.

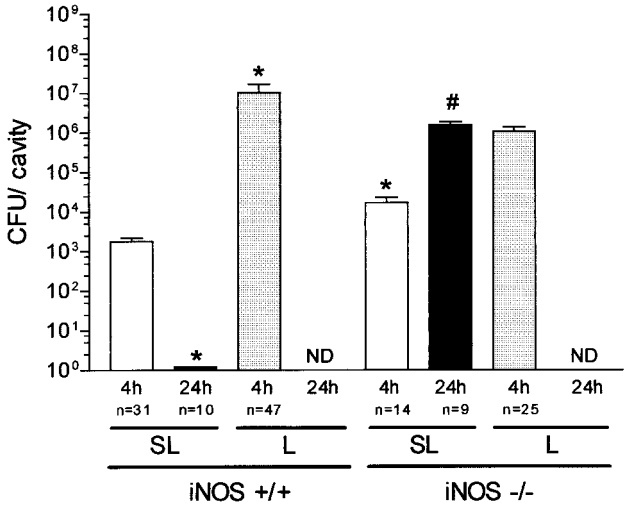

FIG. 4. Bacterial counts in the peritoneal fluid of wild-type and iNOS$^{-/-}$ mice subjected to SL- and L-CLP. Quantification of the amount of bacteria in the peritoneal cavity was performed 4 and 24 h after SL-CLP and 4 h after L-CLP surgery. The number of bacteria present in the peritoneal cavity is expressed as mean CFU per cavity. The numbers of animals in the different experimental groups are indicated below the bars. ND, value not determined because the animals died before this time. *, $P < 0.05$ compared with wild-type SL-CLP group/4 h; #, $P < 0.05$ compared with iNOS$^{-/-}$ SL-CLP group/4 h.

nificantly in the exudates of wild-type and iNOS$^{-/-}$ L-CLP compared with sham-operated mice. The increases in the concentrations of IL-1β and IL-10 were less in iNOS$^{-/-}$ than in wild-type animals.

Leukocyte-endothelium interaction (rolling, adhesion, and transmigration) in animals subjected to CLP. Leukocyte-endothelium interaction (rolling, adhesion, and transmigration) was examined in the mesenteric postcapillary venules with resting diameters ranging from 12 to 18 μm. Figure 6 shows that there was no increase in the rolling of leukocytes in wild-type SL-CLP mice compared with that in the sham-operated mice. Leukocyte rolling in the sham-operated group was probably induced by manipulation during preparation of the tissue for the intravital microscopy assay. However, a significant decrease in the number of rolling cells (rollers) in L-CLP wild-type mice was observed. On the other hand, wild-type L-CLP animals treated with AG (30 mg kg^{-1}) and iNOS$^{-/-}$ L-CLP mice did not present reduced leukocyte rolling. In fact, the latter group had an increase in rolling values compared with the SL-CLP group.

With regard to leukocyte adherence and migration, we observed increases in endothelium-leukocyte adhesion and in extravascular leukocyte accumulation in SL-CLP mice. On the other hand, decreased values for these parameters were found in the L-CLP wild-type mice compared with those in the SL-CLP animals. The values for these parameters were not reduced in the wild-type L-CLP mice treated with AG or in the iNOS$^{-/-}$ L-CLP mice (Fig. 6).

Expression of CD62L and CD18 in blood neutrophils obtained from CLP mice. Since the data described above suggest that NO released during L-CLP surgery mediates the reduction of rolling and adhesion of leukocytes when interacting

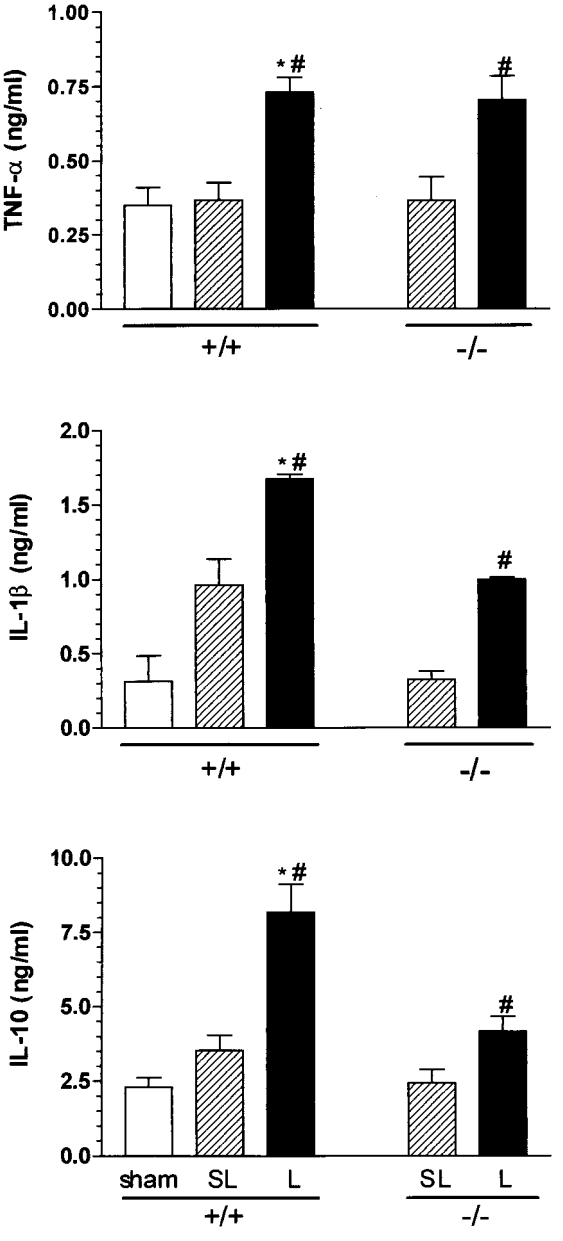

FIG. 5. TNF-α, IL-1β, and IL-10 levels in mice subjected to CLP. The cytokine levels in peritoneal exudates were determined at 4 h after surgery in sham-operated, wild-type, and iNOS$^{-/-}$ CLP mice. Results are expressed as means ± SEM, and each group had 15 mice. *, $P < 0.05$ compared with sham-operated animals; #, $P < 0.05$ compared with SL-CLP animals (analysis by unpaired Student's t test).

with endothelial cells, we examined whether the neutrophils obtained from L-CLP animals showed alteration in the expression of CD62L (L-selectin) and CD18 (β_2 integrin). The analyses were performed in the polymorphonuclear cell gate of the FACSort previously defined by using isolated neutrophils from murine blood.

Figure 7 (left panels) shows that polymorphonuclear cells obtained from wild-type L-CLP mice 2 h after the surgery presented a light reduction but not a significant change in CD62L expression compared to SL-CLP and sham-operated

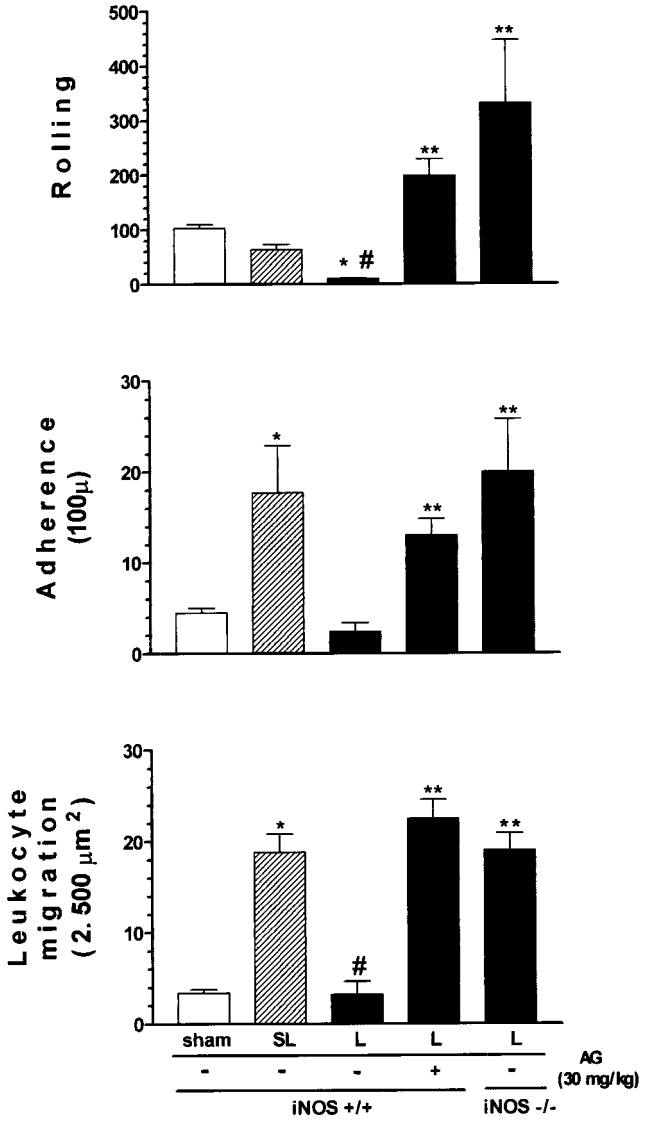

FIG. 6. Leukocyte rolling, adherence, and migration to mesentery in wild-type and iNOS$^{-/-}$ mice subjected to SL- and L-CLP. Bars show the number of rolling, adherent, and migrating leukocytes in postcapillary venules of mesentery, using an in vivo intravital microscopy assay. One group of L-CLP mice received 30 mg of AG kg^{-1} subcutaneously 30 min before surgery. Sham-operated animals served as controls. The parameters were evaluated 3 h after the surgery. The results are expressed as mean numbers of leukocytes ± SEM, and each group had 15 mice. *, $P < 0.05$ compared with sham-operated animals; #, $P < 0.05$ compared with wild SL-CLP group; **, $P < 0.05$ compared with wild L-CLP group (analysis of variance, followed by Bonferroni's test).

animals (second histogram from the top). The polymorphonuclear cells obtained from wild-type L-CLP mice treated with AG and from iNOS$^{-/-}$ L-CLP mice (third and fourth histograms, respectively) also did not display a significant change in the expression of CD62L.

The results for CD18 expression are shown in the right panels of Fig. 7. These data show that CD18 expression on the neutrophil surface increased significantly in SL-CLP wild-type mice compared to sham-operated mice. Moreover, the expression of this molecule on the plasma membrane of neutrophils from wild-type L-CLP mice was significantly reduced compared with sham-operated and SL-CLP (second histogram from the top). This reduction was prevented by treatment of the wild-type L-CLP animals with AG (third histogram), and it was not observed in iNOS$^{-/-}$ mice subjected to L-CLP (fourth histogram).

DISCUSSION

Neutrophil migration to infection loci is extremely important for the local control of bacterial growth and consequently for the prevention of bacterial dissemination. The importance of this phenomenon in the evolution of sepsis has been clearly demonstrated. It has been shown that the failure of neutrophils to migrate to the infection site in lethal sepsis is accompanied by an increased number of bacteria in the peritoneal fluid and in the circulation and by a high mortality rate of the host (5, 36). It seems that NO mediates the failure of neutrophil migration, since it was shown to be prevented by an adequate treatment regimen in CLP-septic animals using a selective inducible NOS inhibitor, AG (30 mg kg^{-1}) (24). Consequently, there was a reduction in the number of bacteria at the infection site and in the circulation and a decrease in the mortality rate. However, the pharmacological inhibition of iNOS with AG (90 mg kg^{-1} and two doses of 30 mg kg^{-1}) for extended periods of time prevented neutrophil migration failure but did not allow the animals to escape death. The possible reason for these apparently conflicting results is that, despite the presence of neutrophils at the infection site, they are unable to kill bacteria due to marked inhibition of NO production (5).

To clarify this apparent paradoxical effect of NO and to determine the mechanism by which it mediates the impairment of neutrophil migration to the infection site, we studied the results of lethal and sublethal sepsis induced in iNOS$^{-/-}$ and wild-type mice. The results demonstrated that NO produced by iNOS mediates the failure of neutrophil migration observed in lethal sepsis, since, in contrast to wild-type mice, iNOS$^{-/-}$ mice subjected to L-CLP surgery did not present with failure of neutrophil migration, determined 4 h after surgery (Fig. 2). Failure of neutrophil migration in wild-type L-CLP mice was also observed at 8, 12, and 24 h after surgery (data not shown).

The mortality in iNOS$^{-/-}$ mice caused by CLP (Fig. 3) could be explained by their inability to control the growth of bacteria at the infection site despite the presence of neutrophils. This was a consequence of the lack of NO production, which is one of the major mediators of neutrophil microbicidal activity (18, 30, 33). In fact, the bacterial number 4 h after surgery in peritoneal exudates of iNOS$^{-/-}$ SL-CLP mice was higher than that observed in wild-type mice. Furthermore, 24 h after surgery, the wild-type SL-CLP mice controlled the infection, whereas the iNOS$^{-/-}$ mice presented elevated numbers of bacteria in the cavity.

Concerning the L-CLP groups, a large number of bacteria were observed in both wild-type and iNOS$^{-/-}$ animals (Fig. 4). Although both groups were not able to control the infection, it seems that the reasons are different. As described above, in iNOS$^{-/-}$ mice, the inability to control the infection is due to the incapacity of the neutrophils present in the infectious site to produce NO. However, the reason in the wild-type mice is the failure of neutrophil migration to the infectious focus.

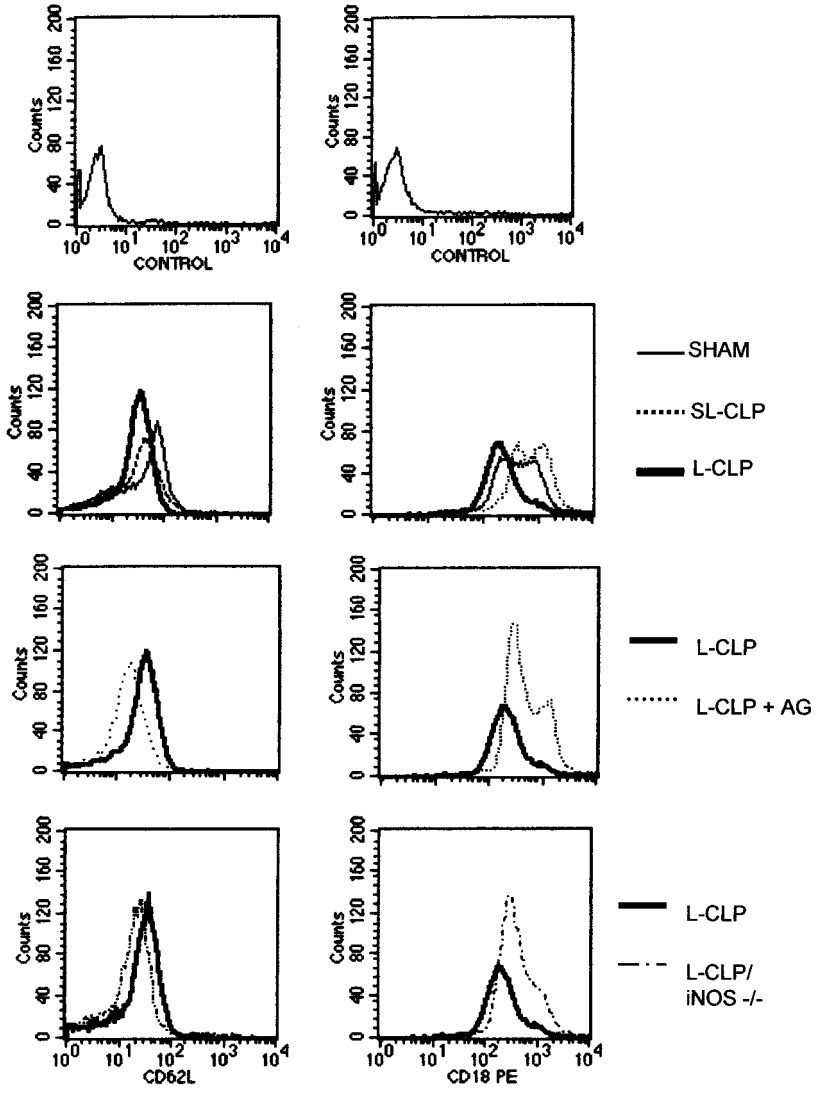

FIG. 7. Flow cytometric analysis of CD62L (L-selectin) and CD18 (β_2-integrin) expression on neutrophils obtained from sham-operated and CLP mice. Three hours after the surgery, whole blood taken from wild-type sham-operated (solid thin line), SL-CLP (dashed line), and L-CLP (thick line) mice were incubated with FITC- and PE-conjugated monoclonal antibodies. These monoclonal antibodies were used to detect the phenotypes of CD62L and CD18, respectively, in neutrophil populations, discriminated in previous experiments. Also, L-CLP mice treated subcutaneously with saline and with 30 mg of AG kg^{-1} 30 min before surgery (dotted line) and $iNOS^{-/-}$ L-CLP mice (dotted/dashed line) were analyzed. The same result was repeated two times, and 10 animals in each group were used to obtain the purified peripheral neutrophils.

Confirming that NO is involved in neutrophil microbicidal activity, it was observed that neutrophils harvested from the peritoneal cavity of SL-CLP mice, which controlled the infection, presented iNOS protein which was active, parameters determined by imunocytochemistry and by L-citrulline assay, respectively (data not shown). Moreover, neutrophils from wild-type but not from $iNOS^{-/-}$ mice showed microbicidal activity against *Staphylococcus aureus* (12).

Together, these data suggest that, despite the importance of NO in the effective microbicidal activity of neutrophils, NO impairs neutrophil migration to the infection site when produced in high concentrations by iNOS of cells in circulation. Failure of neutrophil migration has also been described in other diseases, such as diabetes, cirrhosis, and AIDS, all pathologies associated with high susceptibility to infection (15, 19, 41). However, it has not been investigated yet if NO mediates the observed failure of neutrophil migration in these diseases.

Our results indicate that the neutrophil migration observed in $iNOS^{-/-}$ L-CLP mice is not due to an increased production of TNF-α at the infection site, reported to be a mediator involved in neutrophil recruitment in sepsis (32, 45), and of IL-1β, which is also chemotactic to neutrophils (10). The TNF-α and IL-1β levels in peritoneal exudates of $iNOS^{-/-}$ animals subjected to SL-and L-CLP surgery, which did not present with failure of neutrophil migration, were similar to those observed in exudates of wild-type L-CLP animals, which showed failure of neutrophil migration (Fig. 5). Moreover, treatment of wild-type mice subjected to L-CLP with AG, which also prevented neutrophil migration failure, did not pro-

mote changes in the IL-1β and TNF-α concentrations in the peritoneal exudate (5).

Although the concentration of IL-10 is increased in L-CLP mice (Fig. 5), as a cytokine capable of inhibiting cell migration (40, 51), it does not mediate the failure of neutrophil migration observed in sepsis, since the treatment of wild-type L-CLP mice with AG also did not promote any change in IL-10 production (5).

We next investigated the mechanism by which NO mediates the failure of neutrophil recruitment towards the infection site in CLP-induced sepsis. First, we investigated whether NO inhibits the adhesion of neutrophils to endothelial cells. Earlier in vivo and in vitro results have shown that NOS inhibitors increase the adhesion of neutrophils to endothelial cells (25, 27). In our model using an intravital microscopy assay, we demonstrated that animals subjected to L-CLP surgery had a significant reduction in leukocyte rolling and adherence in postcapillary venules of the mesentery. Consequently, a reduction of leukocyte transmigration to the extravascular tissue was also observed. The reduction of cell rolling was associated with NO production, since the treatment with AG in mice subjected to L-CLP prevented the reduction in leukocyte rolling, adherence and transmigration. Furthermore, iNOS$^{-/-}$ mice subjected to L-CLP surgery presented with high levels of rolling, adherence, and leukocyte transmigration (Fig. 6). Accordingly, iNOS-derived NO reduces leukocyte adhesion and recruitment in mice injected with LPS intravenously due to a direct inhibitory effect on leukocyte rolling, since iNOS$^{-/-}$ animals demonstrated an increase in leukocyte rolling and enhanced adhesion (25, 26).

The rolling of neutrophils on endothelium is mediated by the interaction of neutrophil L-selectin (CD62L) with endothelium adhesion molecules, including P-selectin, E-selectin, and GlyCAM (39). We investigated whether the reduction of leukocyte rolling observed in L-CLP animals by intravital microscopy assay is a consequence of the reduction of L-selectin expression on neutrophils. For this experiment, we purified neutrophils from peripheral blood, and the fluorescence-activated cell sorting analysis demonstrated that only a slight reduction in L-selectin expression on neutrophils occurred in L-CLP mice. This reduction is not apparently sufficient to justify the failure of neutrophil migration towards the infection site, because a similar reduction was observed in iNOS$^{-/-}$ L-CLP mice. Furthermore, AG treatment of wild-type L-CLP mice did not prevent the slight reduction of L-selectin expression in neutrophils (Fig. 7).

The lack of a significant change in expression of L-selectin in the neutrophils from wild-type L-CLP mice indicates that the decrease in rolling observed in these animals could be a consequence of a failure in the expression of adhesion molecules on endothelial cell surfaces. The absence of a reduction in L-selectin expression in neutrophils from L-CLP mice is apparently in contradiction to data showing that neutrophils from septic patients have reduced L-selectin expression due to the shedding of this molecule from the neutrophil surface. However, the authors did not investigate whether the reduction of L-selectin expression correlates with a reduction in the rolling ability of neutrophils from septic patients (37). In accordance with our results, wild-type and iNOS$^{-/-}$ mice treated with LPS presented with similar L-selectin levels in the circulation and expressed on the membranes of leukocytes (26, 27).

The fluorescence-activated cell sorting analysis of CD18 in neutrophils showed an important reduction in the expression of this molecule on the surface of neutrophils from wild-type L-CLP compared with SL-CLP mice. This reduction was prevented by AG treatment of the wild-type L-CLP mice, and it was not observed in iNOS$^{-/-}$ L-CLP mice (Fig. 7). The change in CD18 expression in neutrophils correlates with the change in the adhesion of neutrophils to endothelial cells in animals subjected to CLP. Therefore, the reduction in CD18 expression could be a consequence of an ineffective activation of neutrophils due to a reduction in rolling, leading to low expression of this molecule. It has been well described in the literature that the in vivo expression of β-integrins (CD18) is dependent on the rolling step (31). However, we cannot discount the possibility that NO directly mediates the reduction in the expression of CD18, since NO inhibits NF-κB, which is involved in the expression of adhesion molecules in neutrophils, via induction of Iκ-B synthesis and/or increase of its stabilization (11, 47).

In conclusion, we have demonstrated that NO released by iNOS has a dual effect in sepsis. On the one hand, it is an important mediator for the microbicidal activity of neutrophils present at the infection site. However, on the other hand, it also impairs the recruitment of neutrophils to the infection site. It was also demonstrated that the mechanism by which NO impairs neutrophil migration is due to the reduction in rolling and adhesion of neutrophils to endothelial cells. Furthermore, our results point out the importance of investigating the capacity for neutrophil migration in patients with sepsis to determine a possible correlation between reduction of neutrophil chemotaxis and disease outcome. In the event that the failure of neutrophil migration also occurs in patients, the restoration of the neutrophil chemotactic function could be an appropriate treatment strategy.

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REFERENCES

1. **Astiz, M. E., and E. C. Rackow.** 1998. Septic shock. Lancet **351:**1501–1505.
2. **Baez, S.** 1969. Simultaneous measurements of radii and wall thickness of microvessels in the anesthetized rat. Circ. Res. **25:**315–329.
3. **Bagby, G. J., K. J. Plessala, L. A. Wilson, J. J. Thompson, and S. Nelson.** 1991. Divergent efficacy of antibody to tumor necrosis factor-α in intravascular and peritonitis models of sepsis. J. Infect. Dis. **163:**83–88.
4. **Baker, C. C., I. H. Chaudry, H. O. Gaines, and A. E. Bauer.** 1983. Evaluation of factors affecting mortality rate after sepsis in a murine cecal ligation and puncture model. Surgery **94:**331–335.
5. **Benjamim, C. F., S. H. Ferreira, and F. Q. Cunha.** 2000. Role of nitric oxide in the failure of neutrophil migration in sepsis. J. Infect. Dis. **182:**214–223.
6. **Bhagat, K., A. D. Hingorani, M. Palacios, I. G. Charles, and P. Vallance.** 1999. Cytokine-induced venodilatation in humans in vivo: eNOS masquerading as iNOS. Cardiovasc. Res. **41:**754–764.
7. **Bone, R. C., C. J. Grodzin, and R. A. Balk.** 1997. Sepsis: a new hypothesis for pathogenesis of the disease process. Chest **112:**235–243.
8. **Bone, R. C.** 1996. Immunologic dissonance: a continuing evolution in our understanding of the systemic inflammatory response syndrome (SIRS) and the multiple organ dysfunction syndrome. Ann. Intern. Med. **125:**680–687.

9. **Bone, R. C., R. A. Balk, F. B. Cerra, R. P. Dellinger, A. M. Fein, W. A. Knaus, R. M. Schein, and W. J. Sibbald.** 1992. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. Chest **101:**1644–1655.
10. **Casale, T. B., and E. J. Carolan.** 1999. Cytokine-induced sequential migration of neutrophils through endothelium and epithelium. Inflamm. Res. **48:**22–27.
11. **Connelly, L., M. Palacios-Callender, C. Ameixa, S. Moncada, and A. J. Hobbs.** 2001. Biphasic regulation of NF-κB activity underlies the pro- and anti-inflammatory actions of nitric oxide. J. Immunol. **166:**3873–3881.
12. **Crosara-Alberto, D. P., A. L. C. Darini, R. Y. Inoue, J. S. Silva, S. H. Ferreira, and F. Q. Cunha.** Involvement of NO in the failure of neutrophil migration in sepsis induced by *Staphylococcus aureus*. Br. J. Pharmacol., in press.
13. **Cunha, F. Q., J. Assreuy, D. W. Moss, D. Rees, L. M. C. Leal, S. Moncada, M. Carrier, and C. A. O'Donnell.** 1994. Differential induction of nitric oxide synthase in various organs of the mouse during endotoxemia: role of TNF-α and IL-1β. Immunology **81:**211–215.
14. **Echtenacher, B., W. Falk, D. N. Mannel, and P. H. Krammer.** 1990. Requirement of endogenous tumor necrosis factor/cachectin for recovery from experimental peritonitis. J. Immunol. **145:**3762–3766.
15. **Ellis, M., S. Gupta, S. Galant, S. Hakim, C. Vandeven, C. Toy, and M. S. Cairo.** 1998. Impaired neutrophil function in patients with AIDS and AIDS-related complex: a comprehensive evaluation. J. Infect. Dis. **158:**1268–1276.
16. **Eskandari, M. K., G. Bologos, C. Miller, D. T. Nguyen, L. E. DeForges, and D. G. Remick.** 1992. Anti-tumor necrosis factor antibody therapy fails to prevent lethality after cecal ligation and puncture and endotoxemia. J. Immunol. **148:**2724–2730.
17. **Evans, C. H., and M. Stefanovic-Racic.** 1996. Nitric oxide in arthritis. Methods **10:**38–42.
18. **Fierro, I. M., V. Nascimento-DaSilva, M. A. B. Arruda, M. S. Freitas, M. C. Plotkowski, F. Q. Cunha, and C. Barja-Fidalgo.** 1999. Induction of NOS in rat blood PMN in vivo and in vitro: modulation by tyrosine kinase and involvement in bactericidal activity. J. Leukoc. Biol. **65:**1–7.
19. **Fiuza, C., M. Salcedo, G. Clemente, and J. M. Tellado.** 2000. In vivo neutrophil dysfunction in cirrhotic patients with advanced liver disease. J. Infect. Dis. **182:**526–533.
20. **Fortes, Z. B., S. P. Farsky, M. A. Oliveira, and J. Garcia-Leme.** 1991. Direct vital microscopic study of defective leukocyte-endothelial interactions in diabetes mellitus. Diabetes **40:**1267–1273.
21. **Gazzinelli, R. T., I. Eltoum, T. A. Wynn, and A. Sher.** 1993. Acute cerebral toxoplasmosis is induced by in vivo neutralization of TNF-α and correlates with the down-regulated expression of inducible nitric oxide synthase and other markers of macrophage activation. J. Immunol. **151:**3672–3681.
22. **Granger, D. N., J. N. Benoit, M. Suzuki, and M. B. Grisham.** 1989. Leukocyte adherence to venular endothelium during ischemia-reperfusion. Am. J. Physiol. **257:**G683-G688.
23. **Green, L. C., D. A. Wagner, J. Glogowski, P. L. Skipper, J. S. Wishnok, and S. R. Tannenbaum.** 1982. Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. Anal. Biochem. **126:**131–138.
24. **Griffiths, M. J. D., M. Messent, R. J. MacAllister, and T. W. Evans.** 1993. Aminoguanidine selectively inhibits inducible nitric oxide synthase. Br. J. Pharmacol. **110:**963–968.
25. **Hickey, M. J., and P. Kubes.** 1997. Role of nitric oxide in regulation of leukocyte-endothelial cell interactions. Exp. Physiol. **82:**339–348.
26. **Hickey, M. J., K. A. Sharkey, E. G. Sihota, P. H. Reinhardt, J. D. MacMicking, C. Nathan, and P. Kubes.** 1997. Inducible nitric oxide synthase-deficient mice have enhanced leukocyte-endothelium interactions in endotoxemia. FASEB J. **11:**955–964.
27. **Hickey, M. J.** 2001. Role of inducible nitric oxide synthase in the regulation of leukocyte recruitment. Clin. Sci. **100:**1–12.
28. **Hollenberg, S. M., M. Broussard, J. Osman, and J. E. Parrillo.** 2000. Increased microvascular reactivity and improved mortality in septic mice lacking inducible nitric oxide synthase. Circ. Res. **86:**774–778.
29. **Hollenberg, S. M., R. E. Cunnion, and J. Zimmerberg.** 1993. Nitric oxide synthase inhibition reverses arteriolar hyporesponsiveness to catecholamines in septic rats. Am. J. Physiol. **264:**H660–H663.
30. **Kaplan, S. S., J. R. Lancaster, R. E. Basford, and R. L. Simmons.** 1996. Effect of nitric oxide on staphylococcal killing and interactive effect with superoxide. Infect. Immun. **64:**69–76.
31. **Lidbom, L., X. Xie, J. Raud, and P. Hedqvist.** 1992. Chemoattractant-induced firm adhesion of leukocytes to vascular endothelium *in vivo* is critically dependent on initial leukocyte rolling. Acta Physiol. Scand. **146:**415–421.
32. **Malaviya, R., T. Ikeda, E. Ross, and S. N. Abraham.** 1996. Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-α. Nature **381:**77–80.
33. **Malawista, S. E., R. R. Montgomery, and G. Van Blaricom.** 1992. Evidence for reactive nitrogen intermediates in killing of staphylococci by human neutrophil cytoplasts. A new microbicidal pathway for polymorphonuclear leukocytes. J. Clin. Investig. **90:**631–636.
34. **Martineau, L., and P. N. Shek.** 2000. Peritoneal cytokine concentration and survival outcome in an experimental bacterial infusion model of peritonitis. Crit. Care Med. **28:**788–794.
35. **Matsukawa, A., C. M. Hogaboam, N. W. Lukacs, P. M. Lincoln, H. L. Evanoff, R. M. Strieter, and S. L. Kunkel.** 2000. Expression and contribution of endogenous IL-13 in an experimental model of sepsis. J. Immunol. **164:**2738–2744.
36. **Matute-Bello, G., C. W. Frevert, O. Kajikawa, S. J. Skerrett, R. B. Goodman, D. R. Park, and T. R. Martin.** 2001. Septic shock and acute lung injury in rabbits with peritonitis. Failure of the neutrophil response to localized infection. Am. J. Respir. Crit. Care Med. **163:**234–243.
37. **McGill, S. N., A. A. Ahmed, F. Hu, R. P. Michel, and N. V. Christou.** 1996. Shedding of L-selectin as a mechanism for reduced polymorphonuclear neutrophil exudation in patients with the systemic inflammatory response syndrome. Arch. Surg. **131:**1141–1146.
38. **Numata, M., S. Suzuki, N. Miyazawa, A. Miyashita, Y. Nagashima, S. Inoue, T. Kaneko, and T. Okubo.** 1998. Inhibition of inducible nitric oxide synthase prevents LPS-induced acute lung injury in dogs. J. Immunol. **160:**3031–3037.
39. **Panés, J., M. Perry, and D. N. Granger.** 1999. Leukocyte-endothelial cell adhesion: avenues for therapeutic intervention. Br. J. Pharmacol. **126:**537–550.
40. **Parsons, P. E.** 1998. Interleukin-10: the ambiguity in sepsis continues. Crit. Care Med. **26:**818–819.
41. **Pereira, M. A. A., P. Sannomyia, and J. G. Leme.** 1987. Inhibition of leukocyte chemotaxis by factor in alloxan-induced diabetic rat plasma. Diabetes **36:**1307–1314.
42. **Petros, A., G. Lamb, A. Leone, S. Moncada, D. Bennett, and P. Vallance.** 1994. Effects of a nitric oxide synthase inhibitor in humans with septic shock. Cardiovasc. Res. **28:**34–39.
43. **Rhodin, J. A. G.** 1986. Architecture of the vessel wall, p. 1-31. *In* D. F. Bohr, A. P. Somlyo, and H. V. Sparks (ed.), Handbook of physiology, vol. II: the cardiovascular system. Bethesda, Md.
44. **Rocha, N. P., and S. H. Ferreira.** 1986. Restoration by levamisole of endotoxin-inhibited neutrophil migration, oedema, and increased vascular permeability induced by carrageenin. Eur. J. Pharmacol. **122:**87–92.
45. **Sedgwick, J. D., D. S. Riminton, J. G. Cyster, and H. Körner.** 2000. Tumor necrosis factor: a master-regulator of leukocyte movement. Immunol. Today **21:**110–113.
46. **Silva-Santos, J. E., and J. Assreuy.** 1999. Long-lasting changes of rat blood pressure to vasoconstrictors and vasodilators induced by nitric oxide donor infusion: involvement of potassium channels. J. Pharmacol. Exp. Ther. **290:**380–387.
47. **Spiecker, M., H. Darius, K. Kaboth, F. Hübner, and J. K. Liao.** 1998. Differential regulation of endothelial cell adhesion molecule expression by nitric oxide donors and antioxidants. J. Leukoc. Biol. **63:**732–739.
48. **Stoclet, J.-C., B. Muller, K. Gyorgy, R. Andriantsiothaina, and A. L. Kleschyov.** 1999. The inducible nitric oxide synthase in vascular and cardiac tissue. Eur. J. Pharmacol. **375:**139–155.
49. **Taktak, Y. S., S. Selkirk, A. F. Bristow, A. Carpenter, C. Ball, B. Rafferty, and S. Poole.** 1991. Assay of pyrogens by interleukin-6 release from monocytic cell lines. J. Pharmacol. **43:**578–582.
50. **Tavares-Murta, B. M., F. Q. Cunha, and S. H. Ferreira.** 1998. The intravenous administration of tumor necrosis factor alpha, interleukin 8 and macrophages-derived neutrophil chemotactic factor inhibits neutrophil migration by stimulating nitric oxide production. Br. J. Pharmacol. **124:**1369–1374.
51. **Van der Poll, T., R. W. Malefyt, S. M. Coyle, and S. F. Lowry.** 1997. Anti-inflammatory cytokine responses during clinical sepis and experimental endotoxemia: sequential measurements of plasma soluble interleukin (IL)-1 receptor type II, IL-10, and IL-13. J. Infect. Dis. **175:**118–122.
52. **Walley, K. R., N. W. Lukacs, T. J. Standiford, R. M. Strieter, and S. L. Kunkel.** 1996. Balance of inflammatory cytokines related to severity and mortality of murine sepsis. Infect. Immun. **64:**4733–4738.
53. **Wichtermann, K. A., A. E. Baue, and I. H. Chaudry.** 1980. Sepsis and septic shock: a review of laboratory models and a proposal. J. Surg. Res. **29:**189–201.
54. **Wolkow, P. P.** 1998. Involvement and dual effects of nitric oxide in septic shock. Inflamm. Res. **47:**152–166.
55. **Zhang, X. W., R. Schramm, Q. Liu, H. Ekberg, B. Jeppsson, and H. Thorlacius.** 2000. Important role of CD18 in TNF-α-induced leukocyte adhesion in muscle and skin venules *in vivo.* Inflamm. Res. **49:**529–534.

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