INFECTION AND IMMUNITY, May 1997, p. 1870–1875 Vol. 65, No. 5
0019-9567/97/$04.00+0
Copyright © 1997, American Society for Microbiology

Nitric Oxide Is Required for Effective Innate Immunity against *Klebsiella pneumoniae*

WAN C. TSAI,[1] ROBERT M. STRIETER,[2] DAVID A. ZISMAN,[2] JODI M. WILKOWSKI,[2] KATHY A. BUCKNELL,[2] GWO-HSIAO CHEN,[2] AND THEODORE J. STANDIFORD[2]*

Departments of Medicine[2] and Pediatrics,[1] Divisions of Pulmonary and Critical Care Medicine, University of Michigan Medical School, Ann Arbor, Michigan 48109-0360

Received 12 November 1996/Returned for modification 2 December 1996/Accepted 3 March 1997

Nitric oxide (NO) has been associated with protection against various parasitic and viral infections and may play a similar role in bacterial infections. We studied the role of NO in host defense against *Klebsiella pneumoniae* infection in the lung. Initial studies demonstrated a time-dependent increase in NO production of the lungs of CBA/J mice following the intratracheal administration of *K. pneumoniae* (7×10^2 CFU). To assess the role of NO in *Klebsiella* pneumonia, mice were treated intraperitoneally with either L-NAME (*N*-ω-nitro-L-arginine methylester), a competitive inhibitor of NO synthesis, or D-NAME, an inert enantiomer. The treatment of *Klebsiella*-infected mice with L-NAME resulted in a 10- and 46-fold increase in *K. pneumoniae* CFU in lungs and blood, respectively, at 48 h post-*K. pneumoniae* inoculation compared to treatment of mice with D-NAME. In addition, a greater-than-twofold increase in mortality was evident in L-NAME-treated mice compared to the mortality in control animals. No significant difference in bronchoalveolar lavage inflammatory cell profiles was noted between L-NAME- and D-NAME-treated mice with *Klebsiella* pneumonia. Interestingly, increased levels of tumor necrosis factor, gamma interferon, macrophage inflammatory protein 1α (MIP-1α), and MIP-2 mRNA and protein were noted in infected mice treated with L-NAME compared to the levels in mice treated with D-NAME. Importantly, the in vitro incubation of murine alveolar macrophages with L-NAME, but not with D-NAME, resulted in a significant impairment in both the phagocytosis and killing of *K. pneumoniae*. In total, these results suggest that NO plays a critical role in antibacterial host defense against *K. pneumoniae*, in part by regulating macrophage phagocytic and microbicidal activity.

Nitric oxide has been demonstrated to be a crucial and versatile molecule in the regulation of vascular tone, neurotransmission, acute and chronic inflammation, and host defense mechanisms (24). The widespread expression of inducible nitric oxide synthase (iNOS) following inflammation or infection has been well characterized and accepted as a vital component of the host's adaptive response to noxious stimuli and virulent pathogens (22). This increase in nitric oxide and its role in the control of a variety of intracellular organisms has been described in leishmaniasis (23) and malaria (30) and for trypanasomal (26), viral (4), and fungal (2) infections. However, the role of nitric oxide in bacterial infection has not been clearly defined (5, 14, 28). Despite the obvious significance of the rise in nitric oxide levels in the milieu of infection, the mechanisms by which nitric oxide aids in host defense remain unspecified. Potential mechanisms include direct microbicidal effect via the reaction of nitric oxide with iron or thiol groups on proteins forming iron-nitrosyl complexes that inactivate enzymes crucial in mitochondrial respiration or DNA replication. In addition, nitric oxide has been found to react with superoxide to form reactive oxidants capable of damaging target cells (29). On a cellular level, nitric oxide exerts varied effects on leukocyte cell function, including the induction of macrophage apoptosis (1), the stimulation of macrophage cytoplasmic motility (12), the modulation of neutrophil adhesion (19), and the differential regulation of cytokine synthesis by leukocytes (20).

* Corresponding author. Mailing address: The University of Michigan Medical Center, Department of Internal Medicine, Division of Pulmonary and Critical Care Medicine, 3916 Taubman Center, Box 0360, Ann Arbor, MI 48109-0360. Phone: (313) 764-4554. Fax: (313) 764-4556.

Effective host defense against lung bacterial infection is primarily dependent on the rapid clearance of the organism from the respiratory tract. Early clearance is mediated by a dual phagocytic system involving neutrophils and macrophages which must be vigorously recruited and activated at the site of infection (31). Th-1 type cytokines, such as tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), and interleukin-12 (IL-12), have been shown to exert important antibacterial immune effects by directly or indirectly activating neutrophils and macrophages to release proteases, to commit to the respiratory burst, and to express leukocyte adhesion molecules (18). Furthermore, chemokines, in particular C-X-C chemokine macrophage inflammatory protein 2 (MIP-2), have been found to be relevant mediators of leukocyte influx and/or activation in bacterial pneumonia (15). In contrast, the Th-2-driven immune response appears to be detrimental to the host in the setting of host defense against gram-negative bacterial pneumonia (16). Equally important, these cytokines have been implicated in the regulation of the expression of iNOS and thus in the selective expression of nitric oxide. IFN-γ and TNF-α upregulate nitric oxide synthesis, whereas Th-2 type cytokines IL-4 and IL-10 have been reported to inhibit nitric oxide synthesis (21). These studies raise the possibility that effective cytokine-mediated bacterial host defense may occur in part through regulation of nitric oxide as the end effector molecule.

In this study, we sought to characterize the role of nitric oxide in antibacterial host defense in the setting of pneumonia caused by gram-negative bacteria. Experiments were performed to assess the expression of nitric oxide within the lung during the course of murine *Klebsiella* pneumonia. In addition, we determined the effect of L-NAME (*N*-ω-nitro-L-arginine methylester), an N^G-substituted arginine analog that serves as a competitive inhibitor of nitric oxide synthase, on survival,

Downloaded from http://iai.asm.org/ on November 12, 2019 by guest

bacterial clearance, inflammatory cell recruitment, and cytokine profiles in animals challenged with *Klebsiella pneumoniae*. Furthermore, the effect of nitric oxide depletion on the in vitro phagocytosis and bacterial killing of *K. pneumoniae* cells by murine alveolar macrophages was assessed.

MATERIALS AND METHODS

Animals. Specific pathogen-free CBA/J mice (6- to 12-week-old females; Charles River Breeding Labs, Wilmington, Mass.) were used in all experiments. All mice were housed in specific pathogen-free conditions within the animal care facility at the University of Michigan until the day of sacrifice.

L-NAME and D-NAME treatment. L-NAME and D-NAME were obtained from Sigma Chemical Co. (St. Louis, Mo.) and dissolved in sterile 0.9% saline (vehicle). Each animal was administered 250 μl of a 5×10^{-3} M concentration of either L- or D-NAME intraperitoneally (i.p.) just prior to *K. pneumoniae* administration; the L- or D-NAME injections were repeated at 12-h intervals for the duration of each experiment. L-NAME was previously shown to effectively inhibit the synthesis of nitric oxide both in vitro and in vivo at the dose administered, while D-NAME was shown to be a reliable inert enantiomer (33).

***K. pneumoniae* inoculation.** We chose to use *K. pneumoniae* 43816, serotype 2 (American Type Culture Collection, Rockville, Md.) in our studies, as this strain has been shown to induce an impressive inflammatory response in mice (3, 15, 16). *K. pneumoniae* was grown in tryptic soy broth (Difco, Detroit, Mi.) for 18 h at 37°C. The concentration of bacteria in broth was determined by measuring the amount of absorbance at 600 nm. A standard of absorbancies based on known CFU was used to calculate inoculum concentration. A dose of 7×10^2 organisms/animal was chosen because this dose allowed for the development of substantial inflammation by 36 to 48 h without excessive mortality at that time point. Animals were anesthetized with approximately 1.8 to 2 mg of pentobarbital per animal i.p. The trachea was exposed, and a 30-μl inoculum or saline was administered via a sterile 26-gauge needle. The skin incision was closed with surgical staples.

Nitrite assay. To confirm that nitric oxide production was induced by bacterial infection and to establish the specificity of L-NAME as a competitive substrate inhibitor of iNOS and thus of L-arginine metabolism, stable end products of the nitric oxide reaction were measured in the form of nitrite levels. Mice were anesthetized, and whole lungs were harvested and immediately homogenized in 1.5 ml of 0.9% sterile saline with a tissue homogenizer (Biospec Products, Inc.); the homogenate was sonicated and centrifuged at $1,4000 \times g$ for 10 min. Eighty microliters of the supernatant was placed into each well of a 96-well flat-bottom tissue culture-treated plate (Costar Corp., Cambridge, Mass.). Ten microliters of a 2-mg/ml solution of reduced-form β-NADPH (Sigma) in phosphate-buffered saline (PBS) was then added to each well (20 μg/well) to be converted from nitrate to nitrite. Then, 10 μl of a 1-U/ml solution of nitrate reductase (Sigma) was added, and the plate was shaken on a rocker plate in the dark at 37°C for 1 h. Ten microliters of stopping solution, consisting of 26 mg of potassium ferricyanide and 9 mg of phenazine methosulfate (Sigma) in 10 ml of PBS, was added per well to stop reductase activity. Then, 50 μl of each of the following substrate components was added per well in the order specified: 1% sulfanilamide in 25% (vol/vol) hydrochloric acid prepared by adding 0.1 g of sulfanilamide (Aldrich Chemical Co., Inc., Milwaukee, Wis.) to 10 ml of 25% (vol/vol) hydrochloric acid (Fisher Scientific, Pittsburgh, Pa.) and then 0.0133% *N*-1-naphthylethylenediamine dihydrochloride prepared by adding 2 mg of *N*-1-naphthylethylenediamine dihydrochloride (Sigma) to 15 ml of sterile distilled H_2O. A standard curve was prepared with 100 μl of 100 μM sodium nitrite (Sigma) serially diluted with 50 μl of saline. Twenty-five microliters of solution A, consisting of 2% sulfanilamide in H_2O with 5% phosphoric acid, and then 25 μl of solution B, prepared from 0.2% napthylethylenediamine dehydrochloride in H_2O (Sigma) were added to each standard well. The plate was centrifuged at $1,350 \times g$ for 5 min to eliminate precipitate that may form and then was replated. The nitrite concentrations of the standard and the samples were calculated after spectrometric reading at an optical density of 543 nm (EL 311SX Biotek Instruments Inc.).

BAL. Bronchoalveolar lavage (BAL) was performed to obtain BAL cells. The trachea was exposed and intubated with a 1.7-mm-outside-diameter polyethylene catheter. BAL was performed by instilling PBS containing 5 mM EDTA in 1-ml aliquots. Approximately 5 ml of lavage fluid per mouse was retrieved. Cytospins were then prepared from BAL cells and stained with Diff Quick stain (Baxter, McGaw Park, Ill.), and differential counts were determined.

Lung harvesting for cytokine analysis. At designated time points, the mice were anesthetized by inhalation of methoxyflurane, blood was collected by orbital bleeding or direct cardiac puncture, and the animals were sacrificed. Whole lungs were then harvested for assessment of the various cytokine protein levels. Prior to lung removal, the pulmonary vasculature was perfused with 1 ml of PBS containing 5 mM EDTA via the right ventricle. After removal, whole lungs were homogenized in 3 ml of lysis buffer containing 0.5% Triton X-100, 150 mM NaCl, 15 mM Tris, 1 mM CaCl, and 1 mM MgCl (pH 7.40) with a tissue homogenizer (Biospec Products, Inc.). Homogenates were incubated on ice for 30 min and then centrifuged at $1,400 \times g$ for 10 min. The supernatants were collected, passed through a 0.45-μm-pore-size filter (Gelman Sciences, Ann Arbor, Mich.), and then stored at −20°C for assessment of cytokine levels.

Determination of plasma and lung *K. pneumoniae* CFU. At the time of sacrifice, plasma was collected, the right ventricle was perfused with 1 ml of PBS, and then the lungs were removed aseptically and placed in 3 ml of sterile saline. The tissues were then homogenized with a tissue homogenizer under a vented hood. The lung homogenates were placed on ice, and serial 1:10 dilutions were made. Ten microliters of each dilution was plated on soy base blood agar plates (Difco) and incubated for 18 h at 37°C, and then colonies were counted.

Murine cytokine ELISA. Murine TNF-α, IFN-γ, IL-12, IL-10, MIP-2, and MIP-1α levels were quantitated by a modification of a double-ligand method as previously described (28). Briefly, each well of flat-bottomed 96-well microtiter plates (Immuno-Plate I 96-F; Nunc, Roskilde, Denmark) was coated with 50 μl of rabbit antibody against the various cytokines (1 mg/ml in 0.6 M NaCl–0.26 M H_3BO_4–0.08 M NaOH [pH 9.6]) for 16 h at 4°C and was then washed with PBS (pH 7.5)–0.05% Tween 20 (wash buffer). Microtiter plate nonspecific binding sites were blocked with 2% bovine serum albumin in PBS and incubated for 90 min at 37°C. Plates were rinsed four times with wash buffer, and neat and diluted (1:10) cell-free supernatants (50 μl) in duplicate were added, followed by incubation for 1 h at 37°C. Plates were washed four times, followed by the addition of 50 μl of biotinylated rabbit antibodies against the specific cytokines (3.5 mg/ml in PBS [pH 7.5], 0.05% Tween 20, 2% fetal calf serum), and plates were then incubated for 30 min at 37°C. Plates were washed four times, streptavidin-peroxidase conjugate (Bio-Rad Laboratories, Richmond, Calif.) was added, and the plates were incubated for 30 min at 37°C. Plates were washed again four times, and chromogen substrate (Bio-Rad Laboratories) was added. The plates were incubated at room temperature to the desired extinction, and the reaction was terminated with 50 μl of 3 M H_2SO_4 solution per well. Plates were read at 490 nm in an enzyme-linked immunosorbent assay (ELISA) reader. Standards were 1/2-log-unit dilutions of recombinant murine cytokines from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected murine cytokine concentrations above 25 pg/ml. The ELISA mixture did not cross-react with IL-1, IL-2, IL-4, or IL-6. In addition, the ELISA mixture did not cross-react with members of the murine chemokine family, including murine JE/MCP-1, RANTES, KC, GROα, and ENA-78.

Phagocytic assay. Murine alveolar macrophages (1×10^5 cells) were adherence purified, resulting in adherence of greater than 95% of the macrophage population as described previously (32). The macrophages were then incubated for 2 h at 37°C with Hanks balanced salt solution (HBSS) (control) or L- or D-NAME (5×10^{-5} M) and then coincubated with 5% *K. pneumoniae*-specific immune serum for 5 min at 37°C in 8-well labteks (Nunc, Inc., Naperville, Ill.). *K. pneumoniae* cells (1×10^6 bacteria) were added and incubated for 1 h at 37°C on a rocker plate. Gentamicin was added (final concentration, 10 μg/ml) for an additional 1 h on a rocker plate to eliminate extracellular bacteria. It had been previously reported that gentamicin at higher concentrations can kill intracellular *Listeria* spp. after 72 h of incubation (9). However, the use of a lower dose of gentamicin with a shorter incubation time did not alter intracellular killing, while effectively eliminating extracellular bacteria. Evidence of killing of intracellular organisms was observed when the duration of incubation was increased to longer than 2 h or the concentration of gentamicin was increased to over 10 μg/ml. Importantly, all experimental groups (L-NAME, D-NAME, and HBSS control) were tested under the same conditions. The supernatants were removed, and the cells were washed three times with cold HBSS. As previously described, there were no effects of washing on macrophage adherence (32). The gasket was removed, and the slides were allowed to air dry. Staining with Diff-Quick stain was performed, and 50 cells per well were counted to determine the number of intracellular *K. pneumoniae* bacteria and the percent alveolar macrophages containing bacteria. The mean numbers of *K. pneumoniae* bacteria per alveolar macrophages per condition were then obtained.

Bactericidal assay. Murine alveolar macrophages (2×10^5 cells) were incubated with 5% *K. pneumoniae*-specific immune serum for 5 min at 37°C in 35-mm-diameter plastic culture dishes. Test solutions (L-NAME or D-NAME at 5×10^{-5} M) or an equal volume of HBSS was added for an additional 2 h, *K. pneumoniae* cells (2×10^6 bacteria) were added, and the cultures were incubated for 1 h at 37°C on a rocking plate. Gentamicin at a final concentration of 10 μg/ml was added for an additional 1 h to kill extracellular bacteria. The supernatants were removed, and the cells were washed three times with HBSS. The cells were then lysed by adding 0.5 ml of ice-cold sterile H_2O, disrupting the cells with a cell lifter, and incubating them on ice for 10 min. Then, 0.5 ml of 2× HBSS was added per well (for a total of 1 ml of alveolar macrophage lysate/well) and each sample was serially diluted on blood agar plates. The plates were incubated for 18 h at 37°C, and colony counts were performed. The percent survival of intracellular bacteria was calculated by the following formula: % intracellular killing = 100% − [(CFU of *K. pneumoniae*/ml of macrophage lysate)/total intracellular *K. pneumoniae* CFU] × 100. The total intracellular *K. pneumoniae* CFU is the product of the total number of alveolar macrophages and the mean number of intracellular *K. pneumoniae* CFU per alveolar macrophage.

Statistical analysis. Data were analyzed on a Macintosh II computer with the Statview II statistical package (Abacus Concepts, Inc., Berkeley, Calif.). Survival data were compared by using the chi-square analysis. All other data are expressed as means +/− standard errors of the means and are compared by the two-tailed Student *t* test. Data were considered statistically significant if *P* values were less than 0.05.

Downloaded from http://iai.asm.org/ on November 12, 2019 by guest

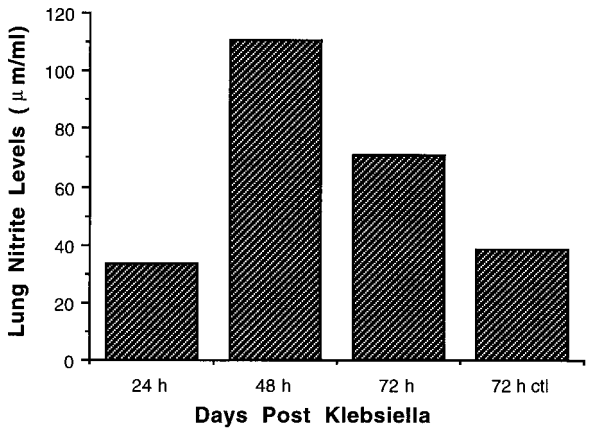

FIG. 1. Time-dependent production of nitrite in lung homogenates after inoculation with *K. pneumoniae* (7×10^2 CFU). There were four mice per group. ctl, control.

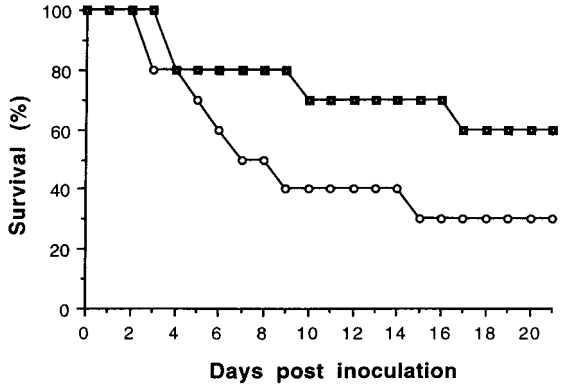

FIG. 2. Effect of inhibition of nitric oxide on survival of mice after inoculation with *K. pneumoniae* (7×10^2 CFU). $P < .05$ at at all time points after day 6. There were 10 mice per group. ○, L-NAME-treated mice; ■, D-NAME-treated mice.

RESULTS

Time-dependent production of nitric oxide after i.t. inoculation with *K. pneumoniae*. The intratracheal (i.t.) administration of *K. pneumoniae* (7×10^2 CFU) resulted in the time-dependent production of pulmonary nitric oxide levels, as determined by its stable end product, nitrite (Fig. 1). Maximal lung nitrite levels peaked at 48 h following bacterial inoculation, the time at which maximal inflammatory response and peak proinflammatory cytokine levels occurred. The levels of nitrite remained elevated at 72 h postinoculation, as compared to levels in noninfected controls. The increases in nitrite levels were compartmentalized to the lungs, as no appreciable increases in plasma nitrite levels in infected animals were noted (data not shown). In addition, L-NAME treatment suppressed nitric oxide synthesis in *K. pneumoniae*-infected animals, with a greater-than-50% reduction in lung nitrite levels in L-NAME-treated animals compared to the levels in the D-NAME-treated group (data not shown). These studies confirm that inoculation with *K. pneumoniae* results in the time-dependent and compartmentalized expression of nitric oxide during *Klebsiella* pneumonia.

Effect of in vivo nitric oxide depletion on survival of mice with *Klebsiella* pneumonia. Our initial experiments were aimed at determining the effect of nitric oxide depletion on survival of mice with *Klebsiella* pneumonia. CBA/J mice were treated i.p. with L-NAME or D-NAME, and then injected i.t. with 30 μl of a saline suspension containing 7×10^2 CFU of *K. pneumoniae*. A significant increase in early lethality was noted in L-NAME-treated infected mice compared to D-NAME-treated mice. In addition, long-term survival in the L-NAME-treated group was significantly decreased, as 70% of the D-NAME-treated mice remained alive at 21 days, whereas only 30% of the L-NAME-treated mice survived to that time point, representing a greater-than-twofold increase in mortality in the L-NAME-treated infected mice (Fig. 2). These results indicate that the inhibition of the endogenous production of nitric oxide significantly decreased survival in mice infected with *K. pneumoniae*.

Effect of nitric oxide inhibition on bacterial clearance in *Klebsiella* pneumonia. We next sought to determine whether the observed decrease in the survival of nitric oxide-depleted mice with *Klebsiella* pneumonia was due to impaired bacterial clearance. Mice were pretreated i.p. with L-NAME or D-NAME and then were administered *K. pneumoniae* (7×10^2 CFU) i.t. Lungs and plasma were harvested 48 h after inoculation. As shown in Fig. 3, treatment of *Klebsiella*-infected mice with L-NAME resulted in a 10-fold increase in *K. pneumoniae* CFU in lungs ($P < 0.05$) compared to that in the lungs of D-NAME-treated mice. An even more impressive 46-fold increase in *K. pneumoniae* CFU was noted in the plasma ($P < 0.01$) of L-NAME-treated animals compared to that in the plasma of mice receiving D-NAME. These results indicate that nitric oxide depletion significantly attenuated effective bacterial clearance in the lung and allowed for greater dissemination of the organism to the bloodstream.

Characterization of the inflammatory cell profile in nitric oxide-depleted mice after i.t. inoculation with *K. pneumoniae*. To determine if the impairment in bacterial clearance in L-NAME-treated mice was due to an alteration in the recruitment of inflammatory cells to the airspace, animals were pretreated with L- or D-NAME and then inoculated with *K. pneumoniae* cells or saline control, and bronchoalveolar lavage was performed 48 h after inoculation. This time point was chosen because the maximum influx of neutrophils in response to the i.t. administration of *K. pneumoniae* cells occurs at 48 h

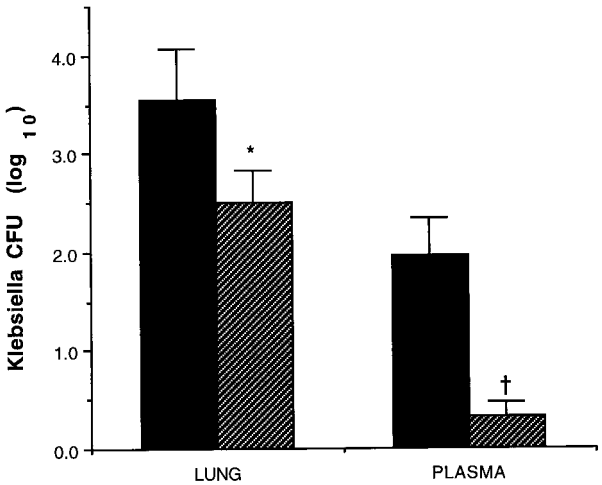

FIG. 3. Effect of nitric oxide depletion on *K. pneumoniae* bacterial clearance in lung homogenates and plasma 48 h following inoculation. *, $P < 0.05$; †, $P < 0.01$ (L-NAME-treated mice compared with D-NAME-treated controls). There were 12 mice per group. Solid bar, treatment with L-NAME and *K. pneumoniae*; hatched bar, treatment with D-NAME and *K. pneumoniae*.

Downloaded from http://iai.asm.org/ on November 12, 2019 by guest

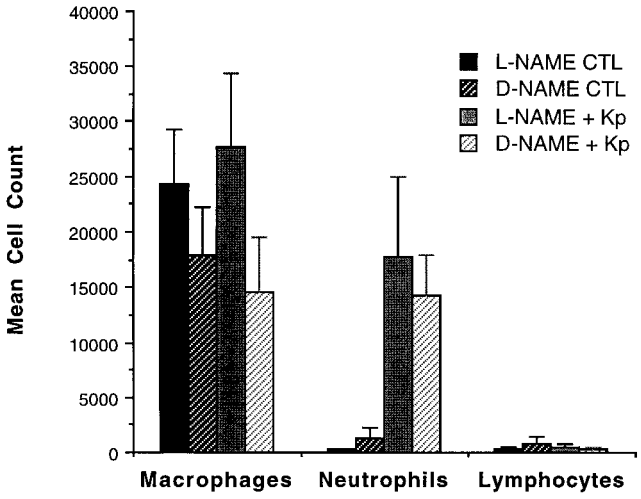

FIG. 4. Effect of inhibition of nitric oxide on BAL cell counts 48 h following *K. pneumoniae* administration (7×10^2 CFU). There were 8 mice per *K. pneumoniae*-infected group and 4 mice per saline control group.

(11, 12). Compared to the results for saline-challenged mice, inoculation with *K. pneumoniae* cells did not result in a change in the total number of alveolar macrophages but did result in a substantial increase in bronchoalveolar lavage fluid (BALF) neutrophils (Fig. 4). Treatment with L-NAME did not alter the numbers of bronchoalveolar lavage fluid (BALF) macrophages or neutrophils in either infected or noninfected mice. These results indicate that the altered host response was not due to an impairment in the recruitment of inflammatory cells in nitric oxide-depleted animals.

Effect of nitric oxide depletion on production of cytokine proteins within the lung after i.t. inoculation with *K. pneumoniae*. Subsequent experiments were performed to determine if the production of important pro- and anti-inflammatory cytokines was altered in nitric oxide-depleted animals. Several cytokines have been shown to be crucial to antibacterial host defense in gram-negative infection, particularly Th-1 phenotype cytokines (TNF-α, IFN-γ, and IL-12), the Th-2 phenotype cytokine (IL-10), and members of the C-X-C and the C-C chemokine families (MIP-2 and MIP-1α, respectively). The i.t. administration of *K. pneumoniae* resulted in maximal expression of the cytokines at 48 h, representing at least a fourfold increase in levels of TNF-α, MIP-1α, MIP-2, and IFN-γ protein compared with those seen in lung homogenates prepared from saline-treated control animals. To determine the role of nitric oxide in the regulation of these cytokines, cytokine profiles from infected L-NAME- and D-NAME-treated mice were compared. Interestingly, there was a significant increase in TNF-α ($P < 0.01$), MIP-1α ($P < 0.02$), and MIP-2 ($P < 0.01$) levels in L-NAME-treated infected mice compared to those in D-NAME-treated infected mice (Fig. 5). There was also an increase in IFN-γ in L-NAME-treated mice compared to the level in D-NAME-treated infected mice, although this difference did not reach the level of statistical significance. Furthermore, a trend toward increased IL-10 levels in L-NAME-treated infected mice was noted, whereas no difference in IL-12 levels was noted between the two groups (data not shown).

Effect of nitric oxide inhibition on alveolar macrophage phagocytic and microbicidal activity in vitro. To determine if nitric oxide directly regulates the phagocytic and bactericidal activity of alveolar macrophages, alveolar macrophages were harvested by bronchoalveolar lavage from uninfected CBA/J mice and were coincubated with L-NAME (5×10^{-5} M) or D-NAME (5×10^{-5} M), and then *K. pneumoniae* cells were added. Importantly, a significant decrease in the phagocytosis of *K. pneumoniae* cells by L-NAME-treated alveolar macrophages compared to that by alveolar macrophages incubated in

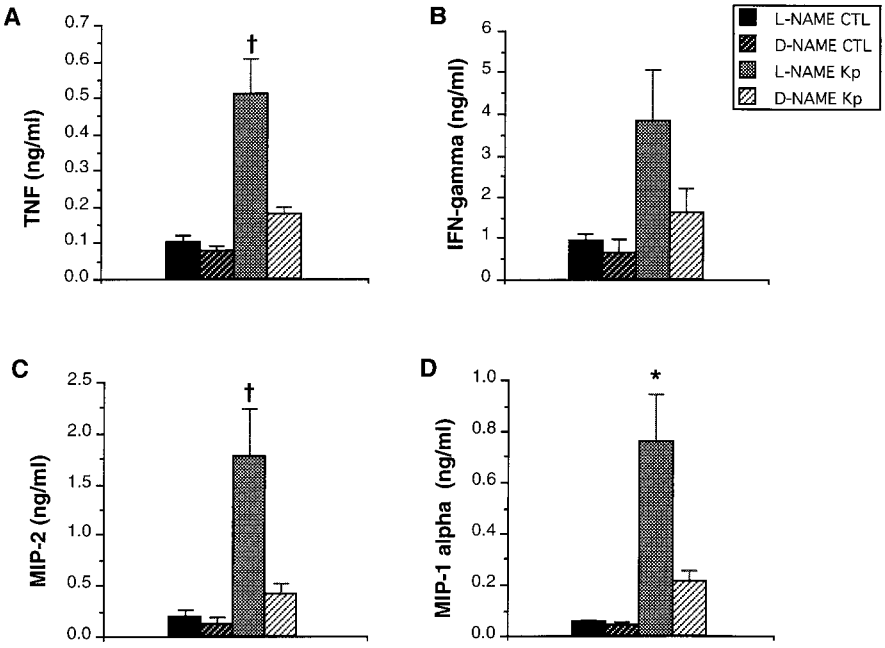

FIG. 5. Effect of nitric oxide depletion on the production of TNF-α (A), IFN-γ (B), MIP-2 (C), and MIP-1α (D) in lung homogenates 48 h after the i.t. administration of saline or *K. pneumoniae* to L-NAME- and D-NAME-treated CBA/J mice. CTL, animals administered saline; Kp, animals administered *K. pneumoniae* (7×10^2 CFU). *, $P < 0.02$; †, $P < 0.01$ (infected L-NAME-treated mice compared with infected D-NAME-treated mice). There were 12 mice per infected group and 4 mice per saline control group.

Downloaded from http://iai.asm.org/ on November 12, 2019 by guest

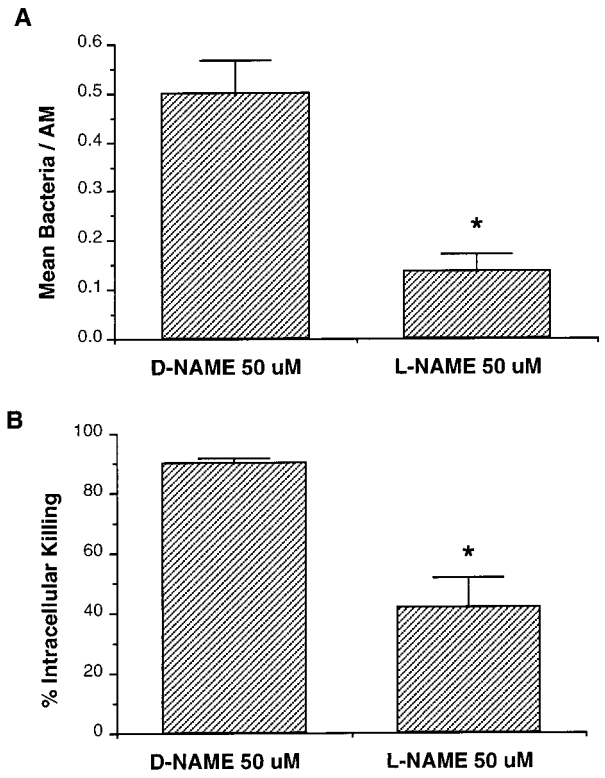

FIG. 6. Effect of nitric oxide inhibition on alveolar macrophage phagocytic (A) and microbicidal (B) activity in vitro. AM, alveolar macrophages. *, $P < 0.01$ (L-NAME-treated macrophages compared to D-NAME-treated macrophages).

D-NAME was noted ($P < 0.01$) (Fig. 6A). In addition, bacterial killing of ingested *K. pneumoniae* cells was significantly attenuated in L-NAME-treated alveolar macrophages compared to that in cells exposed to D-NAME ($P < 0.01$) (Fig. 6B). Incubation of *K. pneumoniae* cells with either L-NAME or D-NAME in the absence of macrophages did not alter bacterial viability (data not shown). These findings indicate that nitric oxide represents a very important regulator of alveolar macrophage phagocytic and microbicidal activity in vitro.

DISCUSSION

The emergence of multidrug-resistant organisms has made the study of mechanisms of innate immunity against bacterial pathogens increasingly important (25). Effective antibacterial host defense requires the generation of a vigorous inflammatory response involving the recruitment and activation of neutrophils and macrophages to effect rapid phagocytosis and bacterial killing (31). Nitric oxide has been implicated as a molecule that may mediate the immune host defense in many types of infections, including bacterial infections. In this study, we examined the contribution of nitric oxide to effective bacterial clearance in a murine model of bacterial pneumonia. We have established the time-dependent expression of nitric oxide within the lung during the course of murine *Klebsiella* pneumonia. Furthermore, the inhibition of nitric oxide synthesis by L-NAME results in significant mortality due to impaired bacterial clearance after challenge with *K. pneumoniae*. To our knowledge, this is the first study to clearly demonstrate that inhibition of endogenous nitric oxide production significantly alters the survival of animals in a murine model of infection by gram-negative bacteria.

Mechanisms whereby nitric oxide mediates effective bacterial clearance have not previously been defined. We observed no significant difference in the numbers of neutrophils or macrophages within the airspaces of infected L-NAME- and D-NAME-treated mice. While *Klebsiella* pneumonia in animal models and in humans is characterized by a substantial influx of neutrophils and macrophages, the presence of which are essential for effective containment and clearance of the organism (15, 16, 18), our studies indicate that nitric oxide appears to play little or no role in the recruitment of these cells to the lung in *Klebsiella* pneumonia. Similarly, Gosselin and colleagues noted that treatment of BALB/c mice with the iNOS inhibitor aminoguanidine did not alter lung inflammatory cell influx in a murine model of *Pseudomonas* pneumonia (14). Furthermore, we found an increase, rather than a decrease, in the expression of cytokines in the nitric oxide-depleted (L-NAME-treated) animals compared to cytokine expression in D-NAME-treated animals. These findings contrast with those of Brieland and colleagues, who observed a decreased expression of TNF in L-*N*-monomethyl-L-arginine (L-NMMA)-treated mice with *Legionella*-related pneumonia (5). In addition, we found that members of both the C-X-C and C-C chemokines were upregulated in nitric oxide-depleted animals. These studies compare to in vitro studies in which treatment with L-NMMA resulted in a dose-dependent increase in IL-8 and ENA-78, members of the C-X-C family, but not in members of the C-C chemokine family during a mixed-lymphocyte reaction. This increase in the expression of several proinflammatory cytokines may have occurred as a direct result of removal of the tonic inhibitory effects of endogenous nitric oxide on cytokine expression (15). Alternatively, enhanced proinflammatory cytokine expression may occur nonspecifically as a result of impaired intra-alveolar bacterial killing and the resultant increased bacterial burden and persistent stimulus for further cytokine induction. While L-NAME-treated mice had a greater increase in cytokine expression than D-NAME treated mice, the opposite effect on anticipated bacterial clearance was observed. Because TNF-α and IFN-γ have been shown to augment nitric oxide production, the direct inhibition of nitric oxide synthesis by L-NAME may render that portion of the cytokine-mediated host defense ineffective, as nitric oxide is no longer present to function as the target effector molecule.

Importantly, nitric oxide does not alter the influx of inflammatory leukocytes in vivo, but in vitro studies suggest that endogenous nitric oxide may activate the inflammatory cells present at the site of infection. Inhibition of nitric oxide synthesis clearly impaired both the ingestion and killing of *K. pneumoniae* cells, suggesting that nitric oxide may play a crucial role in activation of resident alveolar macrophages. The mechanism(s) whereby nitric oxide regulates alveolar macrophage phagocytic and microbicidal activity has not yet been defined. Nitric oxide has been shown to augment macrophage cytoplasmic motility (12), which may be mediated by changes in cell surface expression of adhesion molecules that are also instrumental in the ingestion of bacterial organisms. Alternatively, nitric oxide may directly or indirectly regulate the cell surface expression of other relevant molecules, including the complement and immunoglobulin Fc receptors (27). Nitric oxide has been shown to stimulate the respiratory burst in inflammatory cells (29), which may partially account for the impairment in bacterial killing observed in alveolar macrophages incubated with L-NAME. Studies are ongoing to clearly determine specific mechanisms by which nitric oxide regulates alveolar macrophage phagocytic and microbicidal activity. Our studies indicated that nitric oxide does not modulate neutrophil recruitment in vivo. However, we cannot exclude an effect

Downloaded from http://iai.asm.org/ on November 12, 2019 by guest

of nitric oxide on neutrophil phagocytic and killing function. Conflicting evidence exists on the effect of nitric oxide on neutrophil function. Recent data from Elferink and VanUffelen showed nitric oxide to have a concentration-dependent stimulatory and inhibitory effect on neutrophil migration (10). Furthermore, the phagocytosis and killing of *Candida albicans* cells by rat peritoneal neutrophils has been shown to be dependent upon endogeneous nitric oxide production (11). In contrast, Deitch and colleagues noted in a *Pseudomonas* model that neutrophil bactericidal activity was oxidant rather than nitric oxide dependent (8).

In summary, our studies demonstrate that mice in which nitric oxide is depleted and which are challenged i.t. with *K. pneumoniae* develop an inflammatory response similar to that of their non-nitric-oxide-depleted counterparts but that nitric oxide-depleted mice clearly had impairment in bacterial clearance. We have reported that there are no differences in the recruitment of inflammatory cells and that there is an increased expression of proinflammatory cytokines during the evolution of *Klebsiella* pneumonia in nitric oxide-depleted mice, compared to those animals with intact nitric oxide synthesis. Furthermore, nitric oxide appears to be an important mediator of macrophage phagocytosis and killing in *Klebsiella* pneumonia. The crucial role of nitric oxide in antibacterial host defense should be evident in several pertinent arenas. As treatment of bacterial infections becomes increasingly more difficult, the augmentation of nitric oxide synthesis and/or the local administration of nitric oxide may improve the clinical management of patients with severe bacterial pneumonias (6). In addition, the use of nonselective nitric oxide inhibitors in disease states such as septic shock (17), diabetes (7), or graft-versus-host disease (13) may result in impairment of antibacterial host defense, putting these patients at increased risk for the development of life-threatening nosocomial bacterial infections.

ACKNOWLEDGMENTS

This research was supported in part by National Institutes of Health grants 1P50HL46487, HL50057, HL31693, HL35276, HL58200, CA66180, HL57243 and AA10571 and by the American Heart Association of Michigan.

We thank Alex Carterson for his assistance in the preparation of the manuscript.

REFERENCES

1. **Albina, J. E., S. Lui, R. B. Mateo, and J. S. Reichner.** 1993. Nitric oxide-mediated apoptosis in murine peritoneal macrophages. J. Immunol. **150:** 5080–5085.
2. **Alspaugh, J. A., and D. L. Granger.** 1991. Inhibition of *Cryptococcus neoformans* by nitrogen oxides supports the role of these molecules as effectors of macrophage-mediated cytostasis. Infect. Immun. **59:**2291–2296.
3. **Bakker-Wondenberg, I. A. J. M., A. F. Lokerse, M. T. ten Kate, J. W. Mouton, M. C. Woodle, and G. Storm.** 1993. Liposomes with prolonged blood circulation and selective localization in *Klebsiella pneumoniae* infected lung tissue. J. Infect. Dis. **168:**164.
4. **Bi, Z., and C. S. Reiss.** 1995. Inhibition of vesicular stomatitis virus infection by nitric oxide. J. Virol. **69:**2208–2213.
5. **Brieland, J. K., D. G. Remick, P. T. Freeman, M. C. Hurley, J. C. Fantone, and N. C. Engleberg.** 1995. In vivo regulation of replicative *Legionella pneumophila* lung infection by endogenous tumor necrosis factor alpha and nitric oxide. Infect. Immun. **63:**3253–3258.
6. **Campbell, G. D.** 1994. Overview of community acquired pneumonia. Prognosis and clinical features. Med. Clin. North Am. **78:**1035–1048.
7. **Corbett, J. A., and M. L. McDaniel.** 1992. Does nitric oxide mediate autoimmune destruction of beta-cells? Possible therapeutic interventions in IDDM. Diabetes **41:**897–903.
8. **Deitch, E. A., Y. Haskel, N. Cruz, D. Xu, and P. R. Kvietys.** 1995. Caco-2 and IEC-18 intestinal epithelial cells exert bactericidal activity through an oxidant-dependent pathway. Shock **4:**345–350.
9. **Drevets, D. A., B. P. Canono, P. J. M. Leenen, and P. A. Campbell.** 1994. Gentamicin kills intracellular *Listeria monocytogenes*. Infect. Immun. **62:** 2222–2228.
10. **Elferink, J. G. R., and B. E. VanUffelen.** 1996. The role of cyclic nucleotides in neutrophil migration. Gen. Pharmacol. **27:**387–393.
11. **Fierro, I. M., C. Barja-Fidalgo, F. Q. Cunha, and S. H. Ferreira.** 1996. The involvement of nitric oxide in the anti-Candida albicans activity of rat neutrophils. Immunology **89:**295–300.
12. **Fukushima, T., K. Sekizawa, M. Yamaya, S. Okinaga, M. Satoh, and H. Sasaki.** 1995. Viral respiratory infection increases alveolar macrophage cytoplasmic motility in rats: role of nitric oxide. Am. J. Physiol. **268:**L399–L406.
13. **Garside, P., A. K. Hutton, A. Severn, F. Y. Liew, and A. M. Mowat.** 1992. Nitric oxide mediates intestinal pathology in graft-versus-host disease. Eur. J. Immunol. **22:**2141.
14. **Gosselin, D., J. DeSanctis, M. Boule, E. Skamene, C. Matouk, and D. Radzioch.** 1995. Role of tumor necrosis factor alpha in innate resistance to mouse pulmonary infection with *Pseudomonas aeruginosa*. Infect. Immun. **63:**3272–3278.
15. **Greenberger, M. J., R. M. Streiter, S. L. Kunkel, J. M. Danforth, L. L. Laichalk, D. C. McGillicudy, and T. J. Standiford.** 1996. Neutralization of MIP-2 attenuates neutrophil recruitment and bacterial clearance in murine Klebsiella pneumonia. J. Infect. Dis. **173:**159.
16. **Greenberger, M. J., R. M. Streiter, S. L. Kunkel, J. M. Danforth, R. E. Goodman, and T. J. Standiford.** 1995. Neutralization of IL-10 increases survival in a murine model of Klebsiella pneumonia. J. Immunol. **155:**722.
17. **Hinder, F., M. Booke, L. D. Traber, N. Matsumoto, K. Nishida, S. Rogers, and D. L. Traber.** 1996. Nitric oxide synthase inhibition during experimental sepsis improves renal excretory function in the presence of chronically increased atrial natriuretic peptide. Crit. Care Med. **24:**131–136.
18. **Kolls, J. K., S. Nelson, and W. R. Summer.** 1993. Recombinant cytokines and pulmonary host defense. Am. J. Med. Sci. **306:**330.
19. **Kubes, P., M. Suzuki, and D. N. Granger.** 1991. Nitric oxide: an endogeneous modulator of leukocyte adhesion. Proc. Natl. Acad. Sci. USA **88:**4651–4655.
20. **Liew, F. Y.** 1995. Interactions between cytokines and nitric oxide. Adv. Neuroimmunol. **5:**201–209.
21. **Liew, F. Y.** 1994. Regulation of nitric oxide synthesis in infectious and autoimmune diseases. Immunol. Lett. **43:**95–98.
22. **Liew, F. Y., and F. E. G. Cox.** 1991. Nonspecific defense mechanisms: the role of nitric oxide. Immunol. Today **12:**A17–A21.
23. **Liew, F. Y., Y. Li, D. Moss, C. Parkinson, M. V. Rogers, and S. Moncada.** 1991. Resistance to Leishmania major infection correlates with the induction of nitric oxide synthase in murine macrophages. Eur. J. Immunol. **21:**3009–3014.
24. **Moncada, S., R. M. J. Palmer, and E. A. Higgs.** 1989. Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication. Biochem. Pharmacol. **38:**1709–1715.
25. **Orens, J. B., N. W. Lukacs, S. L. Kunkel, M. D. Burdick, C. A. Wilke, A. Walz, and R. M. Streiter.** 1994. Regulation of chemokine production by the oxidative metabolism of L-arginine in a human mixed lymphocyte reaction. Cell. Immunol. **156:**95–101.
26. **Petray, P., E. Castanos-Velez, S. Grinstein, A. Orn, and M. E. Rottenberg.** 1995. Role of nitric oxide in resistance and histopathology during experimental infection with Trypanosoma cruzi. Immunol. Lett. **47:**121–126.
27. **Schwartz, B. D. (ed.).** 1991. Immunology, p. 98–108. Upjohn Co, Kalamazoo, Mich.
28. **Shi, Y., H. Q. Li, C. K. Shen, J. H. Wang, J. Pan, S. W. Qin, and R. Liu.** 1993. Association between protective efficacy of antibodies to tumor necrosis factor and suppression of nitric oxide production in neonatal rats with fatal infection. Pediatr. Res. **34:**345–348.
29. **Stamler, J. S., D. J. Singel, and J. Loscalzo.** 1992. Biochemistry of nitric oxide and its redox-activated forms. Science **258:**1898–1902.
30. **Taylor-Robinson, A. W., R. S. Phillips, A. Severn, S. Moncada, and F. Y. Liew.** 1993. The role of TH1 and TH2 cells in a rodent malaria infection. Science **260:**1931–1934.
31. **Toews, G. B., G. N. Gross, and A. K. Pierce.** 1979. The relationship of inoculum size to lung bacterial clearance and phagocytic cell response in mice. Am. Rev. Respir. Dis. **120:**559–566.
32. **Van Otteren, G. M., T. J. Standiford, S. L. Kunkel, J. M. Danforth, M. D. Burdick, L. Abruzzo, and R. M. Streiter.** 1994. The expression and regulation of macrophage inflammatory protein 1 α by murine alveolar and peritoneal macrophages. Am. J. Respir. Cell Mol. Biol. **10:**8–15.
33. **Wang, Y. X., I. Poon, and C. C. Y. Pang.** 1993. Vascular pharmacodynamics of N^G-nitro-L-arginine methyl ester in vitro and in vivo. J. Pharmacol. Exp. Ther. **267:**1091–990.

Editor: P. E. Orndorff

Downloaded from http://iai.asm.org/ on November 12, 2019 by guest