INFECTION AND IMMUNITY, Mar. 1994, p. 1089–1100
0019-9567/94/$04.00+0

Vol. 62, No. 3

Nitric Oxide Produced during Murine Listeriosis Is Protective

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Received 9 August 1993/Returned for modification 5 November 1993/Accepted 7 December 1993

Nitric oxide (NO) has been shown to be important for intracellular microbiostasis in vitro. To determine the role of NO in immune function in vivo, groups of C57BL/6 mice were given a sublethal intravenous inoculum of *Listeria monocytogenes* EGD, and their urine was monitored daily for nitrate, the mammalian end product of NO metabolism. Urinary nitrate levels peaked at 5 to 10 times the basal level on days 5 to 6, when spleen and liver *Listeria* counts declined most steeply, and decreased thereafter, when spleens and livers were nearly sterile. Peritoneal macrophages explanted from *Listeria*-infected mice produced nitrite spontaneously, whereas macrophages from uninfected mice did not. The inducible NO synthase mRNA was detectable in the spleens of infected mice on days 1 to 4 of infection. When *Listeria*-infected mice were treated orally throughout the infection with N^G-monomethyl-L-arginine (NMMA), a specific NO synthase inhibitor, they showed no detectable rise in urinary nitrate excretion. Mean *Listeria* counts in the livers and spleens of NMMA-treated mice were 1 to 3 orders of magnitude greater than counts in control mice on days 4 through 8 of infection. Compared with control mice, NMMA-treated mice also showed worse clinical signs of infection, namely, weight loss, hypothermia, decreased food and water intake, and decreased urine output. Histologically, NMMA-treated mice had many more inflammatory foci in their livers and spleens than control mice. The histologic observation that mononuclear cells are present at sites of infection suggests that inhibiting NO production did not block the flux of macrophages into infected viscera. As controls for possible drug toxicity, a group of uninfected mice given NMMA orally showed no detrimental effects on weight, temperature, and food and water intake. These experiments demonstrate that inhibition of NO production in *Listeria*-infected mice results in an exacerbated infection and thus that NO synthesis is important for immune defense against *Listeria* infection in mice.

Following the discoveries that nitric oxide (NO) is produced enzymatically by activated macrophages and that it functions as a cytotoxic effector molecule for tumor target cells (28, 29), NO was postulated to exert antimicrobial effects on pathogenic intracellular microorganisms. This postulate gained support when it was shown that certain bacteria, fungi, and protozoa are sensitive to NO-induced cytostasis and/or killing in vitro (1, 2, 4, 13, 21, 23, 24, 32, 40). Recent attempts have been made to demonstrate the in vivo relevance of these observations and, thus, to establish a role for NO in mammalian cell-mediated immunity. Liew et al. reported exacerbation of cutaneous leishmaniasis in mice upon intralesional injection of the NO synthase (NOS) inhibitor N^G-monomethyl-L-arginine (NMMA) (34). Recently, Green et al. showed that the large nitrate excretion accompanying *Mycobacterium bovis* BCG administration in mice correlated with nonspecific resistance to challenge with the heterologous microorganism *Francisella tularensis* (25). In this system, inhibition of cytokines that are known inducers of NOS both suppressed nitrate excretion and abrogated nonspecific resistance to tularemia. Furthermore, administration of NMMA blocked this nonspecific resistance, suggesting that NO production plays an important role in host resistance under these conditions. Evans et al. observed NOS induction in vivo during cutaneous leishmaniasis (17). They found that inhibiting NOS by oral administration of NMMA exacerbated the cutaneous infection and led to an increase in parasites in regional lymph nodes. Beckerman et al. reported exacerbation of *Listeria monocytogenes* infection in SCID mice after intraperitoneal administration of aminoguanidine, an NOS inhibitor (6), and suggested a role for NO in T-cell-independent host resistance. However, neither of these studies documented in vivo NOS inhibition. Recently, Gregory et al. provided data suggesting that NO suppressed acquired immunity to *Listeria* spp. (27). Mice given a single intravenous dose of NMMA at the time of infection had fewer listeriae in their livers and spleens on days 3 and 7 than controls did. These results conflict with those of Liew et al. (34), Evans et al. (17), and Beckerman et al. (6). Thus, it is not clear what role NO plays during infection in vivo. Studies such as these are complicated by the known multiplicity of physiological actions of NO in vivo, which include effects on vasculature (41) and on neuron (19), platelet (37), and lymphocyte (31) function.

By utilizing methods reported by Green et al. (22) and later by Stuehr and Marletta (48), we reported that *M. bovis* BCG infection in mice led to marked induction of NO synthesis in vivo (20). BCG-infected mice excreted up to 200 times more daily urinary nitrate, the major product of endogenously synthesized NO, than uninfected controls. When NMMA was administered orally to BCG-infected mice by adding the compound to their drinking water, nitrate excretion was inhibited by 90%. This methodology could be used to examine the role of inducible NO synthase (iNOS) in murine listeriosis. This is a relatively short-lived primary infection for which cells known to produce NO, namely macrophages and hepatocytes, are parasitized by *Listeria* spp. Immunoglobulins are not protective, but immunity can be transferred adoptively with T lymphocytes from convalescent animals (35, 36). This report

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documents the induction of NOS in mice at a site of *Listeria* infection, gives measurements of in vivo NO production over the course of infection, and examines the effect of inhibiting NOS on the host organism load in the liver and spleen. The histopathology of these infected organs and clinical signs of illness are also examined.

MATERIALS AND METHODS

Animals. Eight- to nineteen-week-old male or female C57BL/6 mice (Charles River Laboratories, Raleigh, N.C.) weighing 17 to 20 g each were housed four animals per cage in Nalgene (Rochester, N.Y.) rodent metabolic cages. The room ambient temperature was 25 to 28°C, and light sources included a window and overhead fluorescent lights which were turned off at night. Mice were fed a defined L-arginine-deficient amino acid rodent diet (Zeigler Bros., Inc., Gardners, Pa.), which contained no nitrite or nitrate and which was ground into 1- to 2-mm-diameter particles, all as described previously (20). Mice drank sterile, deionized, distilled water which contained no nitrite or nitrate. Mouse weights, food and water intake, and urine and stool output were monitored daily by cage. Individual animal rectal temperatures were taken with a 0.039-in. (0.10-cm)-diameter tissue temperature probe (Yellow Springs Instrument Co., Yellow Springs, Ohio) inserted 2 cm into the rectum. Mouse cages were cleaned daily with Roccal II (National Labs, Montvale, N.J.) diluted 1/500 with tap water and rinsed with tap water. Mice were adapted to this environment for 3 to 7 days before *Listeria* challenge.

In one nonmetabolic experiment, mice were housed five per cage in standard cages containing sawdust bedding at the Duke University Medical Center Vivarium. They ate undefined rodent chow and drank tap water.

***Listeria* infection.** *L. monocytogenes* EGD was grown to mid-log phase in Trypticase soy broth, diluted with phosphate-buffered saline (PBS), and administered to mice in a 0.3-ml volume by tail vein injection. The 50% lethal dose (LD_{50}) for *L. monocytogenes* EGD in C57BL/6 mice is approximately 3×10^4 organisms (43). Tenfold dilutions of the inoculum were plated on Trypticase soy agar, and CFU were counted. The target inoculum for all experiments was $0.1\times$ LD_{50} (standard inoculum). Control mice were injected with 0.3 ml of PBS.

Mice were assayed for live listeriae between 1 and 13 days after infection. On days of sacrifice, mice were anesthetized with ketamine (100 mg/kg of body weight) and xylazine (5 mg/kg) injected intraperitoneally. Blood was collected by heart puncture under sterile conditions with syringes coated with heparin, and 100 μl of whole blood was spread onto Oxford or modified Oxford agar plates (Difco Laboratories, Detroit, Mich.), which are selective for *Listeria* spp. (see below). Plates were incubated at 30°C for 36 to 48 h, and CFU were counted. After heart puncture, mice were killed by cervical dislocation. Spleens and livers were excised under sterile conditions, and a small portion of each was fixed in formalin for pathological analysis. The remainder of each organ was homogenized in 1 ml of PBS with 0.05% Triton X-100 (Sigma Chemical Corp., St. Louis, Mo.) in a glass grinder. Tenfold dilutions of each homogenate were spread onto Trypticase soy agar plates. The plates were incubated at 37°C for 24 h, and CFU were counted. Results were expressed as numbers of listeriae per organ. Cultures of the blood, spleens, and livers of mice not experimentally infected with listeriae were always negative for bacteria.

Twenty-four-hour stool samples from each cage were collected and stored at −30°C. At the time of assay, samples were weighed and a known fraction of each sample (usually not less than one-third of the sample) was put into a glass grinder, to which was added PBS with 0.05% Triton X-100 in a ratio of 1 ml per 100-mg sample. The samples were left to soak at 25°C for 0.5 to 2 h, after which they were homogenized, making sure all stool pellets were dispersed. Tenfold dilutions of each homogenate were spread onto Oxford or modified Oxford agar plates. The plates were incubated at 30°C for 36 to 48 h, and CFU were counted. It was found that viable *Listeria* counts did not decrease during storage at −30°C for 4 weeks.

Modified Oxford agar, which contains the antibiotics moxalactam and colistin, successfully inhibited all microbial growth from mouse stool except for listeriae, which appeared as round, umbilicated gray colonies with a black halo. The identity of *Listeria* spp. was confirmed by demonstration of (i) gram-positive bacilli on Gram stain, (ii) beta-hemolysis activity on sheep blood agar plates (Remel, Lenexa, Kans.), (iii) catalase activity, and (iv) tumbling motility under a phase-contrast microscope. After this initial confirmation, *Listeria* spp. were identified by colony characteristics.

Inhibitor administration. In initial experiments, mice received NMMA (Calbiochem Corp., San Diego, Calif.) in their drinking water (50 mM), their food (100 μmol/g), and via twice-a-day subcutaneous neck skin fold injections (150 mM) for a total dosage of 150 to 350 μmol per mouse per day. In subsequent experiments, NMMA (0.6 M) was instilled directly into the stomachs of mice with 22-gauge straight gavage needles (Perfektum; Popper & Sons, New Hyde Park, N.Y.) twice a day for a total dosage of 120 μmol per mouse per day. Control mice received, by the same routes and at the same dose, L-arginine acetate made from an equimolar mixture of free base L-arginine (Sigma) and glacial acetic acid.

Nitrogen products excreted in urine. The nitrate reductase-containing bacterium *Pseudomonas oleovorans* was prepared and stored as described previously (20). Twenty-four-hour urine samples from each cage were collected on isopropanol (1 ml of isopropanol per approximately 5 ml of urine) to prevent bacterial growth. All samples were centrifuged at $6{,}000 \times g$ for 10 min to remove debris and stored at −30°C until assayed. At the time of assay, samples were diluted at least 1/10 in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (0.2 M, pH 7.4). *P. oleovorans* bacteria were then thawed, diluted 1/20 with phenol-red-free Dulbecco's modified Eagle medium (DMEM) to about 2.5×10^9 CFU/ml, and kept on ice. To a 1.5-ml microcentrifuge tube was added 30 μl of a diluted urine sample, 150 μl of 0.2 M HEPES, and 150 μl of diluted *P. oleovorans*. The tubes were incubated at 37°C for 90 min and then microcentrifuged for 2 min to pellet the bacteria. Sample supernatant (0.4 ml) was assayed for nitrite spectrophotometrically, by using Griess reagents, as described previously (21). The concentration of sample nitrate was calculated from a standard curve of absorbance versus known concentrations of nitrate in spiked nitrate-free mouse urine. The curve was linear between 0 and 100 μM nitrate. All urine samples were serially diluted such that the nitrate concentration fell within 10 and 100 μM, the most accurate range of the assay.

The same urine samples used for urinary nitrate determinations were used for total nitrogen, creatinine, and urea determinations. Total nitrogen was obtained via a modified Kjeldahl procedure, as developed by Brinkmann Instruments, Westbury, N.Y., and outlined by the Association of Official Analytical Chemists (4a). These values were used to calculate the nitrogen balance. Urine creatinine was obtained via the Jaffe rate method by using a Synchron CX3 System (Beckman Instruments, Inc., Brea, Calif.) at the Duke University Medical Center clinical laboratory. Urine urea was obtained via urease-catalyzed hydrolysis by using an Ektachem 700 C Series

Analyzer (Eastman Kodak Co., Rochester, N.Y.) at the Duke University Medical Center clinical laboratory. Urine samples had to be diluted 21 to 42 times for creatinine and urea determinations.

Peritoneal macrophage assay. On the days of sacrifice, after heart puncture of the animals but prior to the removal of livers and spleens, a 2-cm incision was made in the peritoneum and the peritoneal cavity was washed with approximately 5 ml of DMEM. The peritoneal wash fluid was removed, centrifuged, and plated at 3×10^6 cells per 16-mm-diameter tissue culture well. Peritoneal macrophages were allowed to adhere for 45 min at 37°C in a humidified 5% CO_2–95% air incubator, and then nonadherent cells were removed by rinsing three times with PBS. The cells were overlaid with DMEM and allowed to incubate overnight under the conditions listed above. Nitrite was assayed from the culture supernatants after 24 h of incubation as described previously (21).

Detection of NOS mRNA. Total RNA from spleen homogenates (dispersed in guanidine isothiocyanate) was isolated by ultracentrifugation on cesium chloride gradients (10). The RNA yield was calculated on the basis of the A_{260}, and the RNA purity was assessed by comparing the A_{260} with the A_{280} of each sample. RNA preparations (10 to 20 μg per lane) were size fractionated by formaldehyde gel electrophoresis through 1% agarose, transferred by capillary blotting to nitrocellulose filters, and then hybridized with radiolabelled cDNA probe for iNOS.

The probe for iNOS was a 3.4-kb fragment kindly provided by Carl F. Nathan (51). This was excised from the plasmid pUC19 with *Hin*dIII and *Eco*RV, isolated by electrophoresis in low-melting-point agarose, and labelled with [^{32}P]dCTP by random primer extension (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) by the method of Feinberg and Vogelstein (18). The probe for beta-actin was excised from the plasmid pA1 by using *Hin*dIII (11). Hybridization with beta-actin was included as a control to confirm the integrity of RNA in each sample and as a semiquantitative check on the amount of RNA per lane. The specific activities of the probes used were greater than 10^9 cpm/μg of DNA. iNOS mRNA and beta-actin migrated with mobilities of 5.0 and 2.0 kb, respectively.

Histology of livers and spleens. Random slices of livers and spleens taken from infected and control mice on days 4 and 7 were preserved in buffered formalin until they were embedded in paraffin for sectioning. The tissue sections (4-μm thick) were stained with hematoxylin and eosin and mounted under coverslips for microscopic examination and microphotography.

Statistical analysis. *Listeria* counts in organs were compared by using a nonparametric test for statistical significance (Wilcoxon rank sums). Blood culture data were compared by using Student's *t* test.

RESULTS

***L. monocytogenes* EGD infection in C57BL/6 mice.** Mice were challenged intravenously with approximately 3×10^3 bacilli per mouse. Mice were sacrificed on days 1, 3, 5, 7, 9, and 13 of infection, and their livers and spleens were assayed for viable listeriae (Fig. 1A). *Listeria* counts per organ in the mouse livers and spleens rose to a level of about 10^5 in the spleen and 10^4 in the liver by the end of day 1. Organism counts plateaued between days 1 and 3. This plateau phase may represent a protective host response which prevents lethal, overwhelming proliferation of listeriae. Between days 5 and 9, a killing response eliminated listeriae from the spleen and liver. By day 9, the organs were sterile and remained so on day 13.

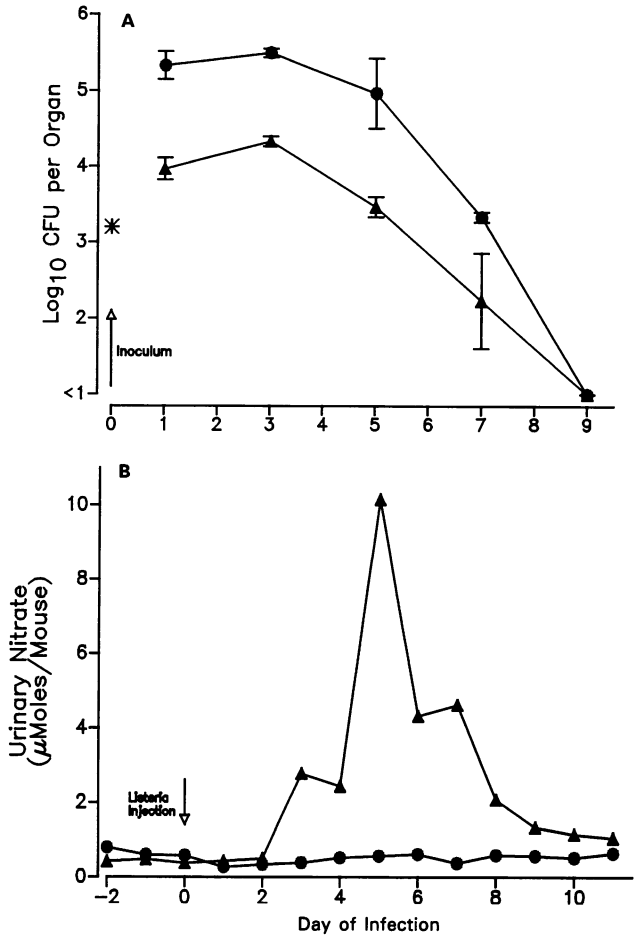

FIG. 1. Correlation between primary response to *Listeria* infection and nitrate excretion in infected mice. (A) C57BL/6 mice were injected intravenously with 2×10^3 *L. monocytogenes* EGD or with PBS on day 0 (asterisk). Their spleens (circles) and livers (triangles) were cultured for listeriae. Each datum point represents the mean count ± standard error for a group of four animals. Uninfected mice had no listeriae in their spleens or livers. Animals sacrificed on day 13 had sterile livers and spleens (not shown). (B) Groups of C57BL/6 mice were adapted to metabolic cages and a nitrate-free diet and injected intravenously with 3×10^3 *L. monocytogenes* EGD (triangles) or with PBS (circles) on day 0. Each datum point represents the per mouse urinary nitrate in a 24-h pool of urine samples from at least four mice.

Correlation of course of *Listeria* infection with urinary nitrate excretion. Mice were adapted to metabolic cages where their water and food intake, urine and stool output, and rectal temperatures were monitored daily and where they were fed a defined nitrate-free rodent diet and given sterilized distilled water. When these mice were infected with the standard *Listeria* inoculum and sacrificed on days 2, 4, and 7, they showed a pattern of liver and spleen organism counts nearly identical to those observed in mice caged in sawdust bedding (data not shown). *Listeria* counts per organ increased to 10^5 in the spleen and 10^4 in the liver by day 2, remained at that level until day 4, and decreased to 10^2 in the spleen and 10^1 in the liver by day 7, suggesting that this experimental environment did not affect the ability of normal mice to control the infection.

Daily 24-h urine samples were collected from infected mice and assayed for nitrate (Fig. 1B). It has been shown that within

TABLE 1. Urinary nitrate excretion correlates with *Listeria* inoculum

No. of expts	Approx size of *Listeria* inoculum (CFU/mouse)	Total urinary nitrate excreted on days 2 to 7 of infection (μmol/mouse)[a]
3	0	3.2 ± 1.85
1	100	5.6
3	1,000–3,000	21.5 ± 1.67
3	5,000–10,000	24.3 ± 1.59

[a] Values are means ± standard errors of the means.

24 h after mice ingest nitrate, 57% of it appears in the urine (20). Another known route of nitrate elimination in rodents is via the feces, in which 20% of the nitrate ingested by germfree rats can be detected (22). It is likely that daily urine assays reflect the nitrate produced over the previous 24 h, and the majority of nitrate entering the circulation is eliminated in the urine.

While on a diet devoid of nitrate or nitrite, mice excreted nitrate at a baseline of about 0.5 μmol per mouse per day in the urine. This amount is consistent with baseline levels excreted by other strains of mice kept on a nitrite- and nitrate-free diet (20) and is thought to be due to endogenous constitutive synthesis by endothelial cells (41) and neurons (33). After *Listeria* challenge, a rise in mouse urinary nitrate excretion was detected by day 3 of infection, with increases of up to 10 μmol per mouse per day by day 5. Nitrate excretion then decreased, returning to near-baseline levels by day 9. This pattern of urinary nitrate excretion during primary listeriosis was highly reproducible, the peak always occurring between days 5 and 6 of infection, which was also the period when *Listeria* counts in the mouse livers and spleens began to decline. The magnitude of the nitrate excretion peak depended on the number of listeriae injected (Table 1). An intravenous injection of about $0.1\times$ LD_{50} of listeriae was determined to be optimal, with the goal of maximizing urinary nitrate excretion and yet minimizing mouse mortality.

Evidence that this increase in urinary nitrate excretion was related at least in part to an increase in NO production by activated macrophages inside the mouse was obtained by culturing ex vivo peritoneal macrophages from sacrificed mice and assaying the culture supernatants for nitrite. Peritoneal macrophages from five of five mice infected with the standard *Listeria* inoculum and sacrificed on days 4 and 7 of infection produced 15 to 25 nmol of nitrite per 10^6 cells when cultured ex vivo for 24 h, whereas peritoneal macrophages from uninfected mice did not.

Twenty-four-hour urine creatinine excretions from mice infected with *Listeria* spp. were not significantly different from those from uninfected mice, suggesting that sublethal listeriosis caused no compromise in renal function.

Correlation between *Listeria* infection and detection of NOS mRNA. Mice were infected with the standard *Listeria* dose. As a positive control, NOS was induced in vitro by gamma interferon and endotoxin in the macrophage-like cell line J774.1 (49) (Fig. 2). iNOS mRNA was not present in uninduced J774 cells or in splenic RNA extracts of an uninfected mouse. The iNOS message was detected on the first day of listeriosis, increased to peak expression on day 4, and then decreased markedly on day 5 to barely detectable amounts on days 6 and 7. The onset of iNOS mRNA synthesis in the spleens of *Listeria*-infected mice followed within 12 h the onset of gamma interferon, tumor necrosis factor alpha (TNF-α), and interleukin-1β mRNA syntheses in these mice, which occurred 15 to 18 h after *Listeria* challenge (43, 44). These cytokines are known inducers of iNOS in macrophages and hepatocytes (12, 15).

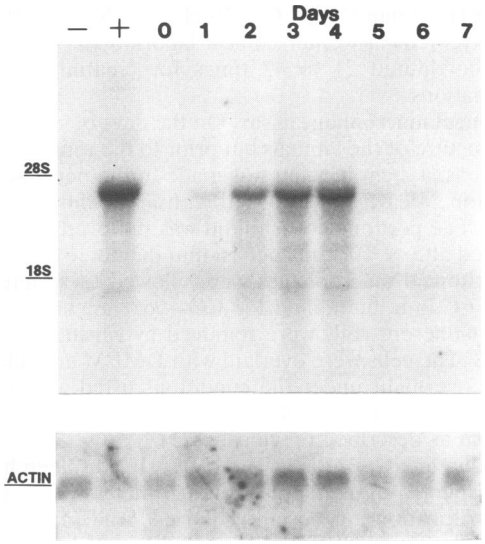

FIG. 2. Effect of *Listeria* infection on the detection of mRNA for the iNOS in the spleens of mice. Northern (RNA) blots on total RNA extracted from spleen on days 0 to 7 of infection with 5×10^3 listeriae are shown. One infected mouse was sacrificed each day. mRNA for iNOS migrates near the 28S rRNA band (marker placed from ethidium bromide-stained gel). The probe was a 3.4-kb fragment labelled with ^{32}P. Each lane was loaded with 20 μg of RNA. The actin probe for the same filter is shown in the inset at the bottom of the figure. Lanes: –, RNA extract from J774.1 macrophage-like cells; +, same cells as those in lane – but treated with gamma interferon (50 U/ml) plus endotoxin (1.0 μg/ml) to induce NOS.

Effect of NMMA administration on the excretion of nitrate by mice during *Listeria* infection. Mice not infected with listeriae were given NMMA to test for side effects. Gavage with 60 μmol of NMMA per mouse twice daily did not alter food and water intake, stool and urine output, or body temperature. In addition, upon sacrifice, the viscera of the animals were normal upon gross examination. However, uninfected mice which received an instilled gastric dosage of 300 μmol of NMMA per mouse per day had significant decreases in food and water intake, and they developed diarrhea (data not shown). These changes were evident within 2 days of administration. When sacrificed after 7 days at this NMMA dosage, the animals were noted uniformly to have inflamed, distended ceca, suggesting typhlitis. Trials with routes of administration other than gavage showed that mixing NMMA in food and water resulted in an undesirable animal-to-animal variation and that the use of subcutaneous NMMA administration and slow-release capsules appeared to be less effective than oral dosing in suppressing urinary nitrate (data not shown). On the basis of these findings, the total dosage of 120 μmol per mouse per day given in two dosages by gavage 12 h apart was chosen for experiments involving *Listeria* infection.

When mice were adapted to metabolic cages, infected intravenously with the standard *Listeria* inoculum, and given NMMA via direct gastric instillation at a total dosage of 120 μmol per mouse per day beginning on day 1 of infection, they showed no surge in urinary nitrate excretion (compared with control mice infected with the same *Listeria* inoculum and given an equivalent dose of L-arginine as a sham drug admin-

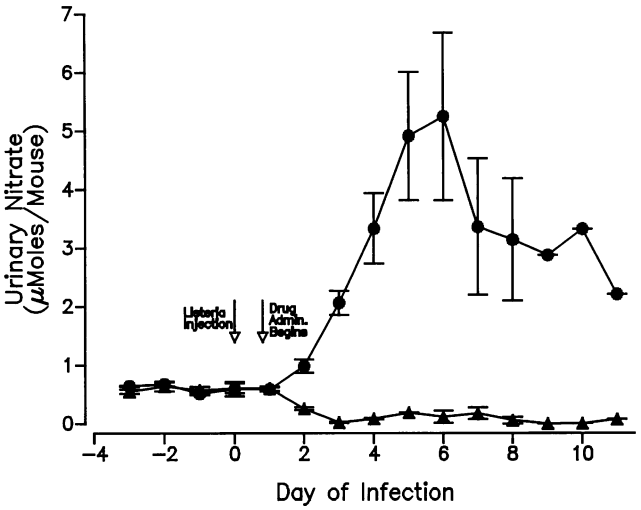

FIG. 3. Effect of oral NMMA administration on urinary nitrate excretion during primary murine listeriosis. Groups of C57BL/6 mice were adapted to metabolic cages and a nitrate-free diet and injected intravenously with 3×10^3 *L. monocytogenes* EGD on day 0. On day 1, animals began to receive 60 μmol of NMMA (triangles) or L-arginine (circles) via direct gastric instillation twice a day. Each datum point represents the mean ± standard error of at least 4 (days 9 to 11) but usually 8 (days 5 to 8) to 12 (days 0 to 4) mice.

istration). In fact, NMMA-treated animals showed an almost undetectable amount of urinary nitrate excretion, dropping from 0.5 μmol per mouse per day to below baseline levels (<0.1 μmol per mouse per day) about 24 h after receiving the first dose of NMMA (Fig. 3). Thus, in vivo NO activity, as monitored by elimination of the end product metabolite of NO in the urine, was suppressed by NMMA given via the gastrointestinal tract.

Effect of NMMA on the course of primary listeriosis. Mice were infected intravenously with the standard *Listeria* inoculum and given NMMA at a dosage of 60 μmol per mouse every 12 h beginning on day 1 of infection or L-arginine (control group) at the same dosage in the same manner. Control mice manifested clinical signs of infection. Mean temperatures increased from 37 to 39°C during days 2 to 4 of infection, and mean food and water intake decreased during days 2 to 5 as mice developed a concomitant loss in body weight. NMMA-treated mice showed reproducibly worse clinical signs of infection than control mice (Fig. 4). Weight loss in infected control mice was minimal and transient, whereas NMMA-treated animals developed sustained weight losses of up to one-fifth of their body weight. NMMA-treated mice also had significant decreases in food and water consumption compared with control infected mice. Beginning on day 6, these decreases began to lessen, but normal intake levels were not reached by day 8. NMMA treatment of infected mice also affected body temperature. This occurred later during infection (days 6 to 8) when subnormal body temperatures were observed. The ear-

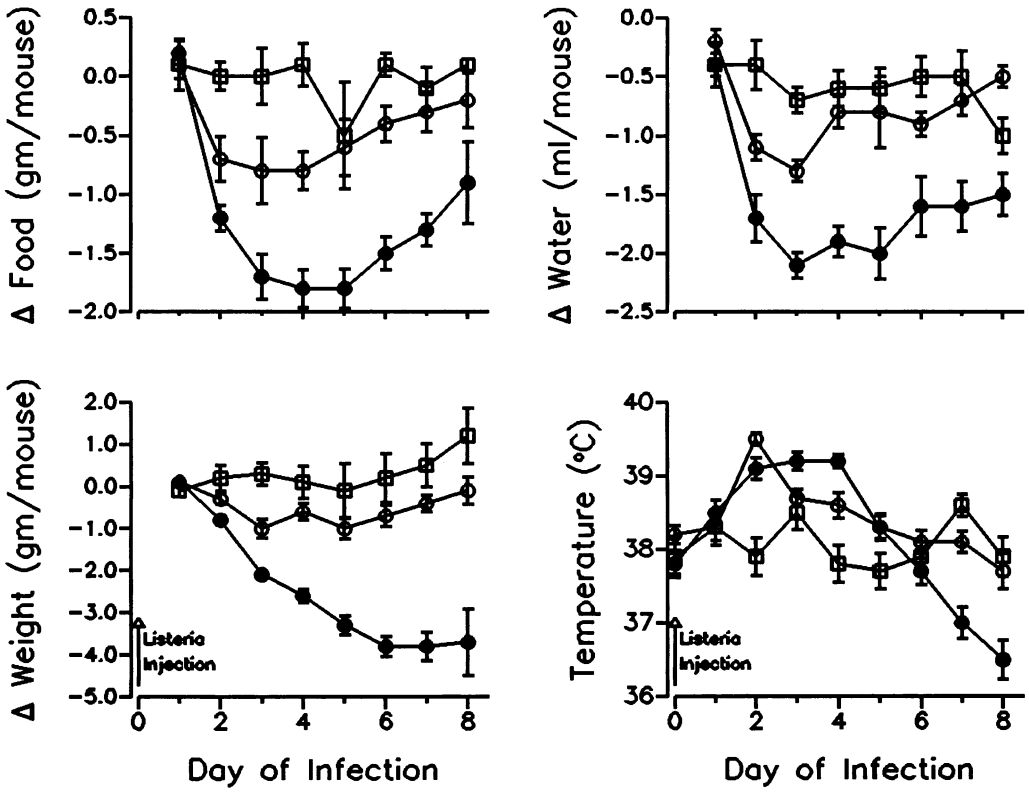

FIG. 4. Clinical assessment of mice given NMMA orally during primary listeriosis. Groups of C57BL/6 mice were adapted to metabolic cages and a nitrate-free diet and injected intravenously with 3×10^3 *L. monocytogenes* EGD organisms (circles) or with PBS (squares) on day 0. On day 1, animals began to receive NMMA (closed circles, open squares) or L-arginine (open circles) via direct gastric instillation (total amount, 120 μmol per mouse per day given in two doses). Each datum point represents the mean ± standard error of at least 11 but usually 17 to 32 mice from five different experiments. Weight, food intake, and water intake are expressed as daily deviations from day 0 (preinfection) values.

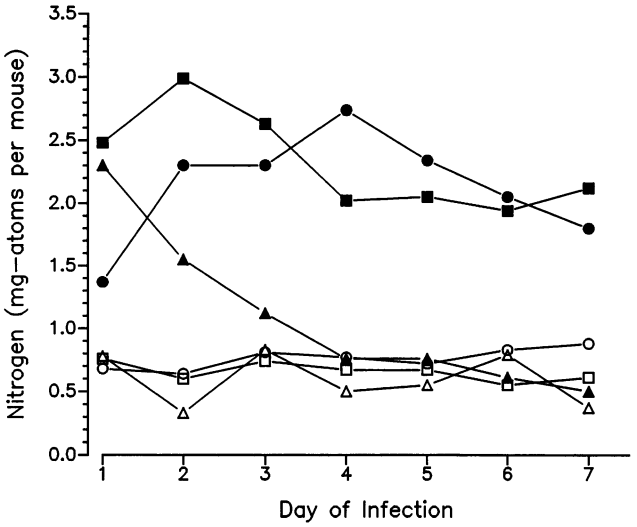

FIG. 5. Daily nitrogen intake and urine excretion of nitrogen during *Listeria* infection. Mice received approximately 3,000 listeriae (triangles, squares) or PBS (circles) intravenously on day 0. One group (squares) was given L-arginine orally, 120 μmol per mouse per day, while the others (triangles, circles) received NMMA orally at 120 μmol per mouse per day. Nitrogen intake (closed symbols) was calculated from food nitrogen assayed by a macro-Kjeldahl method. This measured value agreed with the calculated amount from the diet recipe. Urine nitrogen (open symbols) was assayed by a macro-Kjeldahl method. Urine urea, creatinine, and nitrate concentrations were also determined, and their sum of nitrogen closely approximated Kjeldahl urine nitrogen. Each value represents milligram atoms of nitrogen per mouse.

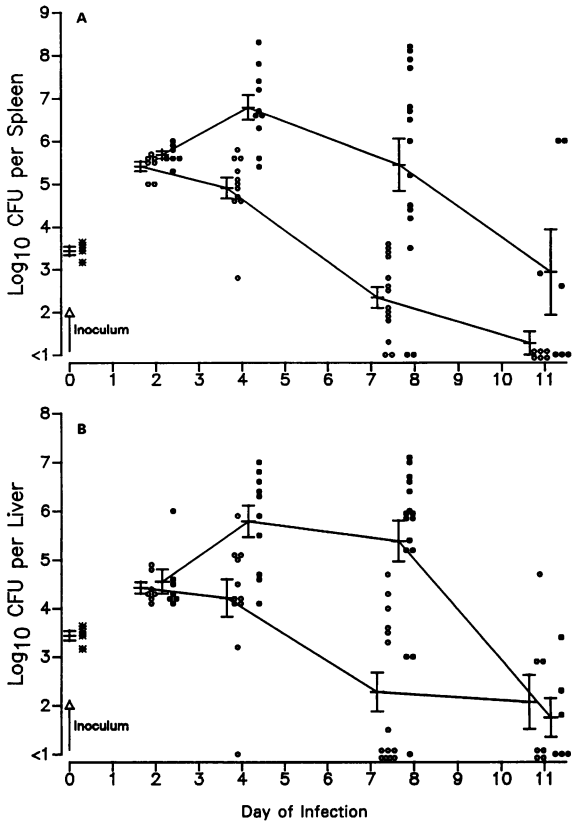

FIG. 6. Effect of oral NMMA administration on spleen and liver organism counts during primary murine listeriosis. Groups of C57BL/6 mice were adapted to metabolic cages and a nitrate-free diet and injected intravenously with 3×10^3 *L. monocytogenes* EGD organisms on day 0 (asterisks). On day 1, animals began to receive NMMA (closed circles) or L-arginine (open circles) orally at a dosage of 120 μmol per mouse per day. For each day of sacrifice, each circle represents a count from a single animal. To the left of each group of circles is the mean ± standard error for that group. *n* ranges from 6 to 21 animals per group. Data from five separate experiments are shown. Comparisons of the two groups are statistically different on days 4 and 7 for both spleen (A) and liver (B) counts (see text).

lier febrile response (days 2 to 4) to infection was not altered by NMMA. In one experiment, nitrogen intake and excretion of nitrogen in urine were measured (Fig. 5). In both uninfected mice treated with NMMA and infected mice receiving L-arginine, daily nitrogen intake and urine nitrogen excretion were fairly constant. Intake exceeded urine excretion in these animals because of the following. (i) The animals were in a state of positive nitrogen balance, being preadult, growing mice. (ii) It is estimated that 10 to 20% of the nitrogen is lost in feces and hair, etc. (iii) Urine nitrogen levels may be underestimated because evaporation may leave some solute coated upon the collecting cones. Nevertheless, NMMA-treated, infected mice had a marked alteration in nitrogen balance. Their urine nitrogen excretion remained about the same despite a significant drop in nitrogen intake such that on days 4 to 7 they entered a state of presumed negative nitrogen balance. This indicates mobilization of lean body mass, leading to inanition. The partitioning of nitrogen excretion was also determined (data not shown). Irrespective of the group, 98 to 99% of excreted nitrogen was urea, and 1 to 2% was creatinine on each of the 7 days. The amount of nitrogen lost as nitrate was a fraction of 1%. Even when nitrate excretion peaked in infected mice, this amounted to only 0.7% of total nitrogen loss. Thus, NOS-derived nitrogen contributes negligibly to total body nitrogen balance.

The results of *Listeria* counts in organs of NMMA-treated mice are compiled from five experiments and presented in Fig. 6. Spleen and liver counts on day 2 showed no difference between NMMA- and arginine-treated groups in two separate experiments (n = 14 mice [total]). On day 4, there was about 100 times more listeriae in the spleens and livers of NMMA-treated mice than in control mice (n = 21 mice from three experiments; $P < 0.0005$, spleen; $P < 0.009$, liver). By day 7 or 8, the mean difference increased up to 3 orders of magnitude, and this was due primarily to decreased spleen and liver counts in arginine-treated animals at a time when NMMA-treated mice failed to clear listeriae from these sites (n = 14 from four experiments; $P < 0.0008$, spleen; $P < 0.0003$, liver). In two experiments extended to day 11, infected, NMMA-treated mice showed variability in outcome. Three of seven mice receiving NMMA cleared their livers and spleens of listeriae by day 11. Another three mice showed heavy infection and/or were clinically premorbid (with wasting or hypothermia) by day 11. One of the seven mice died heavily infected. Six of seven arginine-treated mice had sterile spleens and appeared healthy by day 11. Four of seven had sterile livers. Of the organs that were not sterile in these animals, *Listeria* counts were relatively low (10^2 to 10^4). Thus, the ultimate fate of infected NMMA-treated mice is variable and requires more study.

Blood culture data were obtained on days 4 and 7 at the time that animals were sacrificed. Fifty-two percent of NMMA-treated mice (13 of 25) had *Listeria* bacteremia, while none of

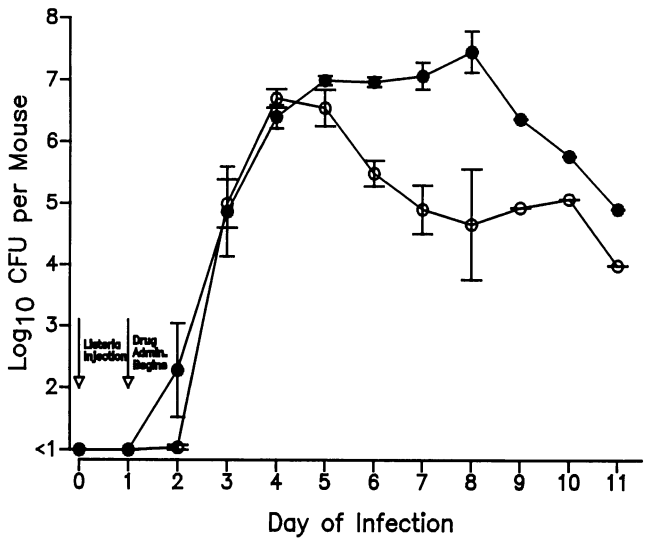

FIG. 7. Effect of oral NMMA administration on excretion of listeriae in stools during primary murine listeriosis. Groups of C57BL/6 mice were adapted to metabolic cages and a nitrate-free diet and injected intravenously with 3×10^3 *L. monocytogenes* EGD organisms on day 0. On day 1, animals began to receive NMMA (closed circles) or L-arginine (open circles) orally at a dosage of 120 μmol per mouse per day. Stool was cultured for listeriae on modified Oxford medium containing selective antibiotics. Each datum point represents the *Listeria* count per mouse in a 24-h pool of stool samples from at least four mice. Uninfected mice had no listeriae in their stools (data not shown).

the arginine-treated mice (0 of 25) had a positive blood culture ($P < 0.0001$).

Listeria counts in stool samples paralleled the rise and fall of organism counts in viscera. Stool samples were collected every 24 h during the infection period, and *Listeria* excretion was calculated as the total number of live bacteria recovered per mouse per day (Fig. 7). Mice began excreting low levels of bacteria in the stool by day 1 of infection, after which excretion increased logarithmically to a peak of about 10^7 per mouse by day 5. Of the two groups, greater shedding of listeriae was found in the stools of NMMA-treated mice. This difference was evident by day 5 and continued throughout the course of infection. In one experiment, *Listeria* excretion in the stool continued after the livers and spleens were sterile, suggesting persistence at another site such as in the intestinal wall or the lymphatics or as part of the fecal flora.

Histopathology of listeriosis in mice treated with NMMA (Fig. 8). NMMA could have multiple effects on the cell-mediated immune response to *Listeria* infection. In addition to inhibiting the production of NO as an antibacterial effector molecule, NMMA could alter proliferation events in the bone marrow and lymph nodes and at sites of infection. NMMA could also inhibit leukocyte immigration into infected tissue. Therefore, it was important to examine the morphology of the livers and spleens in mice treated with this compound. Histologic sections stained with hematoxylin and eosin from mice sacrificed on days 4, 8, and 11 of infection were examined. Uninfected mice treated with 120 μmol of NMMA per day had no histologic changes. Infected mice treated with arginine showed minimal changes, probably because the *Listeria* inoculum was relatively low (approximately 2,000 CFU per mouse). There were small clusters of inflammatory cells in the liver (4 to 16 per 10 low-power fields) on days 4 and 8, but no necrosis was present in the liver or spleen and neutrophils were rare to nonexistent. In contrast, NMMA-treated infected mice had 11 to 40 (per 10 low-power fields) clusters of inflammatory cells in the liver, some of which showed necrosis with hypereosinophilic hepatocytes containing pyknotic nuclei. These areas were surrounded by mononuclear cell infiltrates containing prominent histiocytes. Necrosis was likewise apparent in the spleens of NMMA-treated infected mice. There was exudate over the splenic capsule, and the follicular architecture was disrupted by extensive necrosis and by mononuclear cell infiltration containing masses of histiocytes. In both organs, inflammation showed progression between days 4 and 8. It was clear from the histopathology that NMMA treatment did not block cellular infiltration into sites of infection. The findings are compatible with macrophage immigration into the livers and spleens of NMMA-treated, infected mice.

DISCUSSION

These results show a reproducible induction of iNOS activity during primary listeriosis. The time course of nitrate excretion in primary listeriosis is unique in its abrupt onset and decline. This observation can be correlated with what is known about cytokine secretion during this infection. In the spleen, a major site of organism burden, the mRNAs for gamma interferon, TNF-α, and interleukin-1β are induced maximally on the first day of infection (44). The mRNA for iNOS appears shortly thereafter. Urinary excretion of the NO end product, nitrate, lags by about 24 to 48 h. This lag between message and end product excretion is probably determined by several steps, including (i) synthesis of an active enzyme, which requires several cofactors, (ii) conversion of NO via several intermediates to nitrate, and (iii) excretion of nitrate by the kidneys. Considering all of these factors, the bulk of NO synthesis in the spleen most likely takes place between days 2 and 5 of infection. It seems likely that macrophages in the spleen and that macrophages and hepatocytes (7) in the liver are the sources of the surge of nitrate excretion during primary infection. Other possible sources of induced NO production are endothelial cells (41) and smooth muscle cells (8). The relative contribution of these cells to the surge observed in *Listeria*-infected mice is as yet unknown.

It is clear from Fig. 1 that an exponential expansion of the bacterial population occurs during the first 24 h. This early, rapid expansion may occur in the nonimmune host because listeriae circumvent constitutive defense mechanisms by gaining access to the cytosolic compartment of macrophages and hepatocytes (26, 42). Here they would not be subjected to a hostile phagolysosomal environment, and they would have direct access to nutrients required for rapid replication. If proliferation continued at the rate seen over the first 24 h, there would be 10^9 listeriae per spleen by day 3, a near-lethal number. In reality, *Listeria* counts remained relatively constant from days 1 to 5 at 10^5 in the spleen and 10^4 in the liver. This could be a result of (i) halted bacterial replication, (ii) a rate of bacterial killing which equals bacterial replication, or (iii) a combination of (i) and (ii). Because this plateau phase of infection coincides with the period of NO synthesis, NO may exert its bacteriotoxic effect during this period. Results from several in vitro assays and from studies on the antimicrobial action of reagent NO attest to the microbiostatic activity of this compound (3, 45).

To determine more directly the extent to which NO is important in immune defense against intracellular pathogens, we examined the effect of inhibiting iNOS in vivo during an infection. Substituted arginines have been shown to be sub-

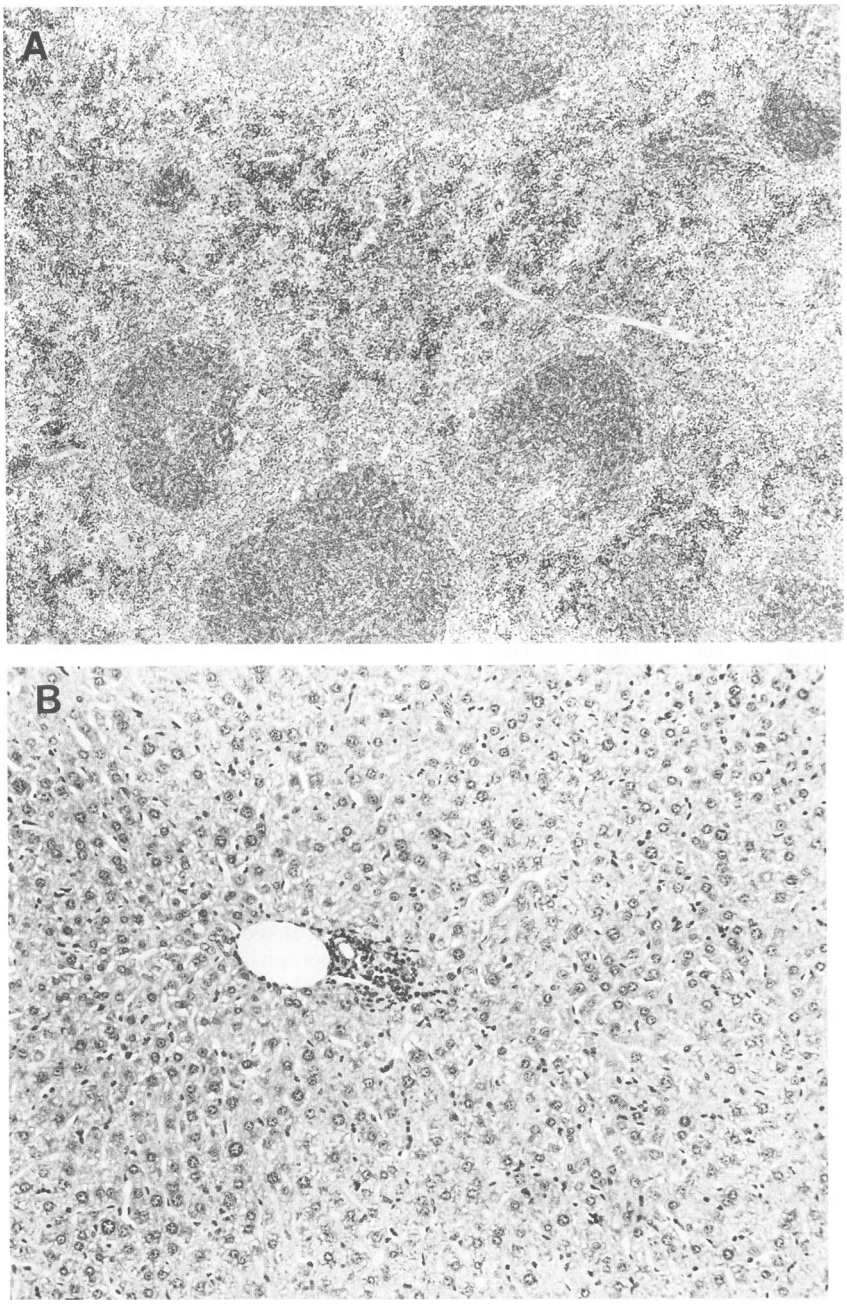

FIG. 8. Effect of oral L-arginine or NMMA administration on spleen and liver histopathology during primary murine listeriosis. C57BL/6 mice were challenged intravenously with 3×10^3 *L. monocytogenes* EGD organisms on day 0, treated daily with oral NMMA or L-arginine beginning on day 1, and sacrificed on day 8 of infection. (A) The spleen from an L-arginine-treated infected mouse shows normal splenic architecture consisting of germinal centers and intervening extramedullary hematopoiesis (dark staining areas between germinal centers). There is no inflammatory infiltrate. Magnification, ×52. (B) The liver from the same animal shows a portal triad with surrounding normal liver parenchyma. Magnification, ×130. (C) The spleen from an NMMA-treated infected mouse shows geographic areas of necrosis outlined by a hyperemic rim of inflammation. Magnification, ×40. (D) Higher-power magnification of the spleen reveals a primary inflammatory infiltrate composed of large masses of histiocytes and lymphocytes with few neutrophils. Magnification, ×520. (E and F) The liver from the same animal shows many large clusters of mononuclear cell inflammation, some of which are necrotic, with hepatocytes containing pyknotic nuclei. Magnifications, ×170 and ×250, respectively.

strate analogs and competitive inhibitors of iNOS in vitro (30). The overall pharmacology of these compounds has not yet been investigated. However, one of them, NMMA, has been shown to have relatively little effect on other arginine biochemical pathways in vitro, namely the urea cycle, protein synthesis, and polyamine synthesis, while at the same time effectively inhibiting the synthesis of NO by cytotoxic macrophages (21). In addition, the potency of NMMA as an iNOS inhibitor is about 10 times greater than its inhibitory effect on the L-arginine transport system of macrophages (19a). These obser-

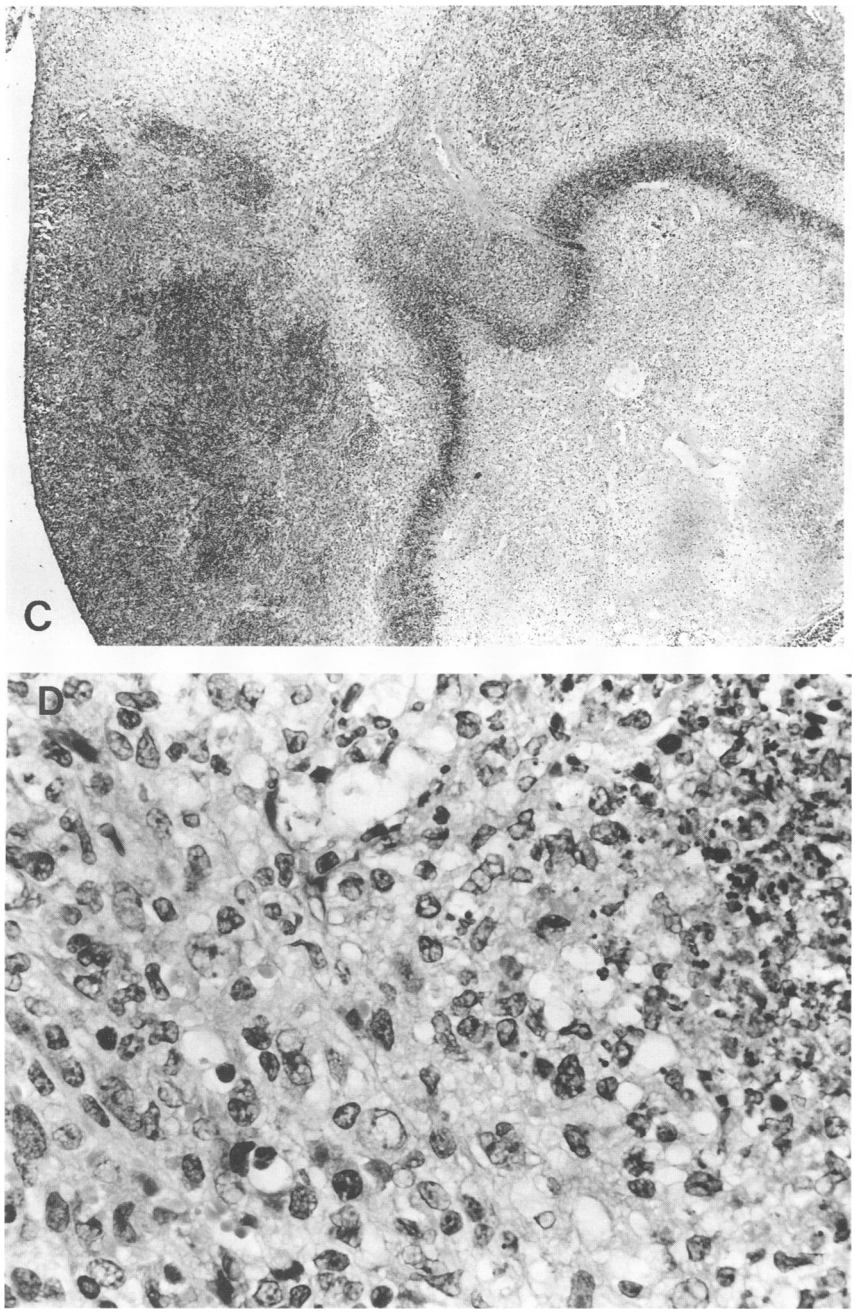

FIG. 8—*Continued*

vations suggest that NMMA is relatively specific for NOS inhibition. Unfortunately, inhibitors with specificity for iNOS without inhibition of constitutive NOS are not yet available. NMMA appears to be rapidly absorbed by the gastrointestinal tract, as shown by the suppression of urinary nitrate excretion within 24 h after NMMA administration (Fig. 3). Data regarding the half-life of its action are lacking. However, experiments by Evans et al., in which NMMA was administered to mice via their drinking water to suppress NO production induced by cutaneous infection with *Leishmania major* and in which NMMA treatment was abruptly stopped, showed that nitrate excretion increased to high levels within 24 h after NMMA discontinuation, suggesting a half-life of less than 24 h (17). Taking these results into account, the twice-daily gastric dosing of NMMA used in our experiments should have been sufficient to suppress NO production around the clock. Drug administration was delayed until 12 to 24 h after *Listeria* injection to let the organisms disperse via an unaltered vasculature because it was presumed that inhibition of endothelial cell NOS would cause vasoconstriction.

Recently, Beckerman et al. treated *Listeria*-infected SCID mice with aminoguanidine, an inhibitor of NOS (6). Listeriosis in SCID mice manifests itself as a chronic, persisting infection, a result of a T-cell-independent host resistance to lethal proliferation of organisms. Aminoguanidine-treated mice showed greater burdens of listeriae in the liver and spleen as

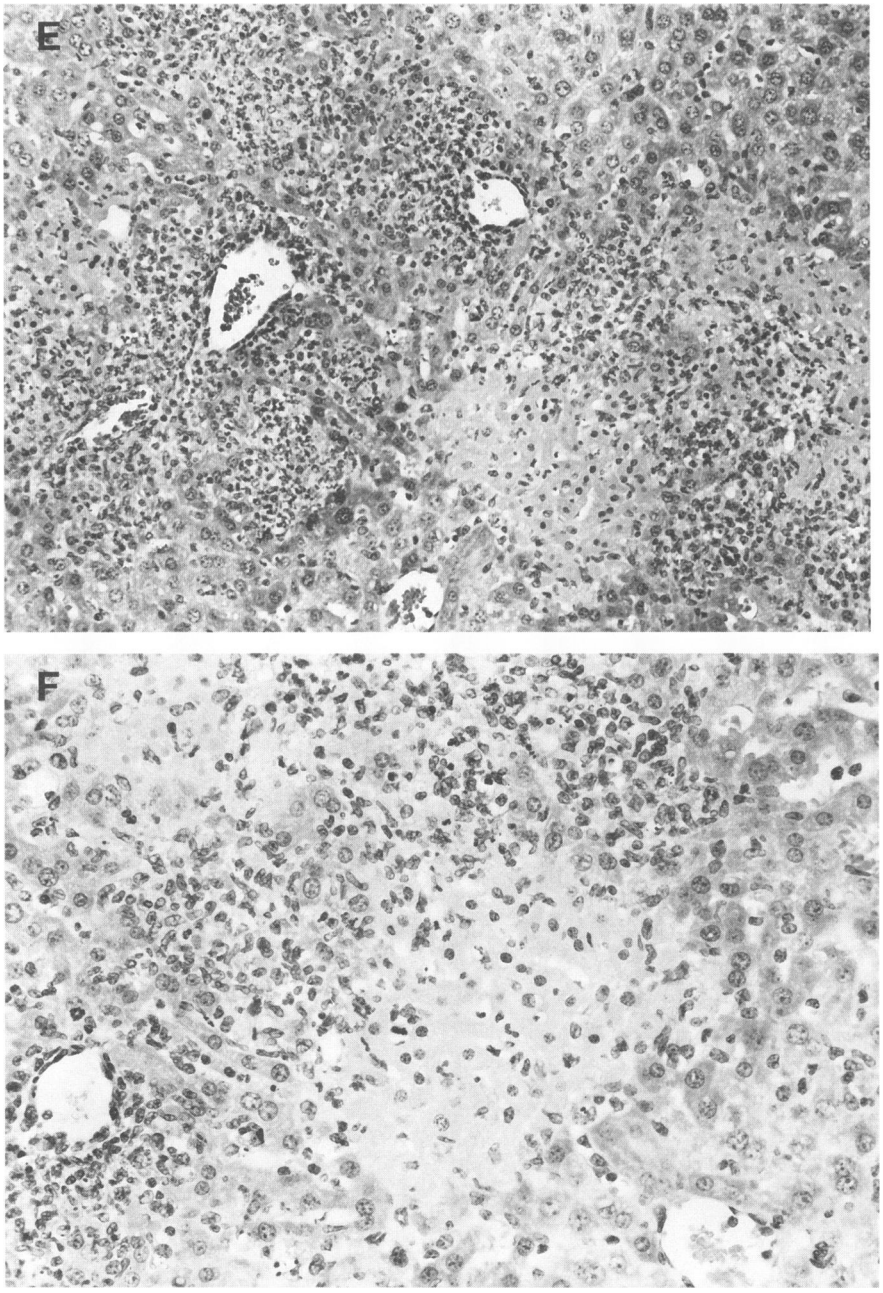

FIG. 8—*Continued*

well as increased mortality compared with untreated mice. Although no measurements of NO production in vivo were provided, the authors propose that iNOS may be involved in T-cell-independent host resistance in *Listeria*-infected SCID mice. This resistance mechanism could involve bacteriostasis without killing, an activity which corresponds with a known in vitro action of NO (3).

Gregory et al. recently treated mice with one intravenous dose of NMMA at the time of *Listeria* challenge (27). Considering the presumed short half-life of NMMA, it is not surprising that there was no effect of this day 0, one-time dose on serum NO_2^- and NO_3^- levels in mice on days 3 and 7 of infection. However, NMMA-treated mice had significantly fewer listeriae in their livers on day 3 of infection and in their livers and spleens on day 7 of infection than untreated infected control mice. The authors attribute this immuno-enhancing effect of NMMA treatment to the suppression of an inhibiting effect of NO on the proliferation of T lymphocytes. The timing and dose of NMMA treatment in their experiments make a comparison of their results with those of our experiments difficult. It may be that NO has immuno-enhancing and immunosuppressive effects at different phases of infection due to its biostatic effects on both microorganisms and T lymphocytes.

Our results demonstrate that total body NO production was nearly completely inhibited by NMMA administration and that NMMA, at the dose used, had no untoward effects on healthy mice. NO inhibition in *Listeria*-infected mice, however, led to

worsened primary infection. On the average, *Listeria* counts were 2 orders of magnitude greater in the livers and spleens of NMMA-treated mice on day 4 and 3 orders of magnitude greater on days 7 and 8 than in control mice. This was corroborated by finding increased *Listeria* shedding in stool samples, frequent bacteremia, and clinical signs reminiscent of uncontrolled infection NMMA-treated mice. The histopathology in the liver and spleen is compatible with the notion that macrophages and other cells at the sites of infection may be less able to restrict *Listeria* multiplication. This may be due to failure of an NO-mediated bacteriostatic host defense mechanism operating early during the course of primary infection. By days 5 to 6, however, once T-cell immunity is acquired, other bactericidal mechanisms may be activated, leading to eradication of listeriae from the liver and spleen. The complete recovery of some NMMA-treated, NO-suppressed, *Listeria*-infected mice by day 11 is compatible with this hypothesis. It is more likely that an NO-independent killing mechanism accounts for the recovery of some NMMA-treated mice than that NOS activity escapes inhibition in some individual animals. This is because pooled urine samples from four mice treated with NMMA consistently contain such small amounts of nitrate that significant NO synthesis by one or two animals in the group seems very unlikely. When anticytokine antibodies are used to immunosuppress mice, survivors are rare. These treatments may inhibit both NO-dependent and NO-independent mechanisms. NMMA treatment might be specific for inhibiting a single effector mechanism and hence produces a less-drastic effect on the cell-mediated immune response. Support for this notion comes from recent experiments which show rapid killing of listeriae in the adoptive immunity situation in the complete absence of NOS induction (36a).

When *Listeria*-infected mice were treated with NMMA, a state of inanition ensued. Mice experienced significant decreases in thirst, hunger, temperature, and body weight. These effects can be produced by administration of recombinant TNF to mice (5, 50). TNF is an important proinflammatory monokine required for an effective host response against *Listeria* infection (39), and it is a co-inducer of iNOS transcription (15, 16). The TNF-like effects seen in infected mice treated with NMMA are probably a result of the increased burden of bacteria present during days 4 through 8. Alternatively, NO produced during infection could be directly involved in regulating TNF secretion. Against the latter possibility is the observation that the loss of hunger and thirst improved on days 6 to 8 (Fig. 4) and yet NO synthesis remained inhibited (Fig. 3). This issue will require further investigation employing an assay to quantitate TNF produced at the major sites of infection (39).

On the basis of our results and those cited from previously published data, it is becoming clear that the induction of NOS is an important component of the cell-mediated immune response to intracellular infections. Future studies are required to investigate specific roles for the products of NOS and to determine the cell types and locations in the body in which iNOS is active. Very little information is available regarding the issue of NOS induction during infection in humans. In vitro studies on human macrophages and monocytes, save for a few reports, have not demonstrated iNOS activity (9, 14, 38, 46, 47). In vivo studies in humans are needed to determine whether, as in the case of rodents, there is an augmentation of NOS activity during intracellular infections. Molecular techniques can be used to detect iNOS transcription in human tissues, and specific antibodies are available which may be used to detect this enzyme by immunohistochemistry. Nitrate excretion studies done in the appropriately controlled clinical setting may reveal inflammatory states which cause urinary nitrate to increase in humans. The challenge then will be to determine what roles NO plays during inflammation.

ACKNOWLEDGMENTS

We thank Denise Paulsen for conscientious technical assistance and Mary Ann Howard and Janet Routten for preparing the manuscript. Carl Nathan, Department of Medicine, Cornell University Medical Center, kindly provided the plasmid in *E. coli* used to detect mouse iNOS mRNA. We also thank Phyllis Snyder of the Duke Medical Center Nutritional Support Service for performing Kjeldahl assays on food and urine samples.

This work was supported by Public Health Service grant AI26188 from the National Institutes of Health.

REFERENCES

1. **Adams, L. B., S. G. Franzblau, Z. Vavrin, J. B. Hibbs, Jr., and J. L. Krahenbuhl.** 1991. L-arginine-dependent macrophage effector functions inhibit metabolic activity of *Mycobacterium leprae*. J. Immunol. **147:**1642–1646.
2. **Adams, L. B., J. B. Hibbs, Jr., R. R. Taintor, and J. L. Krahenbuhl.** 1990. Microbiostatic effect of murine activated macrophages for *Toxoplasma gondii*: role of synthesis of inorganic nitrogen oxides from L-arginine. J. Immunol. **144:**2725–2729.
3. **Alspaugh, J. A., and D. L. Granger.** 1991. Inhibition of *Cryptococcus neoformans* replication by nitrogen oxides supports the role of these molecules as effectors of macrophage-mediated cytostasis. Infect. Immun. **59:**2291–2296.
4. **Anthony, L. S. D., P. J. Morrissey, and F. E. Nano.** 1992. Growth inhibition of *Francisella tularensis* live vaccine strain by IFN-gamma-activated macrophages is mediated by reactive nitrogen intermediates derived from L-arginine metabolism. J. Immunol. **148:**1829–1834.

4a. **Association of Official Analytical Chemists.** 1975. Official Methods of Analysis of the AOAC, 12th ed. Association of Official Analytical Chemists, Arlington, Va.

5. **Bauss, F., W. Droge, and D. N. Mannel.** 1987. Tumor necrosis factor mediates endotoxic effects in mice. Infect. Immun. **55:**1622–1625.
6. **Beckerman, K. P., H. W. Rogers, J. A. Corbett, R. D. Schreiber, M. L. McDaniel, and E. R. Unanue.** 1993. Release of nitric oxide during T cell-independent pathway of macrophage activation. J. Immunol. **150:**888–895.
7. **Billiar, T. R., R. D. Curran, D. J. Stuehr, J. Stadler, R. L. Simmons, and S. A. Murray.** 1990. Inducible cytosolic enzyme activity for the production of nitrogen oxides from L-arginine in hepatocytes. Biochem. Biophys. Res. Commun. **168:**1034–1036.
8. **Busse, R., and A. Mulsch.** 1990. Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. FEBS Lett. **275:**87–90.
9. **Cameron, M. L., D. L. Granger, J. B. Weinberg, W. J. Kozumbo, and H. S. Koren.** 1990. Human alveolar and peritoneal macrophages mediate fungistasis independently of L-arginine oxidation to nitrite or nitrate. Am. Rev. Respir. Dis. **142:**1313–1319.
10. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18:**5294–5299.
11. **Cleveland, D. W., M. A. Lopata, R. J. MacDonald, N. J. Cowan, W. J. Rutter, and M. W. Kirschner.** 1980. Number and evolutionary conservation of alpha- and beta-tubulin and cytoplasmic beta- and gamma-actin genes using specific cloned cDNA probes. Cell **20:**95–105.
12. **Curran, R. D., T. R. Billiar, D. J. Stuehr, J. B. Ochoa, B. G. Harbrecht, S. G. Flint, and R. L. Simmons.** 1990. Multiple cytokines are required to induce hepatocyte nitric oxide production and inhibit total protein synthesis. Ann. Surg. **212:**462–469.
13. **Denis, M.** 1991. Interferon-gamma-treated murine macrophages inhibit growth of tubercle bacilli via the generation of reactive nitrogen intermediates. Cell. Immunol. **132:**150–157.
14. **Denis, M.** 1991. Tumor necrosis factor and granulocyte macrophage-colony stimulating factor stimulate human macrophages to restrict growth of virulent *Mycobacterium avium* and to kill aviru-

lent *M. avium*: killing effector mechanism depends on the generation of reactive nitrogen intermediates. J. Leukocyte Biol. **49:** 380–387.
15. **Ding, A. H., C. F. Nathan, and D. J. Stuehr.** 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. J. Immunol. **141:**2407–2414.
16. **Drapier, J. C., J. Wietzerbin, and J. B. Hibbs, Jr.** 1988. Interferon-gamma and tumor necrosis factor induce the L-arginine-dependent cytotoxic effector mechanism in murine macrophages. Eur. J. Immunol. **18:**1587–1592.
17. **Evans, T. G., L. Thai, D. L. Granger, and J. B. Hibbs, Jr.** 1993. Effect of in vivo inhibition of nitric oxide production in murine leishmaniasis. J. Immunol. **151:**907–915.
18. **Feinberg, A. P., and B. Vogelstein.** 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **137:**266–267. (Addendum.)
19. **Garthwaite, J., S. L. Charles, and R. Chess-Williams.** 1988. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. Nature (London) **336:**385–388.
19a. **Granger, D. L.** Unpublished data.
20. **Granger, D. L., J. B. Hibbs, Jr., and L. M. Broadnax.** 1991. Urinary nitrate excretion in relation to murine macrophage activation. Influence of dietary L-arginine and oral N^G-monomethyl-L-arginine. J. Immunol. **146:**1294–1302.
21. **Granger, D. L., J. B. Hibbs, Jr., J. R. Perfect, and D. T. Durack.** 1990. Metabolic fate of L-arginine in relation to microbiostatic capability of murine macrophages. J. Clin. Invest. **85:**264–272.
22. **Green, L. C., S. R. Tannenbaum, and P. G. Goldman.** 1981. Nitrate synthesis in the germ-free and conventional rat. Science **212:**56–58.
23. **Green, S. J., R. M. Fortier, R. M. Crawford, and C. A. Nacy.** 1991. *Francisella tularensis* is susceptible to nitrogen oxides produced by cytokine-activated murine macrophages, p. 235. *In* J. Oppenheim et al. (ed.), Cellular and cytokine networks in tissue immunity. Wiley-Liss, Inc., New York.
24. **Green, S. J., M. S. Meltzer, J. B. Hibbs, Jr., and C. A. Nacy.** 1990. Activated macrophages destroy intracellular *Leishmania major* amastigotes by an L-arginine-dependent killing mechanism. J. Immunol. **144:**278–283.
25. **Green, S. J., C. A. Nacy, R. D. Schreiber, D. L. Granger, R. M. Crawford, M. S. Meltzer, and A. H. Fortier.** 1993. Neutralization of gamma interferon and tumor necrosis factor alpha blocks in vivo synthesis of nitrogen oxides from L-arginine and protection against *Francisella tularensis* infection in *Mycobacterium bovis* BCG-treated mice. Infect. Immun. **61:**689–698.
26. **Gregory, S. H., L. K. Barczynski, and E. J. Wing.** 1992. Effector function of hepatocytes and Kupffer cells in the resolution of systemic bacterial infections. J. Leukocyte Biol. **51:**421–424.
27. **Gregory, S. H., E. J. Wing, R. Hoffman, and R. L. Simmons.** 1993. Reactive nitrogen intermediates suppress the primary immunologic response to Listeria. J. Immunol. **150:**2901–2909.
28. **Hibbs, J. B., Jr., R. R. Taintor, and Z. Vavrin.** 1987. Macrophage cytotoxicity: role for L-arginine deiminase and iminonitrogen oxidation to nitrite. Science **235:**473–476.
29. **Hibbs, J. B., Jr., R. R. Taintor, Z. Vavrin, and E. M. Rachlin.** 1988. Nitric oxide: a cytotoxic activated macrophage effector molecule. Biochem. Biophys. Res. Commun. **157:**87–94.
30. **Hibbs, J. B., Jr., Z. Vavrin, and R. R. Taintor.** 1987. L-arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. J. Immunol. **138:**550–565.
31. **Hoffman, R. A., J. M. Langrehr, T. R. Billiar, R. D. Curran, and R. L. Simmons.** 1990. Alloantigen-induced activation of rat splenocytes is regulated by the oxidative metabolism of L-arginine. J. Immunol. **145:**2220–2226.
32. **James, S., and J. Claven.** 1989. Macrophage cytotoxicity against schistosomula of *Schistosoma mansoni* involves arginine-dependent production of reactive nitrogen intermediates. J. Immunol. **143:**4208–4212.
33. **Knowles, R. G., M. Palacios, R. M. J. Palmer, and S. Moncada.** 1989. Formation of nitric oxide from L-arginine in the central nervous system: a transduction mechanism for stimulation of the soluble guanylate cyclase. Proc. Natl. Acad. Sci. USA **86:**5159–5162.
34. **Liew, F. Y., S. Millott, C. Parkinson, R. M. J. Palmer, and S. Moncada.** 1990. Macrophage killing of Leishmania parasites *in vivo* is mediated by nitric oxide from L-arginine. J. Immunol. **144:**4794–4797.
35. **Mackaness, G. B.** 1962. Cellular resistance to infection. J. Exp. Med. **116:**381–417.
36. **Mackaness, G. B.** 1964. The immunological basis of acquired cellular resistance. J. Exp. Med. **120:**105–120.
36a. **Maybodi, M.** Unpublished data.
37. **Mellion, B. T., L. J. Ignarro, E. H. Ohlstein, E. G. Pontecorno, A. L. Hyman, and P. J. Kadowitz.** 1991. Evidence for the inhibitory role of guanosine 3′,5′-monophosphate in ADP-induced platelet aggregation in the presence of nitric oxide and related vasodilators. Blood **57:**946–955.
38. **Murray, H. W., and R. F. Teitelbaum.** 1992. L-arginine-dependent reactive nitrogen intermediates and the antimicrobial effect of activated human mononuclear phagocytes. J. Infect. Dis. **165:**513–517.
39. **Nakane, A., T. Minagawa, and K. Kato.** 1988. Endogenous tumor necrosis factor (cachectin) is essential to host resistance against *Listeria monocytogenes* infection. Infect. Immun. **56:**2563–2569.
40. **Nusser, A., J. C. Drapier, L. Renia, S. Pied, F. Miltgen, M. Gentilini, and D. Mazier.** 1991. L-arginine-dependent destruction of intrahepatic malaria parasites in response to tumor necrosis factor and/or interleukin 6 stimulation. Eur. J. Immunol. **21:**227–230.
41. **Palmer, R. M. J., D. S. Ashton, and S. Moncada.** 1988. Vascular endothelial cells synthesize nitric oxide from L-arginine. Nature (London) **333:**664–666.
42. **Portnoy, D. A., T. Chakraborty, W. Goebel, and P. Cossart.** 1992. Molecular determinants of *Listeria monocytogenes* pathogenesis. Infect. Immun. **60:**1263–1267.
43. **Poston, R. M., and R. J. Kurlander.** 1991. Analysis of the time course of IFN-gamma mRNA and protein production during primary murine listeriosis: the immune phase of bacterial elimination is not temporally linked to IFN production in vivo. J. Immunol. **146:**4333–4337.
44. **Poston, R. M., and R. J. Kurlander.** 1992. Cytokine expression in vivo during murine listeriosis: infection with live, virulent bacteria is required for monokine and lymphokine messenger RNA accumulation in the spleen. J. Immunol. **149:**3040–3044.
45. **Rake, J. B., and R. G. Eagon.** 1980. Inhibition, but not uncoupling, of respiratory energy coupling of three bacterial species by nitrite. J. Bacteriol. **144:**975–982.
46. **Schneemann, M., G. Schoedon, S. Hofer, N. Blau, L. Guerrero, and A. Schaffner.** 1993. Nitric oxide synthase is not a constituent of the antimicrobial armature of human mononuclear phagocytes. J. Infect. Dis. **167:**1358–1363.
47. **Sherman, M. P., M. L. Loro, V. Z. Wong, and D. P. Tashkin.** 1991. Cytokine- and Pneumocystis carinii-induced L-arginine oxidation by murine and human pulmonary alveolar macrophages. J. Protozool. **38:**234S–236S.
48. **Stuehr, D. J., and M. A. Marletta.** 1985. Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. Proc. Natl. Acad. Sci. USA **82:**7738–7742.
49. **Stuehr, D. J., and M. A. Marletta.** 1987. Synthesis of nitrite and nitrate in murine macrophage cell lines. Cancer Res. **47:**5590–5594.
50. **Wei, H., K. J. Tracey, K. R. Monogue, H. Nguyen, Y. Gong, D. Hesse, B. Beutler, R. Solomon, A. Cerami, and S. Lowry.** 1987. Cachectin mediates suppressed food intake and anemia during chronic administration. FASEB J. **46:**1338–1342.
51. **Xie, Q., H. J. Cho, J. Calaycay, R. A. Mumford, K. M. Swiderek, T. D. Lee, A. Ding, T. Troso, and C. F. Nathan.** 1992. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science **256:**225–228.