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Cyclophosphamide Decreases Nitrotyrosine Formation and Inhibits Nitric Oxide Production by Alveolar Macrophages in Mycoplasmosis

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We previously reported that congenic C57BL/6 inducible nitric oxide synthase$^{-/-}$ (iNOS$^{-/-}$) mice infected with *Mycoplasma pulmonis* developed higher bacterial numbers and lung lesion scores than C57BL/6 iNOS$^{+/+}$ controls but had similar lung nitrotyrosine levels. The present studies investigated the role of inflammatory cells in nitrotyrosine formation during mycoplasmal infection. iNOS$^{+/+}$ and iNOS$^{-/-}$ mice were injected with cyclophosphamide (CYP) and inoculated with 10^7 CFU of *M. pulmonis*. CYP pretreatment of *M. pulmonis*-infected iNOS$^{+/+}$ and iNOS$^{-/-}$ mice reduced polymorphonuclear cells (PMNs) within bronchoalveolar lavages (BALs) by 88 and 72%, respectively, and whole-lung myeloperoxidase levels by 80 and 78%, respectively, at 72 h postinfection but did not alter the number of alveolar macrophages (AMs) in BALs. CYP treatment also significantly decreased nitrate and nitrite (NOx) levels in BALs and plasma of infected iNOS$^{+/+}$ mice, whereas neither CYP nor mycoplasmal infection altered NOx in iNOS$^{-/-}$ mice. CYP reduced lung nitrotyrosine levels in both iNOS$^{+/+}$ and iNOS$^{-/-}$ mice to uninfected-control levels as shown by immunohistochemical staining and enzyme-linked immunosorbent assay and inhibited mycoplasmal killing by iNOS$^{+/+}$ mice in vivo. CYP inhibited the production of gamma interferon-inducible NOx by iNOS$^{+/+}$ AMs in vitro but did not alter the number of iNOS-positive AMs, as detected by immunocytochemistry. In addition, AMs from CYP-treated iNOS$^{+/+}$ mice had significantly decreased ability to kill mycoplasmas in vitro. These results demonstrate that reactive species generated by inflammatory cells as well as PMN myeloperoxidase are important contributors to nitrotyrosine formation during mycoplasmal infection and that treatment with CYP decreases NO$^{\bullet}$ production by AMs and inhibits mycoplasmal killing.

Mycoplasma pneumoniae accounts for 20 to 25% of pneumonias in all age groups, causes tracheobronchitis with prolonged pulmonary clearance and airway hyperresponsiveness, and may contribute to initiation and persistence of asthma. In addition, studies have shown that 46% of *M. pneumoniae* infections are misdiagnosed and that 10% of the patients correctly diagnosed with this illness are treated inappropriately (5). Host defense mechanisms against mycoplasmas are poorly characterized. However, studies with animal models have demonstrated that innate immunity is extremely important for the control and clearance of pulmonary mycoplasmal infections (20).

Mycoplasma pulmonis infection in mice is the best available model for human respiratory mycoplasmosis. *M. pulmonis* causes a suppurative pneumonia in which infection and disease spread centrifugally in the respiratory tract resulting in the presence of cellular exudates in alveoli indicative of maximum disease severity (4). The airway exudate consists primarily of polymorphonuclear cells (PMNs), while the alveolar exudate is mixed PMNs and macrophages. In the absence of a specific antibody, PMNs are unable to clear mycoplasmas and appear to contribute more to pathology than to the resolution of disease. Activated PMNs secrete a number of proteases, including elastase and myeloperoxidase (MPO), as well as reactive oxygen-nitrogen species including nitric oxide (NO$^{\bullet}$), superoxide ($O_2^{\bullet -}$), and hydrogen peroxide (H_2O_2), which alone or in combination may damage the pulmonary epithelium. Our studies of host defense mechanisms against mycoplasmas have identified the alveolar macrophage (AM) as the primary effector cell in early mycoplasmal clearance (20). Activated AMs also produce NO$^{\bullet}$ via inducible nitric oxide synthase (iNOS) and $O_2^{\bullet -}$ via NADPH oxidase. Interaction of NO$^{\bullet}$ with $O_2^{\bullet -}$ generates the strong oxidant peroxynitrite ($ONOO^-$) (22). We have shown that surfactant protein A (SP-A) mediates mycoplasmal killing via the production of $ONOO^-$ by activated AMs (18). In vivo studies with congenic C57BL/6 iNOS$^{-/-}$ mice demonstrated that iNOS production of NO$^{\bullet}$ by AMs was essential for mycoplasmal clearance. Surprisingly, C57BL/6 iNOS$^{-/-}$ mice had higher bacterial numbers and lung lesion scores than iNOS$^{+/+}$ mice despite significant nitrotyrosine formation, consistent with the production of $ONOO^-$ (18).

Although the alveolar lining fluid contains a number of antioxidant substances (25), in vivo evidence has shown that during inflammation enough reactive oxygen-nitrogen intermediates remain to cause extensive damage to the alveolar epithelium and the surfactant system (15, 16). Tyrosine nitration of proteins is regarded as an indication of the production of reactive nitrogen oxides in vivo, and nitrotyrosine is commonly detected in infectious and inflammatory diseases around areas of PMNs and monocytes (16, 35). Both $ONOO^-$ and reactive intermediates produced by the MPO-catalyzed reac-

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tion of PMN-generated reactive species are capable of nitrating proteins in vitro (2, 37). We designed a series of experiments to investigate the roles of PMNs and iNOS-produced $NO^{\bullet}$ in nitrotyrosine formation during mycoplasmal infection in vivo. C57BL/6 mice lacking iNOS (B6 iNOS$^{-/-}$) and C57BL/6 wild-type control (B6 iNOS$^{+/+}$) mice were treated with cyclophosphamide (CYP) to induce neutropenia and infected with mycoplasmas. We then determined in vivo mycoplasmal killing, development of lung lesions, $NO^{\bullet}$ production, and nitrotyrosine formation at 72 h postinfection (p.i.). In addition, we measured the effects of CYP treatment of B6 iNOS$^{+/+}$ mice on SP-A-mediated killing of mycoplasmas and on NO_3^- and NO_2^- production by AMs in vitro. Our results indicate that PMNs and MPO are important contributors to nitrotyrosine formation during mycoplasmal infection and that CYP inhibits AM iNOS production of $NO^{\bullet}$, a side effect that may have very serious implications for patients on long-term CYP treatment.

MATERIALS AND METHODS

Materials. Phosphate-buffered saline (PBS), Dulbecco modified Eagle medium (DMEM) with L-arginine and 4.5 g of glucose/liter, and Hanks balanced salt solution$^+$ (HBSS$^+$) (containing Ca^{2+} and Mg^{2+}) were from Cellgro (Atlanta, Ga.). Saline was obtained from Abbott Laboratories (Abbott Park, Ill). Horse serum was from Life Technologies (GIBCO BRL, Grand Island, N.Y.). Mycoplasma broth base was obtained from Becton Dickinson (BBL Microbiology Systems, Cockeysville, Md.). Diff Quik stain kits were obtained from Baxter Healthcare (McGaw Park, Ill.). Unless otherwise specified, all other chemicals were from Sigma (St. Louis, Mo.).

Isolation of SP-A. SP-A was purified sterilely from the bronchoalveolar lavages (BALs) of patients with alveolar proteinosis by *n*-butanol extraction as previously described (15). Polyacrylamide gel electrophoresis and Western blot analysis of SP-A were done to ensure the purity of SP-A preparations (17). SP-A was stored at −20°C in 5 mM HEPES, pH 7.4. Aliquots were cultured for aerobic bacteria in BBL brain heart infusion broth (Becton Dickinson, Inc., Sandy, Utah), and only culture-negative aliquots were used in experiments. Each lot of SP-A was tested for endotoxin by the University of Alabama at Birmingham (UAB) Media Preparation Shared Facility (Denise Shaw, director), and only batches of SP-A with <0.5 endotoxin units/ml were used in experiments.

Endotoxin testing. Standard practices were followed to keep endotoxin levels in all media and chemicals as low as possible: PBS, DMEM, HBSS$^+$, and saline were tested and certified to contain <0.5 endotoxin units/ml. Low-endotoxin bovine serum albumin (BSA) was used in all tissue culture experiments. Periodically samples of media used in experiments were submitted to the UAB Media Preparation Shared Facility for endotoxin testing by an amebocyte lysate assay.

Animals. C57BL/6NCr (B6 iNOS$^{+/+}$) mice were obtained from the Frederick Cancer Research and Development Center (National Cancer Institute, Frederick, Md). Breeding pairs of C57BL/6J-*Nos2*^{tm1Lau} (B6 iNOS$^{-/-}$) congenic mice were obtained from The Jackson Laboratory (Bar Harbor, Maine) and bred at UAB. Mice were maintained in autoclaved microisolator cages (Lab Products, Maywood, N.J.) and were provided with autoclaved food (Agway, Syracuse, N.Y.) and water ad libitum. Mice were monitored at the Health Surveillance Facility at UAB and were found to be negative for murine pathogens (13). All mice used in the studies were 8 to 12 weeks of age. Mice were anesthetized for inoculation and euthanasia by injection with ketamine (8.7 mg/100 g of body weight; Aveco, Fort Dodge, Iowa) and xylazine (1.3 mg/100 g; Haver, Shawnee, Kans.). All mouse experiments were performed with the approval of the Institutional Animal Care and Use Committee at UAB in accordance with federal guidelines.

PMN depletion. Mice were injected i.p. with 200 mg of CYP (Bristol-Myers Squibb, Princeton, N.J.)/kg (~4 mg) (26, 34) and again 72 h later with 100 mg of CYP/kg (~2 mg) i.p. Control mice were injected with sterile saline. For in vivo infection studies, mice were inoculated with mycoplasmas at the time of the second injection of CYP.

Mycoplasmas. The UAB CT strain of *M. pulmonis* was used in all experiments (6). For in vivo experiments, a 3×10^7-CFU/ml stock was diluted in broth A (6) to 10^7 CFU per 50 μl. Infected mice were given 10^7 CFU of *M. pulmonis* in 50 μl of broth A, with control mice receiving broth A alone. CFU in all inoculates were confirmed by enumeration after serial dilution and plating on agar plates (8). For in vitro experiments, mycoplasmas were incubated at 37°C for 18 h before use to ensure active growth in logarithmic phase.

Cell counts. At euthanasia, blood samples were taken from transected brachial vessels of each mouse and placed in individual microcentrifuge tubes containing 30 μl of 7.5% EDTA solution to prevent clotting. Total leukocyte counts were made utilizing an electronic Coulter counter, and differential counts were done on Diff Quik-stained smears. Plasma was separated by centrifugation and stored at −20°C for subsequent determination of nitrate and nitrite. Total cell counts on cells from BAL fluid were performed utilizing an electronic Coulter counter and/or a hemocytometer. Differential counts of cells in BAL fluid were performed on Diff Quik-stained cytospin preparations.

Lung MPO assay. Lung MPO was assessed as an index of PMN numbers (30). Briefly, whole unlavaged lungs were homogenized in 2 ml of 50 mM KH_2PO_4, pH 7.4, and centrifuged, and the supernatant was discarded. Pellets were resuspended in 2 ml of 50 mM KH_2PO_4–10 mM EDTA–0.5% hexadecyltrimethylammonium bromide, pH 6, and sonicated. Hydrogen peroxide was mixed with a sample aliquot (50 μl) in assay buffer containing 3,3′,5,5′-tetramethylbenzidine and incubated for 3 min at 37°C. The reaction was terminated by addition of catalase and 0.2 M sodium acetate, pH 3, and read at 655 nm. MPO units were calculated as the change in absorbance over time.

Assessment of lung lesion severity. Lungs were removed and fixed by intratracheal infusion of 10% formalin in 70% ethanol until the lungs reached approximately normal distention. Sections (5 μm thick) of paraffin-embedded tissues were stained with hematoxylin and eosin, coded randomly, and scored subjectively for lesion severity on the basis of characteristic lesions for mycoplasmosis: (i) neutrophilic exudate in airway lumina, (ii) hyperplasia-dysplasia of the mucosal epithelium, (iii) peribronchial and perivascular lymphoid accumulation, and (iv) inflammatory infiltration within the alveoli (4).

ELISA for nitrotyrosine. The nitrotyrosine content of BAL was determined by enzyme-linked immunosorbent assay (ELISA) using polyclonal antinitrotyrosine antibody (Upstate Biotechnology, Lake Placid, N.Y.) and nitrated BSA as a standard. BSA (10 mg/ml in 50 mM KH_2PO_4, pH 7.4) was nitrated by exposure to tetranitromethane (100 μM) in 50 mM KH_2PO_4, pH 8, for 30 min at 37°C. Nitrotyrosine was measured spectrophotometrically at 430 nm, after adjusting the pH to 10 with 3 M NaOH using an extinction coefficient (ε_M) value of 4,400 M^{-1} cm^{-1} as previously described (39).

Quantitative lung cultures. Mice were euthanized at 72 h p.i. and lungs were removed aseptically, individually minced, and sonicated for 1 min in broth A. Tenfold serial dilutions were plated onto mycoplasma agar, and the total number of CFU in the lungs of each animal was determined after incubation for 7 days (4).

Macrophage isolation. BALs were collected as described previously (7, 19). Briefly, mice were anesthetized and the proximal tracheas were exposed surgically. A sterile 19-gauge intravenous catheter was inserted through the wall 5 mm into the lumen of the trachea. Lungs were lavaged in situ with four separate 1-ml washes of sterile saline. Lavage fluids from animals within each experiment were pooled and centrifuged to pellet the cellular fraction. Cells were resuspended in DMEM containing 0.2% BSA, 2.5% HEPES, and 1% L-glutamine, counted using a hemocytometer and trypan blue, and aliquoted into sterile 12- by 45-mm glass vials or onto glass slides. Cells isolated from uninfected mice were >90% viable by trypan blue exclusion and >95% macrophages as differentiated on cytospin preps using Diff Quik stain.

Nitrite and nitrate measurements. Concentrations of NO_3^- and NO_2^- were measured using either the Greiss reaction or by fluorescence utilizing 2,3-diaminonaphthalene (DAN) (27). NO_3^- was first converted to NO_2^- with *Escherichia coli* reductase. For the Greiss reaction, 100 μl of sample was incubated in duplicate with equal volumes of 1% sulfanilamide and 0.1% *N*-1-naphthylethylenediamine dihydrochloride for 10 min and the absorbance was read at 550 nm. For fluorescence measurements, 100 μl of sample was incubated in duplicate with 25 μl of freshly prepared DAN (0.05 mg/ml in 0.62 M HCl) for 10 min. The reaction was stopped by the addition of 25 μl of 2.8 N NaOH, and the signal was measured using a fluorescence plate reader with excitation at 360 nm, emission at 450 nm, and a gain setting of 100%. The NO_2^- concentrations for both methods were determined using a $NaNO_2$ standard.

Immunohistochemistry. Paraffin-embedded lung sections from B6iNOS$^{+/+}$ and B6iNOS$^{-/-}$ mice infected for 72 h were stained for nitrotyrosine as described previously (16, 38). Lung sections were treated with 0.3% hydrogen peroxide for 30 min at 4°C to block endogenous peroxidase activity and washed with PBS, and nonspecific protein binding was blocked with 10% nonimmune serum for 1 h at room temperature. Sections were incubated with rabbit polyclonal immunoglobulin G (IgG) antinitrotyrosine antibody (antibody kindly provided by J. S. Beckman and Y. Z. Ye, UAB [3]) overnight at 4°C, washed in PBS,

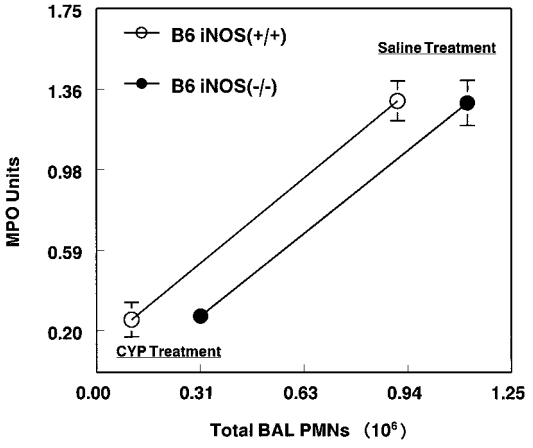

FIG. 1. Effect of CYP on cell counts and MPO. B6 iNOS$^{-/-}$ and control B6 iNOS$^{+/+}$ mice were injected with 200 mg of CYP/kg i.p. at time zero and with 100 mg/kg 72 h later. Control mice were injected with sterile saline. All mice were infected with 1.5×10^7 CFU of *M. pulmonis* at the time of the second CYP injection and euthanized at 72 h p.i. for determination of PMN counts in BAL fluid and MPO levels in whole-lung homogenates. Results are means ± SE (n = 16 to 24 mice).

and incubated with peroxidase-conjugated goat anti-rabbit IgG (EnVision+ System HRP [diaminobenzadine]; DAKO, Carpinteria, Calif.) for 30 min. Slides were developed for 2 min with 3,3′-diaminobenzidine hydrochloride (DAKO) and counterstained with methyl green.

AMs were stained for iNOS as described previously (18). AMs were plated onto Lab-Tek chamber slides (Nunc Inc., Naperville, Ill.), incubated for 1 h, and washed. AMs were activated with gamma interferon (IFN-γ) and/or SP-A and mycoplasmas for 6 h, and the media were collected for NO_3^- and NO_2^- production. AMs were fixed in 4% paraformaldehyde, permeabilized, and stained for iNOS protein using an anti-iNOS antibody (Transduction Laboratories, Lexington, Ky.) as outlined for nitrotyrosine.

Mycoplasmal killing in vitro. AMs (10^5) were used in in vitro assays as described previously (19). Briefly, AMs were adhered to glass vials for 30 min at 37°C, and nonadherent cells were removed by washing. AMs were activated with 100 U of mouse recombinant IFN-γ/ml for 18 h at 37°C, washed once, and resuspended in HBSS$^+$ containing 0.1% BSA and either 25 μg of SP-A/ml or 5 mM HEPES. Samples were incubated for 30 min, washed twice, and infected with 10^{10} viable *M. pulmonis* CFU. Cultures were centrifuged to promote attachment of mycoplasmas to cells, incubated for 15 min at 37°C, and washed. Vials were sonicated to rupture AMs at 0 and 6 h p.i., and viable organisms were determined by quantitative culture. AMs were quantified after the final wash by a modification of the pronase and cetrimide assay (19). Mycoplasmal killing was defined as the difference between the logs of mycoplasmal CFU in the control and experimental groups at each time point. CYP is a prodrug and must be metabolized by the liver to the active form; therefore, to determine the effect of CYP on AM function, mice were pretreated with either CYP or saline (control) prior to AM isolation.

Statistics. All experiments had a minimum of four samples per group for in vitro studies or six mice per group for in vivo studies. All experiments were repeated at least twice to confirm reproducibility. Parametric data were analyzed by analysis of variance (ANOVA) followed by Tukey's multigroup comparison of the means after log conversion or by Kruskal-Wallis ANOVA and Pearson's correlation of the means for nonparametric data (Analytical Software, St. Paul, Minn.). P values of 0.05 or less were considered significant.

RESULTS

CYP depletion of PMNs. Treatment with 200 mg of CYP/kg significantly reduced blood PMN counts in uninfected iNOS$^{+/+}$ mice by 91% (1,257 ± 82.9 to 112 ± 7.7 PMNs/mm^3) after 4 days, with the numbers returning to normal by day 8. A second injection of 100 mg of CYP/kg at 72 h after the first injection maintained total blood PMN levels at 20% of the control values (1,257 ± 82.9 to 246.9 ± 12.5 PMNs/mm^3) out to 7 days. Mice appeared otherwise unaffected by treatment with CYP. Inasmuch as infection with *M. pulmonis* results in an acute suppurative pneumonia with significantly increased PMN recruitment to the lungs by 72 h p.i. (28), we infected B6 iNOS$^{+/+}$ and B6 iNOS$^{-/-}$ mice with *M. pulmonis* (10^7 CFU) at the time of the second injection with CYP (100 mg/kg) and then collected BAL fluid and performed lung and spleen cultures for mycoplasmas 72 h later. CYP significantly reduced total PMN counts in BAL fluid of mycoplasma-infected B6 iNOS$^{+/+}$ control and B6 iNOS$^{-/-}$ mice by 88 and 72%, respectively. Similarly, MPO levels in lung homogenates were significantly reduced by 81% in B6 iNOS$^{+/+}$ mice and by 79% in B6 iNOS$^{-/-}$ mice (Fig. 1). In contrast, the numbers of AMs recovered in BALs were not significantly altered by CYP. Lymphocyte numbers significantly decreased from 4.2×10^4 to 8.0×10^3 lymphocytes/ml in CYP-treated B6 iNOS$^{+/+}$ mice at 72 h p.i. However, consistent with previous reports (28), lymphocyte counts were elevated by mycoplasmal infection in both strains of mice compared to uninfected controls, regardless of CYP treatment (Table 1).

Effects of PMN depletion on nitrotyrosine formation. Immunohistochemical staining of lungs of B6 iNOS$^{+/+}$ and B6 iNOS$^{-/-}$ mice infected with mycoplasma for 72 h demonstrated significant amounts of nitrotyrosine. However, nitroty-

TABLE 1. BAL differential cell counts[c]

Mice	Total cell count (10^5) of indicated cell type for:					
	Uninfected mice (6/group)			*M. pulmonis*-infected mice (18/group)		
	Macrophage	PMN	Lymphocyte	Macrophage	PMN	Lymphocyte
Saline treated						
B6 iNOS$^{+/+}$	0.98 ± 0.31	0.03 ± 0.02	0.01 ± 0.02	1.79 ± 1.07	9.10 ± 9.10[a]	0.42 ± 0.57[a]
B6 iNOS$^{-/-}$	1.25 ± 1.24	0.12 ± 0.23	0.03 ± 0.02	2.32 ± 1.82	11.2 ± 10.30[a]	0.30 ± 0.30
CYP treated						
B6 iNOS$^{+/+}$	1.30 ± 0.25	0.03 ± 0.04	0.01 ± 0.02	1.50 ± 1.02	1.10 ± 9.90[a, b]	0.08 ± 0.10[a,b]
B6 iNOS$^{-/-}$	1.09 ± 0.54	0.06 ± 0.09	0.04 ± 0.01	1.98 ± 0.92	3.10 ± 7.00[a, b]	0.13 ± 0.17

[a] Significant difference between infected and noninfected treated mice of the same strain and treatment group for that cell type.
[b] Significant difference between Cytoxan- and saline-treated mice of the same strain and infection status for that cell type.
[c] B6 iNOS$^{-/-}$ and B6 iNOS$^{+/+}$ mice were injected with either 300 mg of total CYP/kg or sterile saline i.p. Mice were then inoculated with 1.5×10^7 CFU of *M. pulmonis* or sterile broth A and euthanized 72 h later for lung lavage. Total cell counts were determined using a hemocytometer and Coulter counter. Cells were morphologically differentiated after Diff Quik staining of cytospin preparations.

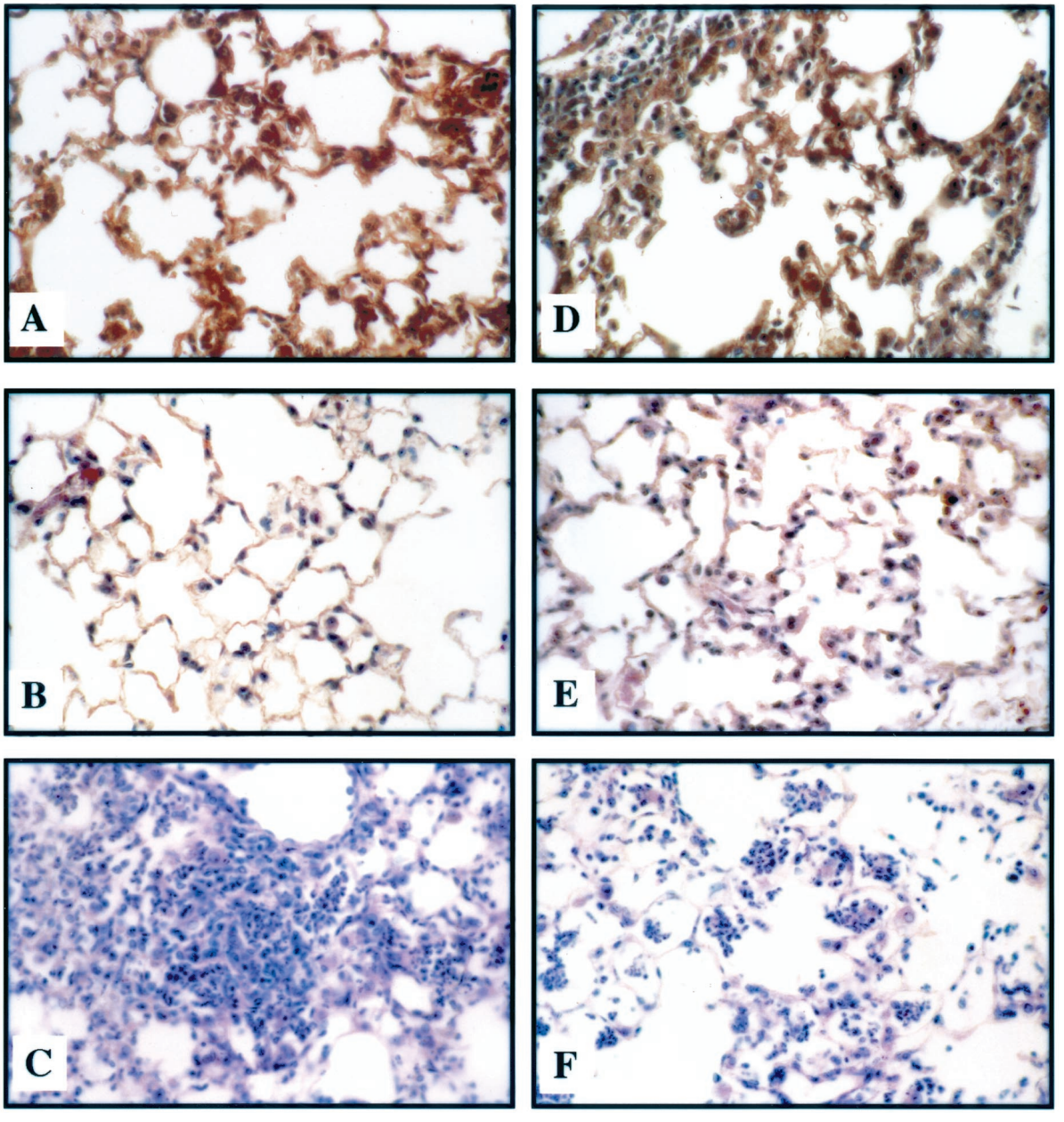

FIG. 2. Nitrotyrosine immunohistochemistry. Visualization of nitrotyrosine residues in the lungs of B6 iNOS$^{+/+}$ or B6 iNOS$^{-/-}$ mice pretreated with CYP or saline and infected with 1.5×10^7 CFU of *M. pulmonis* for 3 days is shown. (A) B6 iNOS$^{+/+}$ nitrotyrosine staining with saline pretreatment. (B) B6 iNOS$^{+/+}$ nitrotyrosine staining with CYP pretreatment. (C) B6 iNOS$^{+/+}$ nitrotyrosine staining of PMN-rich area (same block as panel A) in the presence of excess nitrotyrosine (10 mM). (D) B6 iNOS$^{-/-}$ nitrotyrosine staining after saline pretreatment. (E) B6 iNOS$^{-/-}$ nitrotyrosine staining after CYP pretreatment. (F) B6 iNOS$^{-/-}$ nitrotyrosine staining of neutrophil-rich area (same block as panel D) in the presence of excess nitrotyrosine (10 mM). Pictures are representative ($n \geq 6$ slides per group from separate mice).

rosine levels for the two strains of mice were not significantly different. Treatment of mice with CYP to deplete PMNs prior to infection significantly decreased nitrotyrosine staining (Fig. 2). Nitrotyrosine staining of uninfected lungs was negative (not shown). ELISA measurement of nitrotyrosine in BALs demonstrated significantly increased nitrotyrosine levels in both B6 iNOS$^{+/+}$ and B6 iNOS$^{-/-}$ mice with mycoplasmal infection ($P = 0.0002$). Pretreatment of mycoplasma-infected B6 iNOS$^{+/+}$ and B6 iNOS$^{-/-}$ mice with CYP decreased MPO (Fig. 1) and nitrotyrosine to background uninfected levels (Fig. 3) although plasma PMNs were still significantly elevated compared to the corresponding uninfected controls (Table 1).

Effects of CYP on mycoplasmal infection in vivo. We pretreated B6 iNOS$^{-/-}$ and B6 iNOS$^{+/+}$ mice with CYP and infected them with 10^7 CFU of *M. pulmonis*. Mycoplasma-infected lungs were scored on the basis of severity of characteristic histopathology lesions: (i) neutrophilic exudate in airway lumina, (ii) hyperplasia-dysplasia of the mucosal epithelium, (iii) peribronchial and perivascular lymphoid accumulation, and (iv) inflammatory infiltration within the alveoli. Treatment

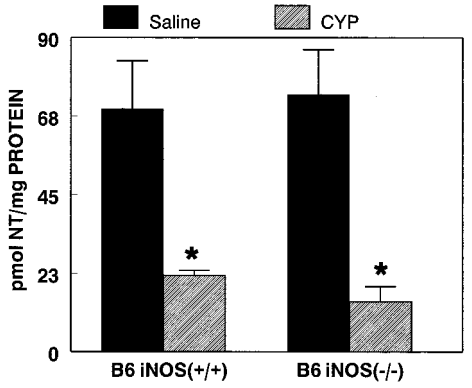

FIG. 3. Quantitation of BAL nitrotyrosine by ELISA. B6 iNOS$^{-/-}$ and control B6 iNOS$^{+/+}$ mice were pretreated with CYP or saline i.p. and infected intranasally with 1.5×10^7 CFU of *M. pulmonis*. All mice were euthanized at 72 h p.i., and their lungs were lavaged with 2 ml of sterile saline. Cells were removed after centrifugation, and supernatants were tested for nitrotyrosine (NT) by ELISA using rabbit anti-nitrotyrosine antibody. ∗, significant difference between CYP- and saline-treated groups. Results are means ± SE (n = 9 to 16 mice).

with CYP significantly reduced neutrophilic exudate in B6 iNOS$^{-/-}$ and B6 iNOS$^{+/+}$ control mice ($P < 0.02$) (Fig. 4). In addition, depletion of PMNs significantly reduced epithelial hyperplasia ($P < 0.002$) and lymphoid accumulation around vessels and airways ($P < 0.001$) in iNOS$^{-/-}$ mice. Parenchymal lesions consisting of histiocytic exudate within alveoli were not affected by treatment with CYP in either B6 iNOS$^{+/+}$ or B6 iNOS$^{-/-}$ mice (Fig. 5).

To determine whether decreased severity of lung lesions was due to decreased PMNs or decreased bacterial loads, we quantified mycoplasma CFU in whole-lung and spleen homogenates at 72 h p.i. Treatment with CYP did not affect mycoplasmal clearance from the lungs of B6 iNOS$^{-/-}$ mice, but B6 iNOS$^{+/+}$ control mice cleared mycoplasmas less efficiently after treatment with CYP (Fig. 6). All spleen cultures were negative at 72 h p.i.

Effects of CYP on NO· production in vivo. We treated B6 iNOS$^{-/-}$ and B6 iNOS$^{+/+}$ mice with CYP as described above, infected them with 10^7 CFU of *M. pulmonis*, and collected plasma samples and BAL fluid at 72 h p.i. As shown in Fig. 7A, B6 iNOS$^{-/-}$ mice had significantly lower plasma NO_2^- and NO_3^- levels than B6 iNOS$^{+/+}$ mice ($P < 0.0001$); however, mycoplasmal infection had no effect on plasma NO_2^- and NO_3^- levels, consistent with culture data indicating that infection was confined to the lungs. Treatment with CYP significantly decreased NO_2^- and NO_3^- in the plasma of B6 iNOS$^{+/+}$ mice to the level of NO_2^- and NO_3^- in B6 iNOS$^{-/-}$ mice (Fig. 7A). Conversely, NO_2^- and NO_3^- levels in BALs were significantly increased with mycoplasmal infection in B6 iNOS$^{+/+}$ mice ($P = 0.037$), an effect that was depressed by treatment with CYP. B6 iNOS$^{-/-}$ mice had low levels of NO_2^- and NO_3^- in BALs, and this remained unchanged by mycoplasmal infection or CYP treatment (Fig. 7B). Pretreatment with CYP did not significantly alter BAL protein content in either mouse strain: saline-treated B6 iNOS$^{+/+}$ mice, 0.30 ± 0.05 mg/ml (n = 21 mice); saline-treated B6 iNOS$^{-/-}$ mice, 0.45 ± 0.05 mg/ml (n = 20 mice); CYP-treated B6 iNOS$^{+/+}$ mice, 0.49 ± 0.04 mg/ml (n = 20 mice); CYP-treated B6 iNOS$^{-/-}$ mice, 0.48 ± 0.10 mg/ml (n = 22 mice) (results are means ± standard errors [SE]; $P = 0.14$).

Effects of CYP on AM function in vitro. B6 iNOS$^{+/+}$ mice were treated with CYP or saline, and AMs were isolated from BALs. AMs were activated with IFN-γ and infected with mycoplasmas in the presence or absence of SP-A. SP-A significantly enhanced the killing of mycoplasmas by AMs from saline-treated mice at 6 h p.i. ($P = 0.03$). Treatment of mice with CYP prior to AM isolation inhibited SP-A-mediated mycoplasmal killing (Fig. 8). These experiments were not repeated with AMs from iNOS$^{-/-}$ mice since they do not kill mycoplasmas (18).

To understand the mechanism involved in the ability of CYP treatment to inhibit SP-A-mediated mycoplasmal killing, AMs were isolated from iNOS$^{+/+}$ mice treated with CYP or saline and plated onto Lab-Tek chamber slides. AMs were activated with IFN-γ and treated with SP-A in the presence or absence of mycoplasmas. Media was collected at 6 h for the determination of NO_2^- and NO_3^- levels, and cells were fixed for iNOS determination. NO_2^- and NO_3^- levels were significantly decreased in AMs from CYP-treated mice ($P = 0.037$) (Fig. 9). However, immunohistochemical staining demonstrated similar levels of iNOS in activated AMs from CYP-treated and saline-treated mice (Fig. 10). In the absence of mycoplasmas and SP-A, AMs did not stain positively for iNOS (not shown). Likewise, we have previously demonstrated that AMs isolated from mycoplasma-infected B6 iNOS$^{-/-}$ mice stain negatively for iNOS and do not kill mycoplasmas (18).

DISCUSSION

Nitrotyrosine formation has been detected in a variety of lung and systemic inflammatory diseases and is considered a marker of NO·-derived species (16, 35). Furthermore, a variety of studies have detected nitrated proteins in the alveolar lining fluid and plasma of patients with acute lung injury and have shown that nitration of at least two proteins, α_1-proteinase inhibitor and SP-A, leads to decreased function (14, 40). In previous studies we found by immunohistochemical staining that significant amounts of nitrotyrosine were present in the lungs of both B6 iNOS$^{-/-}$ and B6 iNOS$^{+/+}$ control mice at 72 h after infection with *M. pulmonis* (18). Nitrotyrosine was detected mainly in areas of neutrophilic inflammation. Herein we show that pretreatment of B6 iNOS$^{+/+}$ and B6 iNOS$^{-/-}$ mice with the nitrogen mustard CYP reduced BAL and total lung nitrotyrosine formation following mycoplasma infection. Nitrotyrosine formation may be caused by either $ONOO^-$ or reactive intermediates formed by the reaction of MPO with H_2O_2 and NO_2^-, the stable end product of NO· metabolism (2, 11). The actions of CYP may be due to either (i) reduced production of reactive oxygen-nitrogen species by inflammatory cells or (ii) decreased numbers of PMNs.

CYP is a prodrug that requires in vivo metabolism to form the reactive components acrolein and phosphoramide mustard (1). These toxic metabolites have been shown to reduce lung microsomal enzyme activity and decrease antioxidant defenses (29). Studies with *Pseudomonas aeruginosa* demonstrated that pretreatment of mice with CYP did not affect the ability of isolated AMs to phagocytize bacteria (34). AMs isolated from B6 iNOS$^{+/+}$ mice treated with CYP and activated with IFN-γ

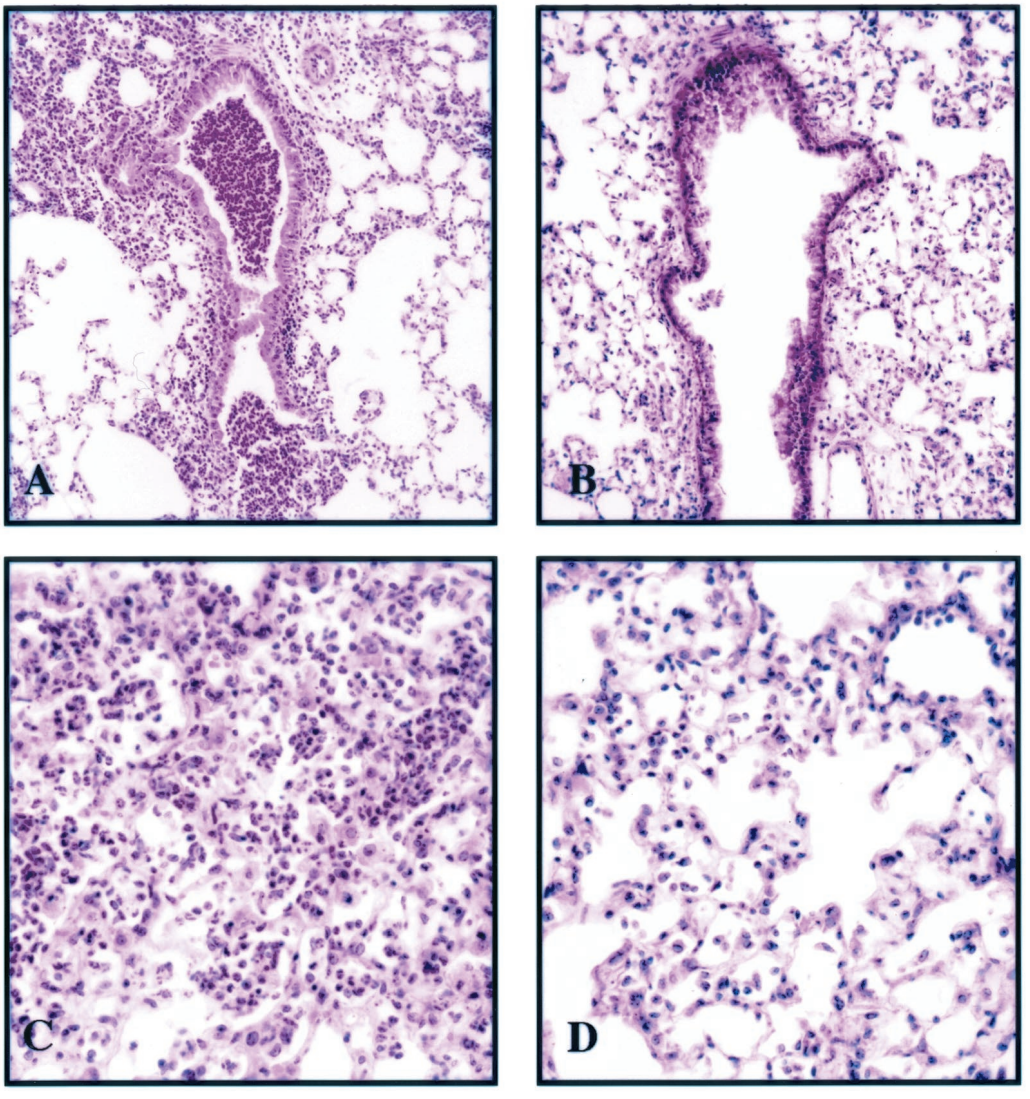

FIG. 4. Effect of CYP on lung histopathology. Shown are hematoxylin- and eosin-stained lung sections from B6 iNOS$^{-/-}$ mice treated with CYP or saline and infected for 72 h with 1.5×10^7 CFU of *M. pulmonis*. (A) Large-airway, saline-treated; (B) large-airway, CYP-treated; (C) alveoli with many PMNs, saline treated; (D) alveoli with rare PMNs, CYP treated. Pictures are representative of sections made for lung lesion analysis.

had normal levels of iNOS protein, as detected by immunocytochemistry, but significantly lower levels of NO_3^- and NO_2^- production than AMs from saline-treated controls. iNOS requires several cofactors for the production of NO$^\bullet$, including flavones, NADPH, and tetrahydrobiopterin. The present data suggest that, because iNOS levels are not decreased, CYP may block NO$^\bullet$ production through depletion of one of these cofactors or by direct damage to the protein. Similar findings have been reported for the antineoplastic drug methotrexate, which inhibits NO$^\bullet$ production without affecting iNOS mRNA or protein levels (31). CYP may decrease NO$^\bullet$ production by activated PMNs in a similar fashion. Thus, decreased levels of NO$^\bullet$ production by inflammatory cells may result in lower $ONOO^-$ formation and consequently lower nitrotyrosine levels.

Our data indicate that infection of B6 iNOS$^{-/-}$ mice with mycoplasmas resulted in levels of lung nitrotyrosine comparable to those in the lungs of B6 iNOS$^{+/+}$ mice. Furthermore, pretreatment of B6 iNOS$^{-/-}$ and B6 iNOS$^{+/+}$ mice with CYP reduced nitrotyrosine levels to the level in uninfected controls. Since CYP treatment did not reduce AM numbers in either mouse strain and since AMs from B6 iNOS$^{-/-}$ mice do not produce iNOS, our findings indicate that NO$^\bullet$ production via iNOS did not play a significant role in nitrotyrosine formation during mycoplasma infection. However, one may argue that infection with mycoplasmas may have significantly upregulated the endothelial and neuronal forms of NOS in inflammatory cells of B6 iNOS$^{-/-}$ mice and that these alternative forms of NOS could then compensate for the lack of iNOS by significantly increasing the production of NO$^\bullet$ and thus $ONOO^-$. This is an unlikely possibility since B6 iNOS$^{-/-}$ mice had significantly lower plasma and BAL NO_3^- and NO_2^- levels than B6 iNOS$^{+/+}$ mice prior to and after infection with *M. pulmonis*. These data suggest that other isoforms of NOS do

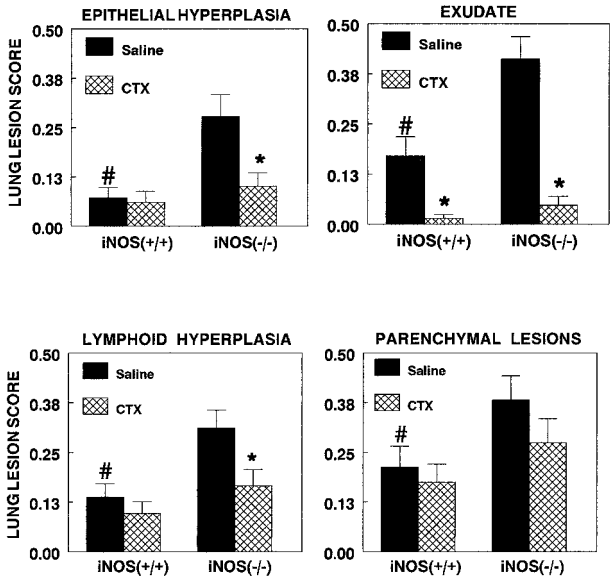

FIG. 5. Effect of CYP on lung lesion indices. Hematoxylin- and eosin-stained lung sections from B6 iNOS$^{-/-}$ and control B6 iNOS$^{+/+}$ mice treated with CYP or saline and infected for 72 h with 1.5×10^7 CFU of *M. pulmonis* were coded randomly and scored subjectively on the basis of characteristic lesions for respiratory mycoplasmosis: (i) neutrophilic exudate in the airway lumen (exudate); (ii) hyperplasia-dysplasia of the airway epithelium (epithelial hyperplasia); (iii) peribronchiolar and perivascular lymphoid accumulation (lymphoid hyperplasia); (iv) inflammatory infiltration of alveoli (parenchymal lesions). Asterisk, significant difference between CYP- and saline-treated groups ($P < 0.05$); pound sign, significant difference between saline-treated B6 iNOS$^{-/-}$ and control B6 iNOS$^{+/+}$ mice ($P < 0.05$). Results are means ± SE (n = 13 to 19 mice).

not compensate for the absence of iNOS production of NO· under these conditions. Thus, our data indicate that CYP reduced nitrotyrosine levels in the lungs of both B6 iNOS$^{+/+}$ and B6 iNOS$^{-/-}$ mice by decreasing the numbers of PMNs.

Mouse PMNs are derived from multipotential stem cells in the bone marrow; they are short lived and are recruited constantly throughout life. After release from the bone marrow, PMNs have half-lives of 6 h to 10 days in the circulation and 24 h to 4 days in extravascular locations (26). CYP causes acute damage to the murine blood-forming tissues in bone marrow, resulting in a transient reduction in circulating PMNs. In the absence of infection, we found that injection with 200 mg of CYP/kg reduced blood PMN levels maximally by 4 days, followed by rapid recovery to normal levels by 8 days. A second injection of 100 mg/kg was required to maintain maximal PMN depletion out to 6 days. Activated PMNs produce H_2O_2 via the respiratory burst and release MPO in azurophilic granules. Eiserich et al. have proposed that HOCl formed by the MPO-catalyzed reaction of H_2O_2 and chloride reacts with NO_2^- to form intermediates capable of both nitrating and chlorinating proteins (10). Furthermore, PMN inactivation of angiotensin-converting enzyme in vitro was exacerbated by the addition of 5 to 25 μM nitrite, presumably by chlorinating and nitrating

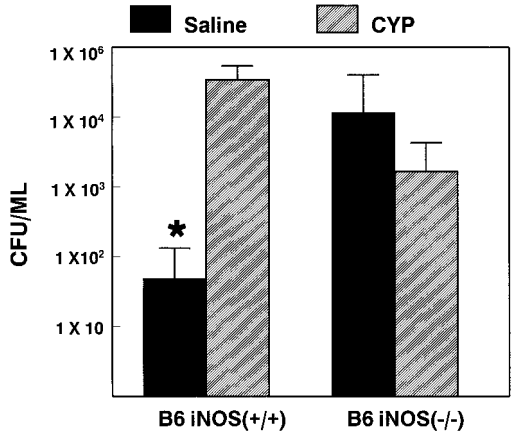

FIG. 6. Effect of CYP on mycoplasma killing in vivo. B6 iNOS$^{-/-}$ and control B6 iNOS$^{+/+}$ mice were treated with 300 mg of total CYP/kg and infected with 1.5×10^7 CFU of *M. pulmonis*. All mice were euthanized at 72 h p.i., and the mean numbers of CFU (total recoverable mycoplasmas) on whole-lung homogenates were determined. Asterisk, significant difference from all other treatment groups ($P < 0.05$). Results are means ± SE (n = 20 to 24 mice).

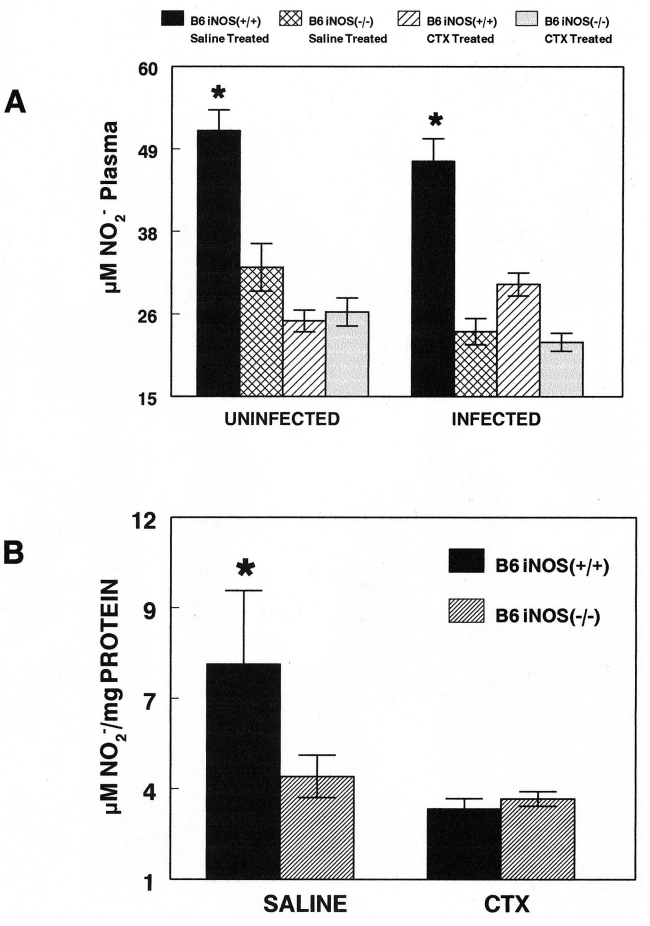

FIG. 7. Effect of CYP on plasma and BAL NO_3^- and NO_2^- levels. B6 iNOS$^{-/-}$ and control B6 iNOS$^{+/+}$ mice were treated with CYP or saline i.p. and infected intranasally with 1.5×10^7 CFU of *M. pulmonis* or sterile mycoplasma broth. All mice were euthanized at 72 h, plasma was collected, and their lungs were lavaged with 2 ml of sterile saline. (A) Plasma NO_2^- levels (n = 18 mice for all groups). (B) BAL NO_2^- levels (n = 20 or 21 mice). NO_2^- levels were determined using the Greiss reagent after conversion of NO_3^- to NO_2^- with *E. coli* reductase. Asterisk, significant difference from all other treatment groups ($P < 0.05$). Results are means ± SE.

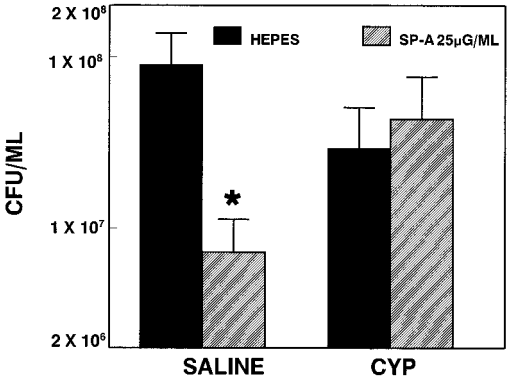

FIG. 8. Effects of CYP on SP-A-mediated killing of *M. pulmonis* by AMs in vitro. B6 iNOS$^{+/+}$ mice were treated with CYP or saline, and AMs were collected by BAL. AMs were activated with 100 U of IFN-γ/ml and infected with 10^{10} CFU of *M. pulmonis* in the presence or absence of SP-A (25 μg/ml). AMs were incubated at 37°C for 6 h and ruptured by sonication, and the remaining CFU were determined by quantitative culture. Results are means ± SE, with 7 to 11 data points per group. Asterisk, significant difference between control and experimental group ($P < 0.05$).

key residues (11). The physiological significance of this reaction has been questioned because of the very high reactivity of the HOCl. For example, Sampson et al. (33) reported that HOCl plus NO_2^- nitrated free tyrosine in simple solutions but failed to nitrate proteins in heart homogenates. However, those authors clearly demonstrated significant nitration of protein-bound tyrosine by mixtures of MPO, NO_2^-, and H_2O_2, showing that nitration can occur in the presence of antioxidants and in the absence of chloride. Furthermore, Jiang and Hurst (23) have shown extensive nitration of phenolic compounds by secreted MPO and NO_2^-. Since NO_3^- and NO_2^- levels in B6 iNOS$^{-/-}$ mice do not increase in mycoplasmosis, our data indicate the importance of MPO or another protease

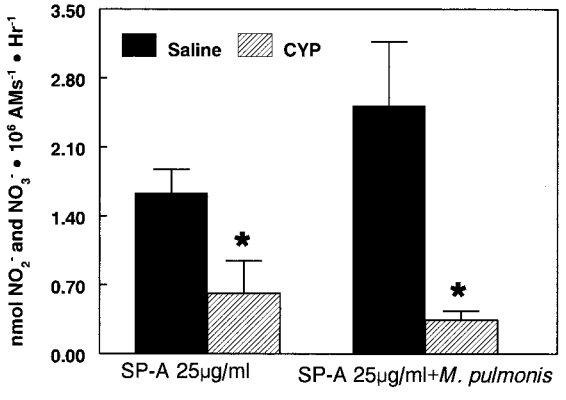

FIG. 9. Effects of CYP on NO_3^- and NO_2^- production. B6 iNOS$^{+/+}$ mice were treated with CYP or saline, and AMs were collected by BAL. AMs (10^5) were plated onto Lab-Tek chamber slides, activated with 100 U of IFN-γ/ml, and treated with SP-A (25 μg/ml) in the presence or absence of *M. pulmonis* (10^{10} CFU). AMs were incubated at 37°C for 6 h, and media were collected for NO_2^- and NO_3^- measurements. NO_2^- levels were determined using DAN after conversion of NO_3^- to NO_2^- with *E. coli* reductase. Results are means ± SE ($n = 6$ to 8). Asterisk, significant difference between CYP-treated group and saline-treated control group ($P < 0.05$).

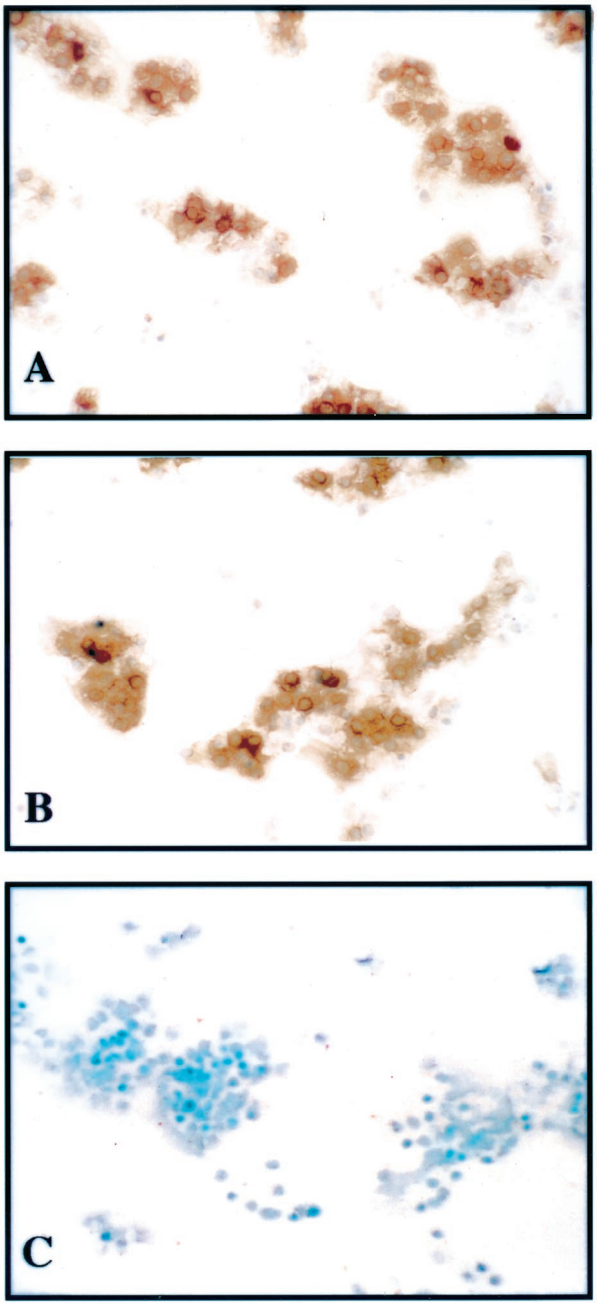

FIG. 10. Effects of CYP on iNOS staining. B6 iNOS$^{+/+}$ mice were treated with CYP or saline, and AMs were collected by BAL. AMs were plated onto Lab-Tek chamber slides, activated with 100 U of IFN-γ/ml, and treated with SP-A (25 μg/ml) in the presence or absence of *M. pulmonis* (10^{10} CFU). AMs were incubated at 37°C for 6 h, and cells were fixed with paraformaldehyde for iNOS determination. (A) iNOS staining after saline pretreatment and treatment with SP-A and mycoplasmas. (B) iNOS staining after CYP pretreatment and treatment with SP-A and mycoplasmas. (C) iNOS staining with nonspecific mouse IgG as the primary antibody, after saline pretreatment and treatment with SP-A and mycoplasmas. Pictures are representative ($n \geq 6$ slides).

in catalyzing reactive intermediates capable of nitrating protein-bound tyrosine. Taken as a whole, these studies indicate that the reactive species and proteases secreted by PMNs are mainly responsible for lung nitrotyrosine formation in myco-

plasmosis. In addition, MPO may have enhanced nitration by catalyzing $ONOO^-$-induced tyrosine nitration (32).

Interestingly, B6 iNOS$^{-/-}$ and CYP-treated B6 iNOS$^{+/+}$ mice had plasma NO_2^- levels comparable to those found in plasma from healthy humans (40) and rats (36). Also similar to what we found in humans, NO• by-products were detected almost entirely (>90%) as NO_3^- (40). This is probably due to the oxidation of NO_2^- by HOCl or its two-electron oxidation by MPO to form $ONOO^-$ (which decomposes to NO_3^-). In addition, NO_2^- may be oxidized by oxyhemoglobin (9), which is omnipresent in plasma and which is a contaminant of BAL.

During mycoplasmal infection, the numbers of PMNs in lungs are significantly increased by 72 h p.i. (28). During the course of these experiments we determined that pretreatment with CYP significantly decreases the ability of B6 iNOS$^{+/+}$ mice to clear mycoplasmas in vivo. These data suggested that (i) PMNs contribute significantly to early mycoplasmal clearance or (ii) CYP treatment affects early mycoplasmal clearance by AMs. Indeed the studies of Klebanoff (24) have shown that MPO enhances *E. coli* killing by H_2O_2 and NO_2^- mixtures. However, the susceptibilities of different pathogens to reactive oxygen-nitrogen species vary widely. Previous studies with mycoplasmas have indicated that PMNs require a specific antibody for effective mycoplasmal killing (21), while the rate of significant clearance of mycoplasmas is maximal by 8 h p.i., long before the production of the specific antibody or the infiltration of tissues by PMNs (28). On the other hand, depletion of AMs by intratracheal instillation of liposomes containing dichloromethylene bisphosphonate greatly diminished mycoplasma killing in vivo (20). Our previous data indicate that the killing of mycoplasmas by AMs requires production of reactive oxygen-nitrogen intermediates and phagocytosis (19). Thus, these data indicate that PMNs do not contribute substantially to early mycoplasma killing in vivo. In addition to decreased nitrotyrosine formation, B6 iNOS$^{-/-}$ mice had decreased hyperplasia of the mucosal epithelium and lymphoid cell accumulation around the airways and vessels. The possibility that these effects may be a direct result of CYP treatment rather than an indirect effect of decreased PMN infiltration cannot be ruled out.

CYP is an immunosuppressive agent used in the long-term treatment of arthritis, systemic lupus erythematosus, scleroderma, glomerulonephritis, interstitial pneumonia, hepatitis, multiple sclerosis, and other chronic inflammatory diseases (1). CYP causes depletion of lymphoid tissues, effectively decreasing the ability of the host to raise an adequate specific immune response. Our data suggest that CYP also impacts the innate immune system by decreasing AM production of NO• by iNOS. The importance of NO• in bacterial killing is widely recognized (12). Furthermore, our recent data show that AMs from patients with acute respiratory distress syndrome (ARDS), but not those of healthy volunteers, immunostain positive for iNOS during the inflammatory period of the disease (35) and that significant levels of NO_3^- and NO_2^- occur in the BAL and edema fluid of patients with ARDS (35, 40). The ability of CYP to inhibit NO• production likely has serious implications for resistance against bacterial infections in patients on long-term CYP therapy.

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