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Antihistoplasma Effect of Activated Mouse Splenic Macrophages Involves Production of Reactive Nitrogen Intermediates

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The mechanism by which recombinant murine gamma interferon (rMuIFN-γ) and bacterial lipopolysaccharide (LPS) activate mouse resident splenic macrophages to inhibit the intracellular growth of the fungus *Histoplasma capsulatum* was examined. Growth inhibition depended on L-arginine metabolism. The growth inhibitory state normally induced by rMuIFN-γ and LPS in resident splenic macrophages did not occur when the macrophages were cultured in the presence of N^G-monomethyl-L-arginine, a competitive inhibitor of L-arginine metabolism. Resident splenic macrophages treated with rMuIFN-γ and LPS produced nitrite (NO_2^-), an end product of L-arginine metabolism. When macrophages were cultured in the presence of N^G-monomethyl-L-arginine together with rMuIFN-γ and LPS, only baseline levels of NO_2^- were detected. Spleen cells from *H. capsulatum*-infected mice produced high levels of NO_2^- in culture. The production of NO_2^- correlated with in vitro inhibition of the intracellular growth of *H. capsulatum*. Anti-tumor necrosis factor alpha antibody did not block NO_2^- production by the immigrant splenic macrophages and did not abolish the antihistoplasma activity.

The dimorphic fungus *Histoplasma capsulatum* is a facultative intracellular pathogen of mononuclear phagocytes of susceptible animals (33). Thus, the yeast cells of the fungus reside within macrophages, where they survive and multiply for a time. In experimental sublethal infections, animals recover (34).

The ability of an animal sublethally infected with *H. capsulatum* to rid itself of the fungus correlates with endogenously produced cytokines (31–33). Recombinant murine gamma interferon (rMuIFN-γ) activates peritoneal macrophages to suppress intracellular growth of the fungus (32, 33). However, induction of a similar suppression in murine red pulp macrophages requires two signals (19). Experiments were undertaken to analyze the molecular basis of the histoplasmastasis of activated splenic macrophages.

In this report, we will show the following: (i) that activated resident splenic macrophages cultured in the presence of N^G-monomethyl-L-arginine (N^GMMA), a competitive inhibitor of L-arginine metabolism, are not histoplasmastatic after stimulation with rMuIFN-γ and lipopolysaccharide (LPS); (ii) that increased levels of nitrite (NO_2^-), an end product of L-arginine metabolism, are detected after activation of resident splenic macrophages to the antihistoplasma state; (iii) that whole spleen cells produce NO_2^- in vitro at 1, 2, and 3 weeks after sublethal infection with *H. capsulatum*; (iv) that activated macrophages from the spleens of infected animals are responsible for the NO_2^- production; and (v) that splenic macrophages from infected mice produce high levels of NO_2^- which correlate with the antihistoplasma activity of these cells.

MATERIALS AND METHODS

Mice. Inbred female C57BL/6 mice were purchased from Jackson Laboratory, Bar Harbor, Maine. Age-matched 6- to 8-week-old mice were used for all experiments.

Fungus. *H. capsulatum* 505, which has been used in experiments described previously (14, 31), was employed in these studies. Yeast cells were grown on blood-cysteine-glucose agar slants at 37°C for 48 to 72 h prior to use.

Reagents and media. Splenic macrophage monolayers were cultured in modified Eagle's medium (MEM; Gibco Laboratories, Grand Island, N.Y.) containing 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, Utah) and supplemented with 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 15 mM glucose, 100 U of penicillin per ml, and 100 μg of streptomycin per ml (SMEM). Phenol-extracted LPS from *Escherichia coli* serotype O11:B4 and arginase were purchased from Sigma Chemical Co., St. Louis, Mo. The N^G-monomethyl-L-arginine monoacetate salt (i.e., N^GMMA) was purchased from Calbiochem Corp., La Jolla, Calif. The rMuIFN-γ was supplied by Genentech, Inc., South San Francisco, Calif.

Cell preparation. Resident splenic macrophages from normal mice and splenic macrophages from infected animals were isolated by a method described previously (18, 24). Spleen cells were suspended at a concentration of 4×10^7 per ml, and 100 μl was seeded onto 13-mm-diameter Thermonox coverslips (Nunc, Inc., Naperville, Ill.) in 24-well tissue culture plates (Corning Glass Works, Corning, N.Y.) and incubated at 37°C in a 5% CO_2 atmosphere for 2 h. The coverslips were then vigorously washed with prewarmed Hanks' balanced salt solution to remove nonadherent cells. A confluent monolayer of splenic macrophages (determined by expression of the macrophage-specific F4/80 antigen) was obtained by this protocol.

Adherent and nonadherent subpopulations of spleen cells from infected mice were fractionated by the following protocol. Splenocytes were suspended at a concentration of 4×10^7/ml, 100 μl was seeded into 24-well tissue culture plates, and the plates were incubated for 2 h. The nonadherent cells were then washed off, collected, and passed over nylon wool to ensure complete removal of any macrophages which may have washed off. The cells were then collected and adjusted to 10^7 cells per ml and incubated at 37°C. Supernatants were collected every 24 h and analyzed for NO_2^-.

Infection of mice. Mice were injected intravenously with 2×10^5 yeast cells of *H. capsulatum* (3). This inoculum size results

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in a sublethal infection which the mice cleared in approximately 8 weeks (3). The mice were killed 1, 2, and 3 weeks postinfection. In different experimental protocols, either all of the spleen cells were used or macrophages were isolated by the method described previously (19, 24).

Assay for macrophage activation. Splenic macrophages were isolated and seeded onto 13-mm Thermonox coverslips as described above and used immediately for the macrophage activation assay. Resident splenic macrophage monolayers were incubated in 1 ml of SMEM with or without gamma interferon (IFN-γ) and LPS alone or in combination with 1.2 mM N^GMMA for an 18-h preincubation period (P_{18}). At the completion of the preincubation period, unopsonized yeast cells of *H. capsulatum* were washed, and approximately 2×10^5 to 3×10^5 cells were added to the macrophage monolayer. Two hours were allowed for phagocytosis, and then extracellular yeasts were washed away. Two coverslips that were not exposed to activating agents were fixed in methanol to determine the number of yeast cells per macrophage at the start of the test period of incubation. Control studies showed that preexposure to rMuIFN-γ and LPS alone or in combination with N^GMMA did not increase phagocytosis and that the macrophages, in the presence of factors, had nearly the same number of yeast cells per macrophage at the start of the preincubation period as did those not exposed to any of the factors (data not shown). The remaining monolayers were overlaid with either medium alone or medium containing the factors corresponding to the preincubation protocol and incubated for an additional 18-h test period (T_{18}) at 37°C. After the second incubation period, the monolayers were washed and fixed in methanol. Monolayers were stained with periodic acid-Schiff stain (EM Diagnostic Systems, Inc., Gibbstown, N.J.), and the mean number of yeast cells within infected macrophages was determined by counting the yeast cells in a minimum of 100 infected cells. The net growth was calculated by subtracting the mean number of yeast cells per macrophage at zero time from the mean number of yeast cells per macrophage at the conclusion of the experiment. The percent inhibition of growth was calculated from the following formula: [1 − (growth within macrophages in various test media/growth within macrophages in control medium)] × 100.

NO_2^- assay. Resident splenic macrophages from normal mice were seeded onto Thermonox coverslips by the method described above. The splenic macrophages were then treated with SMEM with or without rMuIFN-γ and LPS alone or in combination with 1.2 mM N^GMMA for the P_{18}. At the completion of this time, supernatants from the macrophage monolayers were removed and analyzed for NO_2^-. These samples were designated P_{18} NO_2^- levels. After exposure to yeast cells of *H. capsulatum*, the monolayers were incubated for an additional 18 h in SMEM alone or in SMEM containing the factors to which the monolayers were previously exposed, and the supernatants were collected and analyzed for NO_2^- levels. These samples were designated T_{18} NO_2^- levels.

Macrophages isolated from the spleens of infected mice were seeded onto Thermonox coverslips as described above. The macrophage monolayers were incubated in SMEM with or without 1.2 mM N^GMMA and incubated for 18 h (P_{18}). Prior to the addition of yeast cells of *H. capsulatum*, supernatants were collected for NO_2^- analysis. These samples were designated P_{18} NO_2^- levels as described before. After exposure to yeast cells, the monolayers were exposed for an additional 18 h in SMEM alone or in combination with 1.2 mM N^GMMA. At the completion of this incubation period (T_{18}), supernatants were analyzed for NO_2^-. These NO_2^- samples were designated T_{18} NO_2^- levels.

TABLE 1. Correlation of NO_2^- production and antihistoplasma activity of IFN-γ- and LPS-activated splenic macrophages

Treatment	No. of yeast cells/infected macrophage[a]	Growth[b]	% Reduction in growth[c]	NO_2^- concn (μM)[d]
SMEM	7.3 ± 1.7	5.4	0	1.6 ± 1.3
IFN-γ (2,000 U/ml) + LPS (0.1 μg/ml)	3.0 ± 1.1	1.1	80	7.6 ± 0.7[e]
IFN-γ (2,000 U/ml) + LPS (0.1 μg/ml) + 1.2 mM N^GMMA	6.5 ± 0.1	4.6	15	2.9 ± 1.2

[a] The results are means ± standard deviations for two experiments with duplicate wells for each experiment (*n* = 4). The mean ± standard deviation at zero time was 1.9 ± 0.5 yeast cells per infected macrophage.

[b] Growth is recorded as the net growth, which was calculated by subtracting the mean number of yeast cells per infected macrophage at zero time from the mean number of yeast cells per infected macrophage after 18 h of incubation at 37°C.

[c] Percent reduction represents the reduction of the growth of yeast cells within infected macrophages incubated in IFN-γ plus LPS or in IFN-γ plus LPS and N^GMMA compared with the growth of yeast cells within infected macrophages in control medium.

[d] NO_2^- levels determined prior to the addition of yeast cells. The NO_2^- assay was performed as described in Materials and Methods.

[e] Significance was determined by the Student *t* test for the amount of NO_2^- produced by IFN-γ- and LPS-treated macrophages versus NO_2^- produced by macrophages exposed to medium alone. P, ≤0.0001.

The NO_2^- concentration of the macrophage supernatants was determined by modifications to a colorimetric assay described previously (4, 8). Sample supernatants and ice-cold 350 mM NH_4Cl (pH 9.6; 0.25 ml each) were combined in glass culture tubes (12 by 75 mm); vortexed briefly, and placed on ice in dim light. After the tubes were allowed to equilibrate in the dark, 0.5 ml of ice-cold color reagent [a 1:1:3 mixture of 5 mM sulfanilic acid (Sigma), 5 mM *N*-(1-naphthyl)ethylenediamine (Sigma), and glacial acetic acid (Fisher, Pittsburgh, Pa.)] was added to each tube. Samples were then vortexed and incubated in the dark for 10 min at room temperature. The NO_2^- concentration of each sample was determined by comparing the optical density at 555 nm with a standard curve (1 to 40 μM) generated from a freshly prepared solution of $NaNO_2$ (Fluka, Chemika-BioChemika, Buchs, Switzerland). The absence of interference in the assay by medium components was ascertained by comparing standard nitrite aqueous solutions with one made up in culture medium. The results were recorded as a micromolar concentration per well.

RESULTS

The antihistoplasma activity of stimulated resident splenic macrophages correlates with the production of NO_2^-. We have previously shown that treatment of resident splenic macrophages with rMuIFN-γ and LPS activates those cells to inhibit the intracellular growth of *H. capsulatum* (19). NO_2^- levels were recorded for both the P_{18} and T_{18} time periods. The data in Table 1 indicate that activated splenic macrophages produced significant levels of NO_2^- at P_{18} compared with unstimulated macrophages. Interestingly, lower levels of NO_2^- were produced by stimulated macrophages at T_{18} than were detected at P_{18} (data not shown). Inclusion of N^GMMA, a competitive inhibitor of L-arginine metabolism, abolished the inhibition of intracellular growth. Moreover, only baseline levels of NO_2^- were detected in supernatants from stimulated macrophages exposed to N^GMMA. These data suggest that

FIG. 1. Spleen cells from *H. capsulatum*-infected mice produce NO_2^-. Spleens were removed from mice injected with MEM alone or with *H. capsulatum* at 1, 2, and 3 weeks postinfection to measure NO_2^- production in vitro. Spleen cells were adjusted to a concentration of 10^7/ml and incubated for 24 h in a 5% CO_2 atmosphere at 37°C. At the completion of this incubation, cell-free supernatants were collected and NO_2^- concentrations were determined as described in Materials and Methods. The data represent means ± standard deviations of two experiments with duplicate determinations for each group (n = 4). Symbols: solid bars, NO_2^- levels from *H. capsulatum*-infected animals; hatched bars, NO_2^- levels from mice injected with MEM alone.

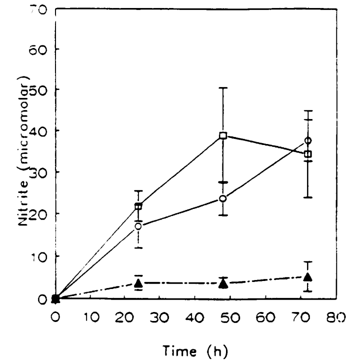

FIG. 2. Macrophages from *H. capsulatum*-infected mice are responsible for production of NO_2^-. Spleen cells from infected animals 2 weeks postinfection were fractionated into adherent and nonadherent subpopulations and cultured in SMEM alone for 72 h. At 24-h intervals, cell-free supernatants were measured for NO_2^- as described in the text. The data represent the means ± standard deviations of three experiments with duplicate determinations for each group (n = 6). Symbols: squares, total cells; circles, adherent cells; solid triangles, nonadherent cells.

activation of resident splenic macrophages to inhibit the intracellular growth of *H. capsulatum* is dependent on the generation of NO˙ from L-arginine. Furthermore, these data suggest that production of NO_2^- by activated macrophages prior to the addition of yeast cells of *H. capsulatum* is important to subsequent intracellular inhibition. The levels of NO_2^- at different time points was more thoroughly explored with immune macrophages from mice recovering from a sublethal infection (see Table 3). A corollary of these results occurred in our studies with peritoneal macrophages. In this system, rMuIFN-γ did not activate macrophages suspended in an arginine-free medium and N^GMMA had no effect on *H. capsulatum* growth in the absence of arginine (35).

Production of NO_2^- by spleen cells of *H. capsulatum*-infected mice. Since the antihistoplasma activity of activated resident splenic macrophages correlated with the production of NO_2^-, we set about to determine whether the generation of NO_2^- by splenic macrophages from infected mice also corresponded with their in vitro effect. A preliminary set of experiments was performed to determine whether spleen cells from infected mice produced NO_2^-. Mice were infected intravenously with 2 × 10^5 yeast cells of *H. capsulatum* and were killed 1, 2, and 3 weeks postinfection. The spleens were removed, whole spleen cell populations from infected mice and mice injected with MEM alone were cultured in SMEM without any additional stimuli for 24 h, and NO_2^- levels were measured. The data in Fig. 1 show that spleen cells from infected mice produced high levels of NO_2^-. The levels of NO_2^- increased gradually from 1 week to 3 weeks postinfection. Production of NO_2^- by splenocytes from MEM-injected animals was extremely low (Fig. 1). These data indicated that *H. capsulatum* infection resulted in populations of spleen cells that produced high levels of NO_2^- when tested in vitro.

Splenic macrophages are responsible for NO_2^- production from *H. capsulatum*-infected mice. To determine the population of spleen cells from the infected mice which were responsible for NO_2^- production, spleen cells from mice were isolated 2 weeks postinfection and fractionated into adherent and nonadherent populations as described in Materials and Methods. The subpopulations of cells were then cultured in SMEM alone for 72 h, and NO_2^- levels were measured every 24 h. The data in Fig. 2 indicate that only adherent cells, representing activated macrophages, produced NO_2^-. The amount of NO_2^- production by splenic macrophages increased over time, with the highest levels occurring after 72 h in culture. These data clearly showed that splenic macrophages from *H. capsulatum*-infected mice were activated to produce high levels of NO˙.

NO_2^- production by splenic macrophages from infected mice correlates with inhibition of intracellular growth. Having demonstrated that splenic macrophages from *H. capsulatum*-infected mice generated NO˙, we next determined whether the growth inhibitory effect of stimulated macrophages correlated with the production of NO_2^-. Splenic macrophages were isolated from infected animals 2 weeks postinfection and cultured in SMEM without activating factors. The data in Table 2 indicate that these macrophages inhibited the intracellular growth of *H. capsulatum* and that the production of NO_2^- correlated with the antihistoplasma activity (Table 2, row 1). The presence of N^GMMA throughout the entire experiment (P_{18} and T_{18}) blocked the generation of NO_2^- and, consequently, the antihistoplasma effect (Table 2, row 2). Inclusion of arginase blocked the inhibition of intracellular growth and NO_2^- production (Table 2, row 5). The data in Table 2 (row 3) show that when splenic macrophages from infected mice were incubated with N^GMMA prior to addition of *H. capsulatum*, low levels of NO_2^- were produced at the P_{18} time point. When yeast cells were added in SMEM alone and the macrophages were incubated for an additional 18 h without N^GMMA, the macrophages generated NO_2^- and inhibited the intracellular growth of the fungus (Table 2, row 1). In contrast, when N^GMMA was present only during the test period (T_{18}), there were only low levels of NO_2^- produced and the yeast cells multiplied within the macrophages (Table 2, row 4). These data suggest that NO_2^- production must occur during the test period for inhibition of intracellular growth to occur and that production of NO_2^- correlates with antihistoplasma activity of splenic macrophages.

TABLE 2. Antihistoplasma activity of splenic macrophages isolated from mice 2 weeks after sublethal infection with *H. capsulatum*

Row	Treatment (time of treatment)	No. of yeast cells/ macrophage[a]	Growth[b]	% Reduction in growth[c]	NO_2^- concn (μM)	
					P_{18}[d]	T_{18}[e]
1	SMEM	1.8 ± 0.2	0	100	15.6 ± 2.6	6.9 ± 3.3
2	1.2 mM N^GMMA (P_{18} and T_{18})[f]	5.8 ± 1.0	3.6	8	2.7 ± 1.8	1.4 ± 0.3
3	1.2 mM N^GMMA (P_{18})[g]	2.2 ± 0.3	0	100	4.3 ± 4.2	17.3 ± 3.6
4	1.2 mM N^GMMA (T_{18})[h]	5.7 ± 1.8	3.5	10	16.5 ± 2.9	3.3 ± 2.9
5	50 U of arginase per ml	6.1 ± 0.2	3.9	0	2.4 ± 1.6	0[i]

[a] The results are means ± standard errors for three experiments with duplicate wells for each experiment ($n = 6$). The mean ± standard error at zero time was 2.2 ± 0.2 yeast cells per macrophage.
[b] Calculated as described in Table 1, footnote *b*.
[c] Calculated as described in Table 1, footnote *c*.
[d] P_{18} is the preincubation period (see Materials and Methods).
[e] T_{18} is the test period after preliminary treatments (see Materials and Methods).
[f] N^GMMA was present throughout the entire experiment (P_{18} and T_{18}).
[g] N^GMMA was present only during the 18-h preincubation period (P_{18}) prior to the addition of yeast cells of *H. capsulatum*.
[h] N^GMMA was added with yeast cells of *H. capsulatum* and was present for the remaining 18 h of incubation.
[i] Undetectable.

DISCUSSION

Ingestion of opsonized yeast cells of *H. capsulatum* by mouse peritoneal macrophages results in the production of reactive oxygen intermediates, and yet the fungus is able to replicate within such phagocytes (14, 29). Moreover, rMuIFN-γ-treated peritoneal macrophages inhibit the intracellular growth of *H. capsulatum* but do not express an oxidative burst (28). Therefore, activation of mouse macrophages to an antihistoplasma state is not directly related to the respiratory burst. Activated macrophages produce reactive nitrogen intermediates, i.e., nitric oxide ($NO^·$) and other oxides of nitrogen, which kill tumor cells (26) and animal parasites such as *Leishmania major* (11) and *Schistosoma mansoni* (15) and inhibit the growth of *Toxoplasma gondii* (1) and *Cryptococcus neoformans* (10). Activated macrophages produce inducible nitric oxide synthase which cleaves the guanidino nitrogen group from L-arginine to generate $NO^·$ and L-citrulline (13). $NO^·$ inactivates iron-containing enzymes which function in DNA synthesis, the citric acid cycle, and mitochondrial respiration (16, 26). It is thought that $NO^·$ disrupts the activity of the enzymes by forming a complex with the iron present in the Fe-S prosthetic groups (17). Ferrous iron reverses the $NO^·$-mediated macrophage cytotoxicity against tumor cells (7), *S. mansoni* (15), and *Leishmania enterretti* (21).

The spleen is one site of infection after intravenous infection of mice with yeast cells of *H. capsulatum* (3, 27, 34). Clearance of the fungus from an infected mouse can be quantified by recording a reduction in the number of yeast cells in their spleens. Therefore, we studied the interaction of yeast cells of *H. capsulatum* with both resident splenic macrophages and splenic macrophages from *H. capsulatum*-infected mice to identify the mechanism by which activated splenic macrophages inhibit the intracellular growth of *H. capsulatum*. The results reported herein show that resident splenic macrophages are activated by exposure to rMuIFN-γ and LPS to produce NO_2^- from L-arginine. The production of NO_2^- by activated resident splenic macrophages correlates with the inhibition of intracellular growth of *H. capsulatum*. Inclusion of the L-arginine analog N^GMMA abolished the antihistoplasma activity and blocked the generation of NO_2^-. Furthermore, we have previously demonstrated that the IFN-γ- and LPS-induced antihistoplasma state of resident splenic macrophages can be reversed by iron (19). Thus, these data indicate that resident splenic macrophages are activated to an antihistoplasma state through the generation of $NO^·$ from L-arginine.

A recent study by Al-Ramadi et al. (2) demonstrated that splenic macrophages from mice injected with an attenuated strain of *Salmonella typhimurium* produced high levels of NO_2^-. Since the production of NO_2^- by rMuIFN-γ-plus-LPS-stimulated resident splenic macrophages correlated with the antihistoplasma state, we were interested in determining whether splenic macrophages isolated from sublethally infected mice produced NO_2^-. Spleen cells from infected animals produce high levels of NO_2^- in vitro in the absence of any added stimulating factors. Activated splenic macrophages were responsible for the production of NO_2^-. Increasing levels of NO_2^- were produced over a 3-week period by spleen cells from sublethally infected animals (Fig. 1). In a previous report from our laboratory, Wu-Hsieh demonstrated significant clearance of *H. capsulatum* from the spleens of sublethally infected animals over a similar period of time (30). It is interesting to speculate that clearance of the organism from the animal is related to NO_2^- production by macrophages. However, further studies are required to define the molecular bases of a host's recovery from *H. capsulatum* infection.

The antihistoplasma activity of splenic macrophages from infected animals 2 weeks postinfection was assessed in vitro. The production of NO_2^- correlated with the antihistoplasma activity of splenic macrophages. The production of NO_2^- appeared to be important in growth inhibition only during and the period after the addition of yeast cells of *H. capsulatum*. However, the data in Table 2, row 1, show that high levels of NO_2^- are produced during the P_{18} time period, lower levels are produced at the T_{18} time period, and yet there is inhibition of intracellular growth. These data are similar to the results obtained with resident macrophages activated with IFN-γ and LPS. We are currently investigating the kinetics of NO_2^- production by activated macrophages versus the antihistoplasma activity of these cells. The presence of anti-tumor necrosis factor alpha (TNF-α) antibodies did not affect either the generation of NO_2^- or the inhibition of intracellular growth (data not shown). These data support our earlier observations that, although TNF-α is important in host defense, it does not have a role in macrophage activation to an antihistoplasma state (19).

Our laboratory has reported that recombinant human IFN-γ-treated human monocyte-derived macrophages are not activated to an antihistoplasma state (9). Newman and Gootee (23) have recently reported an inhibition of intracellular growth of *H. capsulatum* within human macrophages after a

24-h exposure to interleukin 3, granulocyte macrophage-colony stimulating factor, or macrophage-colony stimulating factor. Inclusion of N^GMMA did not affect the antihistoplasma activity nor were levels of NO_2^- detected in the supernatants of activated macrophages (23). There is conflicting evidence as to whether human monocytes/macrophages can be activated to produce $NO^{\cdot}$. Human Kupffer cells treated with interleukin 1, TNF-α, IFN-γ, and LPS do not release significant levels of NO_2^- or NO_3^- (25). Cameron et al. (5) have reported that human alveolar and peritoneal macrophages did not require L-arginine to inhibit the in vitro replication of *C. neoformans* and did not produce detectable levels of NO_2^- or NO_3^- (5). These results suggest that human macrophages do not produce $NO^{\cdot}$. However, there is one recent report that human monocyte-derived macrophages treated with TNF and granulocyte macrophage-colony stimulating factor produce high levels of NO_2^- which correlate with inhibition of growth of virulent *Mycobacterium avium* (6). $NO^{\cdot}$ has been implicated as the effector molecule responsible in killing of *Trypanosoma cruzi* by IFN-γ plus TNF-treated human macrophages (22) and in human neutrophil killing of *Staphylococcus aureus* (20). Thus, the role of induced $NO^{\cdot}$ in human host defense mechanisms remains unclear, but currently, there is no evidence that human monocytes/macrophages generate $NO^{\cdot}$. Further work is necessary to determine the antimicrobial effector molecule in human macrophages activated to histoplasmostasis by appropriate stimulants (23).

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