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Cryptococcus neoformans Fails To Induce Nitric Oxide Synthase in Primed Murine Macrophage-like Cells

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Nitric oxide (NO) is a microbiostatic gas generated by activated murine macrophages. Cytokine signals, gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) act synergistically to induce production of a macrophage nitric oxide synthase (NOS). A variety of intracellular pathogens, when recognized by macrophages primed with IFN-γ, induce NOS by eliciting TNF-α secretion, which then functions as a positive autocrine signal. In cell culture assays, a murine macrophage cell line (J774), primed with IFN-γ, was tested for NOS induction upon challenge with virulent *Cryptococcus neoformans*. *C. neoformans* failed to induce macrophage NOS as measured by nitrite production. This was true irrespective of the *C. neoformans*-to-J774 ratio. Other nonpathogenic *Cryptococcus* species likewise failed to induce NOS, yet *Saccharomyces cerevisiae*, *Histoplasma capsulatum*, and *Candida albicans* were efficient inducers of NOS. Conditions which promoted attachment and/or phagocytosis of *C. neoformans* did not lead to NOS induction (including opsonization with specific antibodies against *C. neoformans*). Assays for transcriptional repressors of NOS were negative. Tests for consumption of nitrite by measurement of additional products of NOS induction were negative. No TNF-α was detected by enzyme-linked immunosorbent assay in supernatants from *C. neoformans*-J774 cocultures. A mutant *C. neoformans* strain with a minimal, but visible, polysaccharide capsule also failed to induce NOS; however, several nonencapsulated mutants of *C. neoformans* did induce NOS. Failure of *C. neoformans* to act as an inducer of NOS may be related to the virulence of this pathogen in mice; *C. neoformans* is a unique example of a facultative intracellular pathogen which fails to induce NOS in primed macrophages. The mechanism appears to involve the failure of TNF-α secretion once the macrophage comes in contact with the fungus. The presence of the polysaccharide capsule appears to mask the signal necessary for TNF-α secretion and, ultimately, NOS induction.

Cryptococcus neoformans is an encapsulated facultative intracellular fungus which is pathogenic for humans and other animals. This microorganism produces an overwhelming, fatal infection in mice (6). It has been demonstrated that macrophages are important in the immunologic defense against cryptococcal infection; activated macrophages inhibit replication of *C. neoformans* in vitro (17, 21, 35). In addition, in vivo experiments have shown that at sites of cryptococcal infection, the majority of inflammatory cells responding to the infection are macrophages. One effector molecule involved in the inhibition of *C. neoformans* is nitric oxide (NO) (2). NO is a microbiostatic, highly reactive gas produced by activated murine macrophages by oxidation of L-arginine to L-citrulline and NO (38). The enzyme catalyzing this reaction, nitric oxide synthase (NOS), is induced by sequential cytokine signals. The first signal, gamma interferon (IFN-γ), is produced by activated lymphocytes. The second signal for NOS induction is tumor necrosis factor alpha (TNF-α), produced by the macrophage itself (13, 14, 37, 40, 46). Several intracellular pathogens, when recognized by macrophages primed with IFN-γ, induce NOS by eliciting TNF-α secretion. Examples include *Listeria monocytogenes* (3, 5), *Mycobacterium bovis* (18, 50), *Leishmania major* (23, 24, 36), and *Francisella tularensis* (16). Lipopolysaccharide (LPS) from gram-negative bacteria is also a potent inducer of NOS in primed macrophages (38, 50). The focus of the present research was to examine the ability of *C. neoformans* to induce NOS in primed murine macrophages in vitro. We observed that NOS was not induced in IFN-γ-primed J774.1 macrophage-like cells challenged with virulent *C. neoformans*. Hence, a study was undertaken to examine the variables which might be responsible for this lack of induced enzyme activity.

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MATERIALS AND METHODS

Cell culture assays. A murine macrophage cell line, J774.1, was used in cell culture experiments. J774.1 cells were grown in a 5% carbon dioxide incubator at 37°C and passaged every 3 to 4 days in sterile culture plates (Falcon, Lincoln Park, N.J.). The medium used was Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, N.Y.) with added glucose (final concentration, 15 mM), sodium bicarbonate (25 mM), buffer (25 mM MOPS [morpholinepropanesulfonic acid], pH 7.4), gentamicin (10 μg/ml), and 10% fetal bovine serum (Hyclone Laboratories, Logan, Utah). J774.1 cells were washed from the cell culture plates and placed into 16-mm-diameter culture wells (Costar, Cambridge, Mass.) at a final concentration of 500,000 cells per well in 1.0 ml of medium. Recombinant murine IFN-γ (kindly provided as a gift from Genentech, San Francisco, Calif.) was added to each experimental well at a concentration of 200 U/ml. After overnight incubation, the medium was changed with fresh Dulbecco's modified Eagle's medium containing the above-listed additives, 10% fetal bovine serum, and 200 U of IFN-γ per ml. Log-phase yeasts were counted with a Coulter ZB_i counter (Coulter Electronics, Hialeah, Fla.) and added at various concentrations, as indicated in the figure legends. LPS, a phenol extract of *Escherichia coli* O128:B12 (Sigma Chemical Co., St. Louis, Mo.), was added to the positive control wells at a concentration of 10 ng/ml. The cultures were incubated for approximately 40 h.

Fungal cultures. Yeast cells were inoculated from yeast nitrogen agar plates into Dulbecco's modified Eagle's medium containing 25 mM sodium bicarbonate, 25 mM MOPS (pH 7.4), 15 mM glucose, and 10 μg of gentamicin per ml without serum. Yeast cells were passaged in this LPS-free medium in small culture flasks (25 ml; Falcon) at least three times prior to use in experiments. All yeasts were grown in a 5% CO_2 incubator at 37°C except non-*C. neoformans* cryptococci, which were grown in the absence of CO_2 at 30°C.

Strain H99 is a clinical isolate of *C. neoformans* serotype A; it is virulent and

has a prominent polysaccharide capsule when cultured in 5% CO_2 (20). Strain C3D is derived from H99. C3D possesses a much thinner polysaccharide capsule than that of H99 when cultured in 5% CO_2 and is markedly attenuated in virulence (20). Strain 602 is an acapsular mutant of *C. neoformans* (32). Strains C305, C307, C309, C322, C325, and C326 are also acapsular mutants of *C. neoformans*. Strains 3501 and 3502 are the wild-type parents of strains C305, C307, C309, C322, C325, and C326, produced by treatment with a mutagen (28).

Avirulent species of the genus *Cryptococcus* were grown at 30°C. These strains were all environmental isolates. *Rhodotorula rubra*, like *C. neoformans*, is a basidiomycete and was tested because of its phylogenic relatedness to the genus *Cryptococcus*.

The *Candida albicans* used in these experiments is a clinical isolate. Initial attempts to use live *C. albicans* in cell culture were unsuccessful, as this species killed the J774.1 cells in culture. Therefore, *C. albicans* used for experiments was passaged as with other yeasts but was killed before use by heating to 70°C for 15 min. Killing was verified by lack of growth on Sabouraud agar plates. *Histoplasma capsulatum* strain 505a and a laboratory strain of *Saccharomyces cerevisiae* were also used.

Cell culture assay for the presence of transcriptional inhibitors of NOS. Cultures containing 5×10^5 J774.1 cells per ml were preincubated for 24 h under three conditions: wells containing J774.1 cells plus IFN-γ, wells containing J774.1 cells plus IFN-γ plus *C. neoformans* H99 (in a 1:1 ratio with J774.1), and J774.1 cells only. Supernatants from these three conditions were designated, respectively, supernatants 1, 2, and 3 (SNF1, SNF2, and SNF3, respectively). These supernatants were removed from the original cell cultures and added to fresh cultures with J774.1 cells. Preliminary experiments demonstrated that between 10^4 and 10^5 U of TNF-α per ml was required to induce NOS in IFN-γ-primed J774.1 cells (data not shown). We therefore chose concentrations of 400 U of IFN-γ per ml and 2×10^4 U of TNF-α per ml, which were added to each well containing preincubated supernatant (either SNF1, SNF2, or SNF3) with fresh J774.1 cells. The wells were assayed for the presence of nitrite by using Greiss reagents after the standard incubation period (see below).

Preparation of rabbit anti-*C. neoformans* antibody. The method discussed here has been previously published elsewhere (27). Briefly, male New Zealand White rabbits were inoculated intracisternally with approximately 5×10^7 CFU of *C. neoformans* H99 in 0.3 ml of phosphate-buffered saline (PBS). On day 3 of infection, cerebrospinal fluid (CSF) was obtained from the subarachnoid space of anesthetized animals and then pooled and centrifuged to remove cells. The CSF was stored at 85°C until just prior to use. Immunoglobulin G was then purified from the CSF by using a protocol published previously (39). Ten milliliters of CSF was unfrozen and diluted with 40 ml of 60 mM acetate (pH 4.0). The pH of the resulting mixture was adjusted to 4.5 with 1.0 N sodium hydroxide. Caprylic acid (25 μl/ml) was added dropwise with thorough mixing for 30 min. Insoluble material was removed by centrifugation (Sorvall RC-5B, rotor model SS-34; at 10,000 × *g* for 30 min at 4°C). The resulting supernatant was mixed with concentrated PBS, and the pH was adjusted to 7.4 with 1.0 N sodium hydroxide. The supernatant was cooled to 4°C, to which 0.277 g of ammonium sulfate per ml was added. This was stirred for 60 min and then centrifuged at 5,000 × *g* for 15 min (Sorvall; at 4°C). The supernatant was discarded, and the pellet was resuspended in 1 ml of PBS and subsequently dialyzed against 300 ml of PBS overnight. The dialyzed sample was heated to 55°C for 20 min and then centrifuged at 5,000 × *g* (Sorvall; at 4°C) for 20 min. The supernatant was filter sterilized and stored at −70°C until just prior to use.

NOS activity measured by nitrite and nitrate production. Supernatants from J774.1 cell cultures were removed and centrifuged for 60 s (Beckman Microfuge TJ6-R; 10,000 × *g* at room temperature) to remove yeast cells. Aliquots (400 μl) of each supernatant were placed in 4.5-ml cuvettes, and 800 μl of each Greiss reagent (1% sulfanilamide in 2.5% phosphoric acid and 0.5% naphthylethylenediamine in 2.5% phosphoric acid) was added. The resulting colorimetric reaction was measured at 540 nm with a Shimadzu model UV-240 spectrophotometer; nitrite concentration was calculated from a standard curve which was linear between 0 and 100 μM sodium nitrite.

The total nitrite and nitrate concentration was determined for each sample to assess the stoichiometric relationship of NO_2^- plus NO_3^- to citrulline. This assay was performed by mixing 300 μl of sample supernatant, 150 μl of 0.2 M HEPES (*N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, pH 7.4), and 150 μl of a *Pseudomonas oleovorans* preparation to reduce NO_3^- to NO_2^- (5). The mixture was vortexed and incubated at 37°C for 90 min and then centrifuged at 10,000 × *g* (Beckman Microfuge TJ6-R) for 2 min. From the resulting supernatant, 400 μl was removed and placed into a 4.5-ml cuvette; 800 μl of each Greiss reagent was added. A_{540} was measured on a spectrophotometer and compared with nitrite and nitrate standards.

NOS activity measured by citrulline production. Three milliliters of fetal bovine serum was dialyzed against 400 ml of sterile PBS to remove urea which would interfere with the citrulline assay. The dialysate was changed every 24 h for 3 days. The resulting dialyzed fetal bovine serum was then filter sterilized for use. Phenol red-free medium was used so as not to interfere with the colorimetric measurement of citrulline. The medium was prepared with dialyzed fetal bovine serum at a final concentration of 10%. J774.1 cells (5×10^5 cells per ml) were plated in this medium as described previously. Supernatants were removed and centrifuged (Beckman Microfuge TJ6-R). The colorimetric assay for citrulline is based on the reaction of the carbamido group of citrulline with diacetyl monoxime to form a chromophore with maximal A_{490} (25). Briefly, each supernatant was placed in a glass test tube into which 10 U of urease per ml was added. In addition, redox buffer (ferric ammonium sulfate plus ferrous ammonium sulfate in 1 N sulfuric acid), an acid mixture (concentrated phosphoric acid plus 1 N sulfuric acid), and diacetyl monoxime were added to each sample. The samples were mixed and heated in a boiling water bath in the dark for 20 min and then chilled in cold water for 5 min. The samples were read immediately in the spectrophotometer at 490 nm. The citrulline concentration was calculated from L-citrulline standards.

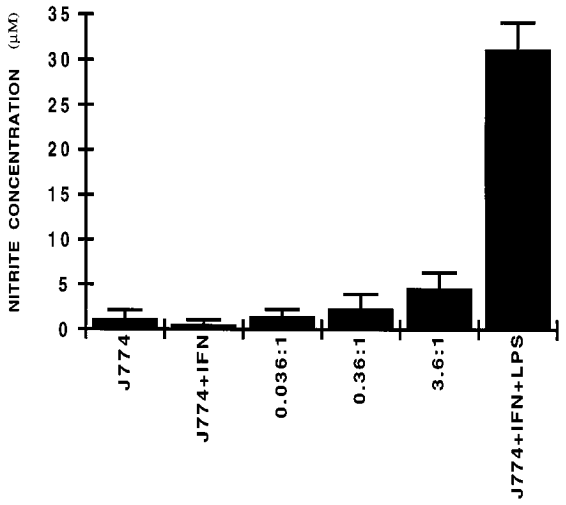

FIG. 1. Lack of NO production by primed macrophages challenged with *C. neoformans*. Shown are nitrite concentrations in cell culture wells containing 500,000 J774 macrophage-like cells and *C. neoformans* H99 cells. The first bar on the left represents culture with J774 only; all other wells received a total of 100 U of IFN-γ per ml. Ratios indicate relative proportions of *C. neoformans* to J774 cells. In this and subsequent figures, wells with J774 plus IFN-γ plus LPS are positive controls. Results shown are from a representative experiment which was repeated 20 times.

TNF-α ELISA. The presence of TNF-α in cell culture supernatants was detected by murine TNF-α enzyme-linked immunosorbent assay (ELISA) (Endogen, Boston, Mass.).

RESULTS

Lack of induction of NOS by H99. The induction of NOS by *C. neoformans* H99, as measured by the production of nitrite in the cell culture medium, was examined in J774.1 cells primed with 50 to 200 U of IFN-γ per ml. In these experiments, ratios of H99 to J774.1 cells were varied by 10-fold dilutions of H99 from 0.036:1 to 3.6:1. As shown in Fig. 1, no appreciable nitrite was produced compared with the negative control wells. The positive control with added LPS (10 ng/ml) showed significant nitrite production. By phase-contrast microscopy, H99 cells were not phagocytized by J774.1 cells under these conditions where no opsonins were added.

Lack of induction of NOS by non-*C. neoformans* cryptococci. The five isolates of nonpathogenic cryptococci (*C. laurentii*, *C. albidus* var. *diffluens*, *C. albidus* var. *albidus*, *C. uniguttulatus*, and *C. terreus*) and the noncryptococcal basidiomycete *R. rubra* were shown to be encapsulated by microscopic examination with India ink. NOS induction assays using twofold dilutions of yeast cells were performed, beginning with three yeast cells to one J774.1 cell and diluting to 0.325 yeast cell per J774.1 cell. Data shown in Fig. 2 represent a yeast-to-J774.1 ratio of 1.5:1. No significant amounts of nitrite were produced in any of the dilutions of experimental yeast cells. The nitrite concentration measured in these wells was, in fact, below that of the negative control (J774.1 plus IFN-γ). By phase-contrast microscopy, approximately 50% or more of the added yeast cells were

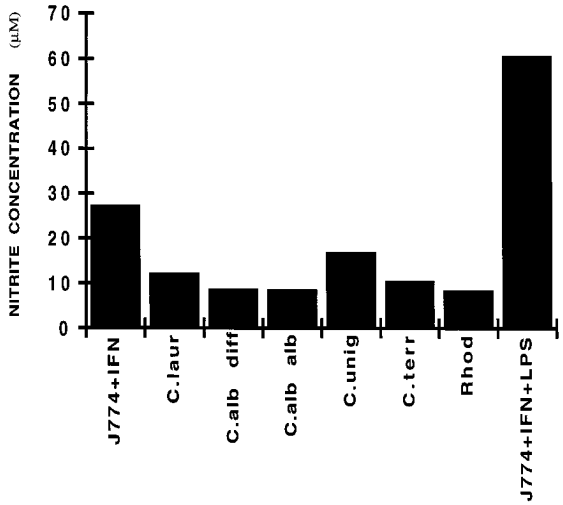

FIG. 2. *Cryptococcus* spp. fail to induce NO production by primed macrophages. Shown are nitrite concentrations in cell culture wells containing 500,000 J774 cells and nonpathogenic cryptococci in a 1:1.5 ratio. All wells received a total of 200 U of IFN-γ per ml. Results shown are from replicates of four culture wells for each yeast species. Abbreviations: C. laur, *C. laurentii*; C. alb diff, *C. albidus* var. *diffluens*; C. alb alb, *C. albidus* var. *albidus*; C. unig, *C. uniguttulatus*; C. terr, *C. terreus*; Rhod, *R. rubra*, a noncryptococcal basidiomycete.

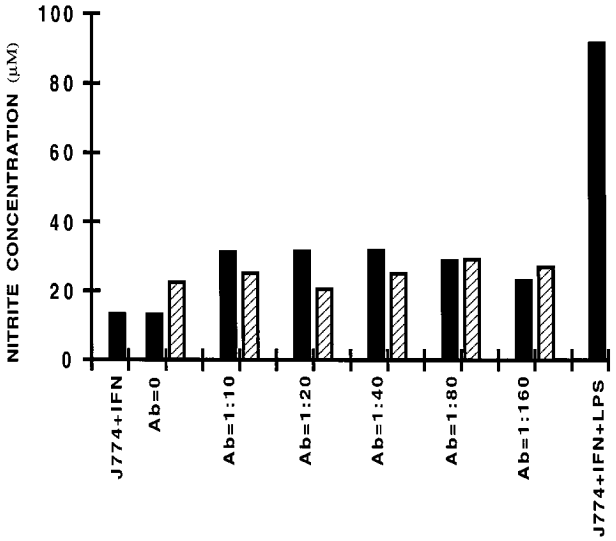

FIG. 4. Effect of anticryptococcal immunoglobulin G on macrophage NO production. Shown are nitrite concentrations in cocultures of 500,000 J774 cells per well and IFN-γ (200 U/ml) with rabbit anti-*C. neoformans* antibodies (Ab). Dilutions of Ab shown. Solid bars represent culture of J774 plus IFN-γ plus Ab; striped bars represent addition of *C. neoformans* (strain H99 at a 1:1 yeast-to-J774 ratio). Results shown are from duplicate culture wells for each condition.

phagocytized and were inside J774.1 cells by the final day of the assay. The yeast cells remained viable, as demonstrated by significant growth of organisms in supernatants taken from experimental wells and incubated at 30°C.

Induction of NOS by fungi other than cryptococci. Fungi other than cryptococci were assayed under the same conditions as those used for the previous experiments. *S. cerevisiae*, heat-killed *C. albicans*, and *H. capsulatum* were counted with a Coulter counter and added to J774.1 cultures primed with IFN-γ, as described previously. As shown in Fig. 3, all three yeast species induced significant production of nitrite by the J774.1 cells ($P = 0.0015$ for *S. cerevisiae*, 0.014 for *C. albicans*, and 0.00025 for *H. capsulatum* by analysis of variance).

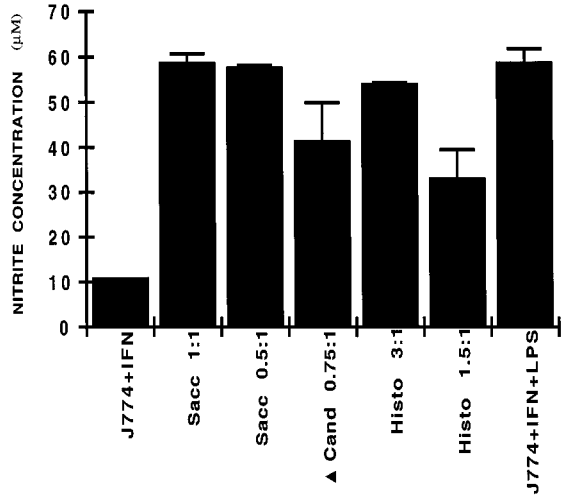

FIG. 3. Pathogenic and nonpathogenic fungi induce NO production in primed macrophages. Shown are nitrite concentrations in cell culture wells containing 500,000 J774 cells and other species. The first bar on the left represents culture with J774 only without added fungus; all wells received a total of 200 U of IFN-γ per ml. Results shown are from duplicate culture wells for each fungus-to-macrophage ratio. Abbreviations: Sacc, *S. cerevisiae*; ▲ Cand, heat-killed *C. albicans*; Histo, *H. capsulatum*.

Lack of induction of NOS by H99 cultured with rabbit anti-H99 antibodies. Given the lack of NOS induction in the previous experiments, an antiserum raised against H99 whole cells was added to cultures of J774.1 cells primed with 200 U of IFN-γ per ml and challenged with H99. The ratio of H99 to J774.1 cells was 1:1. The concentration of antibody was varied by using twofold dilutions beginning at a 1:10 dilution of stock antibodies. Figure 4 demonstrates that no significant nitrite production was measured in the wells containing antibody plus H99. In fact, the supernatants from these wells contained less nitrite than did wells with antibody but no added H99. Phase-contrast microscopic examination revealed that the presence of antibody increased phagocytosis of H99 compared with conditions excluding H99-specific antibody.

Assay for the presence of transcriptional inhibitors of NOS. Transcriptional inhibitors of NOS, such as interleukin 4, interleukin 10, and transforming growth factor β, have been described (12, 43, 48). To test the hypothesis that H99 induced a transcriptional inhibitor which blocked NOS transcription, supernatants from J774.1 cells primed with IFN-γ and challenged with H99 were examined for inhibitory activity (see Materials and Methods). J774.1 cells were primed with IFN-γ and challenged with H99. The resulting supernatant was used as the medium for fresh cultures of J774.1 cells, which were then induced to produce nitrite with known inducers of NOS (IFN-γ and TNF-α). If H99 induced a transcriptional inhibitor, the putative inhibitor might be present in the supernatant medium and might therefore suppress NOS induction in the fresh cultures with known NOS inducers. As shown in Fig. 5, no evidence was found for the presence of a transcriptional inhibitor. The nitrite concentration in the experimental wells (containing SNF2), preincubated with J774.1 cells primed with IFN-γ and challenged with H99, was not signifi-

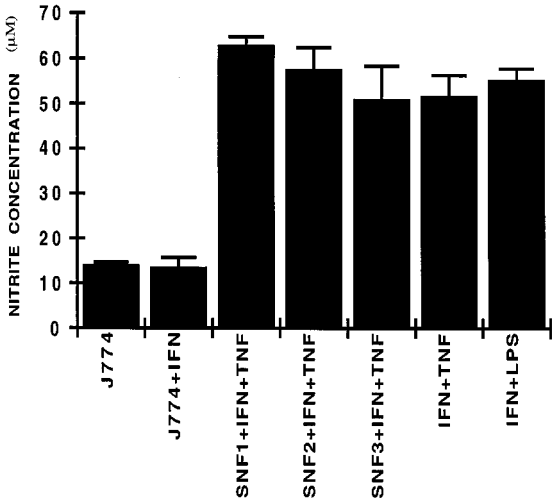

FIG. 5. Assays for the presence of transcriptional inhibitors of NOS in primed macrophages challenged with *C. neoformans*. Cultures with 500,000 J774 cells per well were preincubated for 24 h with either SNF1 (J774 plus IFN-γ), SNF2 (J774 plus IFN-γ plus *C. neoformans*), or SNF3 (J774 only). Subsequently, 200 U of IFN-γ per ml and 20,000 U of TNF-α per ml were added to wells cocultured with SNF1, SNF2, or SNF3. The presence of nitrite equal to or above that in positive control wells (J774 plus IFN-γ plus LPS) indicates the absence of an inhibitor of NOS. Results shown are from replicates of three culture wells for each condition.

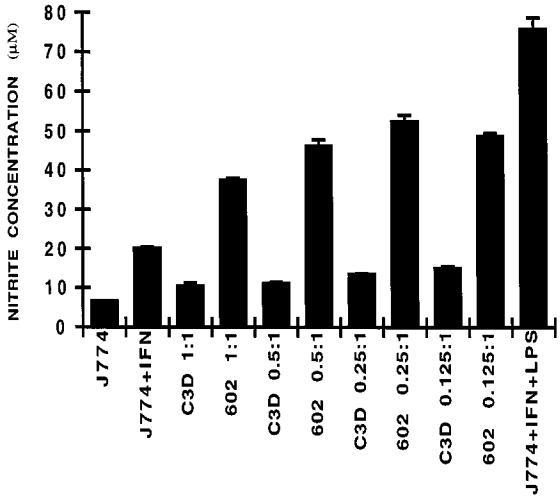

FIG. 6. *C. neoformans* acapsular mutant strain induces NO production by primed macrophages. Shown are nitrite concentrations in wells containing 500,000 J774 cells, 200 U of IFN-γ per ml, and either C3D, a hypocapsular mutant of H99, or 602, an acapsular mutant of *C. neoformans*. Ratios of yeast cells to J774 cells are shown. Results shown are from duplicate culture wells from a representative experiment. The results were repeated and confirmed five times.

cantly less than the nitrite concentrations in positive control wells.

Assay for the presence of citrulline in supernatants of H99 cell culture experiments. The measurement of nitrite concentration in the cell medium is dependent upon the conversion of NO to nitrite. To determine if NO was produced, but because its reactivity was consumed on the surface of H99 cells, citrulline concentrations were measured in culture supernatants. NO and citrulline are produced in equimolar amounts, but because citrulline is neither reactive nor metabolized to other products, it accumulates in the medium over NO-producing cells (19). Accumulation of citrulline in the absence of a significant nitrite concentration would suggest that NO was being consumed.

Assays for citrulline with supernatants from cultures of J774.1 cells primed with IFN-γ and challenged with H99 (ratio of 1:1) were performed. Concentrations of citrulline were obtained and compared with citrulline standards; the values shown in Table 1 were calculated from a standard curve which was linear through the range shown. No citrulline was detected in experimental supernatants, suggesting that NO was neither produced nor consumed by the fungus.

TABLE 1. Assay for products of NO in culture supernatants

Addition to cell culture wells	Nitrate + nitrite concn (μM)[a]	Citrulline concn (μM)[b]
J774 + *C. neoformans*[c]	3.73	0
J774 + IFN-γ[d]	3.73	0
J774 + *C. neoformans* + IFN-γ[d]	1.82	0
J774 + IFN-γ + LPS[e]	70.34	48.44

[a] Calculated from a standard curve which was linear between 0 and 100 μM. Results are means of triplicate cultures.
[b] Calculated from a standard curve which was linear between 0 and 50 μM.
[c] Strain H99 at a 1:1 yeast-to-J774.1 cell ratio.
[d] IFN added at 200 U/ml.
[e] LPS added at 10 ng/ml, IFN added at 200 U/ml.

Assay for the presence of TNF-α in supernatants of H99 cell culture experiments. To determine whether the necessary second signal, TNF-α, was being produced by the J774.1 cells, the TNF-α concentration in supernatants was measured by using a murine TNF-α ELISA. Although TNF-α concentrations were very high in positive control wells primed with IFN-γ plus 10 ng of LPS per ml added (>15.0 ng of TNF per ml without H99 and 14.9 ng of TNF-α per ml with H99), primed J774.1 cells challenged with H99 produced no significant levels of TNF-α (data not shown). Controls (J774.1 without additives and J774.1 plus 200 U of IFN-γ per ml) likewise did not result in significant TNF-α production (data not shown).

Acapsular *C. neoformans* mutants induce NOS, but a hypocapsular mutant does not. *C. neoformans* C3D is a hypocapsular mutant of the encapsulated strain, H99 (20). It cannot up-regulate capsule polysaccharide synthesis in response to environmental signals. Under all conditions, it shows a visible but very narrow capsule by India ink examination. Strain 602 is an acapsular mutant of *C. neoformans* (32). In the standard cell culture assay, nitrite concentrations were measured in supernatants from J774.1 cells primed with IFN-γ and challenged with either C3D or 602 at various concentrations of yeast cells. As shown in Fig. 6, for each concentration of yeast cells, strain C3D failed to induce NOS. In contrast, strain 602, the acapsular mutant, did induce NOS, as evidenced by significant nitrite production ($P = 0.00018$). This was true over a broad range of 602:J774.1 ratios. By phase-contrast microscopic examination, both C3D and 602 were avidly phagocytized and could be seen inside J774.1 cells by the final day of the assay.

As shown in Fig. 7, other acapsular *C. neoformans* strains, C305, C307, C309, C322, C325, and C326, under the standard cell culture conditions, induced NOS, as evidenced by significant nitrite production. By contrast, the parent strains of these acapsular mutants, 3501 and 3502, did not induce NOS.

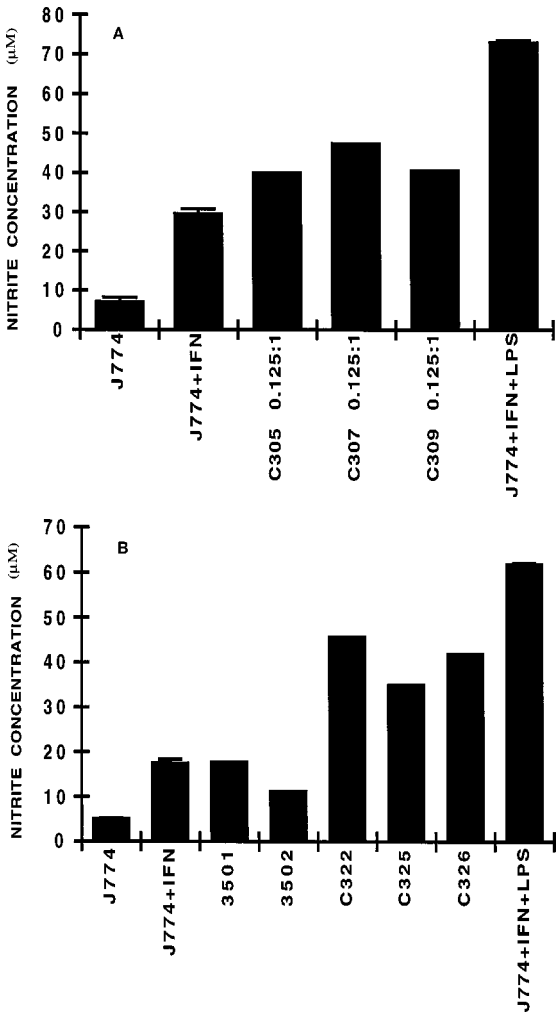

FIG. 7. (A) A series of acapsular mutant strains induce NO in primed macrophages. Nitrite concentration in wells containing 500,000 J774 cells, 200 U of IFN-γ per ml, and acapsular mutants (C305, C307, and C309) at the yeast-to-macrophage ratios shown. Results shown are from replicates of four culture wells. The results were repeated and confirmed in one subsequent experiment. (B) Nitrite concentration in wells containing 500,000 J774 cells, 200 U of IFN-γ per ml, and either wild-type *C. neoformans* (strains 3501 and 3502) or acapsular *C. neoformans* mutants (C322, C325, and C326). Data shown are for a yeast-to-macrophage ratio of 0.125:1. Results shown are from a representative experiment which was repeated, and the results were confirmed.

DISCUSSION

Previous investigators have established that NOS is induced by IFN-γ-primed murine macrophages challenged with a variety of intracellular organisms. Bacteria (3, 5, 16, 51), fungi (11), and protozoan and metazoan parasites (23, 24, 29, 36) have been reported to induce NOS by this pathway. Nitrite has also been detected in cell cultures of macrophages harvested from mice infected with *Trypanosoma musculi*, an extracellular pathogen (53). However, a notable exception may be a strain of the intracellular protozoan pathogen, *Toxoplasma gondii*, which, upon infection of IFN-γ-primed mouse macrophages, requires the addition of LPS to induce a toxoplasmastatic effect (1). The work presented here focused on NOS induction by yeasts, particularly *C. neoformans*.

Our results indicate that the *C. neoformans* strains, H99, 3501, and 3502, which possess prominent polysaccharide capsules, fail to induce NOS in IFN-γ-primed murine macrophages. Other encapsulated species of the genus *Cryptococcus* similarly fail to induce NOS. However, several other fungal species do induce NOS under identical experimental conditions. Yeast induction of NOS has been reported previously. Cenci et al. (11) found that NOS was induced in murine macrophages primed with IFN-γ and challenged with *C. albicans*. Our experiments duplicated this finding. A study by Nakamura et al. (42), in contrast to our work, failed to show induction of nitrite with IFN-γ-primed RAW 264.7 cells challenged with *H. capsulatum*. The difference in results may arise from the cell line used. RAW 264.7 cells are very sensitive to stimulation by IFN-γ alone, generating near-maximal levels of nitrite with as little as 10 U of IFN-γ per ml. J774.1 cells produce very little nitrite with large doses of IFN-γ alone (52). Nakamura et al., by using 10,000 U of IFN-γ per ml, may have reached a plateau for nitrite production such that infection with *H. capsulatum* would not induce further nitrite production. Our data suggest that encapsulated *Cryptococcus* species do not induce NOS, whereas certain other fungi, such as *C. albicans* and *H. capsulatum*, are sufficient stimuli for NOS induction in IFN-γ primed macrophages.

We investigated various possibilities for the lack of NOS induction by *C. neoformans*. Under our standard experimental conditions, J774.1 cells did not phagocytize strain H99. The addition of specific antibodies against H99 did facilitate phagocytosis but did not result in NOS induction. A transcriptional inhibitor of NOS produced in experimental cell cultures was searched for, but no evidence of a transcriptional inhibitor of NOS was found. In addition, we did not detect citrulline in supernatants from primed J774.1 cells challenged with H99, suggesting that NO was not being consumed in cell culture. These findings indicated that NO was not produced in J774.1-*C. neoformans* cocultures, because the necessary signals for NOS induction were not present. This was supported by the observation that TNF-α was not detected in J774.1-*C. neoformans* cell culture supernatants.

TNF-α induction is typically initiated by the organism itself (15) but may also be triggered by mitogens (15), LPS (4), and phorbol esters (49). In addition, anaphylatoxin C5a increases LPS-stimulated TNF-α release from human macrophages (10). The mechanisms of transcriptional and translational control of TNF-α synthesis have not been fully explained. LPS induction of TNF-α is dependent on a protein kinase C pathway (31) and is inhibited by prostaglandin E_2 and cyclic AMP (44). However, transcription of TNF-α varies with the concentration and timing of exposure to LPS. Low-dose exposure to LPS induces tolerance; later LPS exposure causes less TNF-α to be released (47). Furthermore, other factors may modify the release of TNF-α following transcription. Haslberger et al. (26) reported that high extracellular potassium concentrations antagonized release of LPS-stimulated TNF-α, although TNF-α mRNA production was not inhibited. Calcium may also affect TNF-α release (30, 49). It is clear that TNF-α production is complex and regulated at several intermediate steps.

In our experiments, strain H99 failed to induce TNF-α release from stimulated macrophages. This failure, whether through lack of binding a receptor, failure to complete the signal transduction cascade, or, perhaps, posttranscriptional regulation, requires further study. We speculated that the polysaccharide capsule prevents the transmission of signals necessary for NOS induction. In support of this hypothesis, we found that acapsular mutants derived from parent strains with a prominent capsule do induce NOS, whereas hypocapsular mutants do not. This appears not to be due to differences in phagocytosis of the yeast strains. The hypocapsular as well as

the acapsular strains appear to be efficiently phagocytized, as judged by examination by phase-contrast microscopy.

The importance of NO in *C. neoformans* fungistasis has been established by previous experiments in our laboratory. Nitrite production was found to be correlated with fungistasis of *C. neoformans* in vitro (19). In these and other experiments, murine peritoneal macrophages were primed with IFN-γ and challenged with LPS, a potent inducer of NOS (54). These activated macrophages inhibited growth of *C. neoformans* and produced significant quantities of nitrite and nitrate. When fungistasis experiments were carried out in LPS-free conditions, fungistasis did not occur (22). This is consistent with the results reported here.

The polysaccharide capsule of *C. neoformans* is a highly regulated structure and is necessary for virulence (6, 20). Acapsular mutants are greatly attenuated in virulence (32). The capsule has several known immunologic effects. It potently activates the alternate complement pathway (34) and induces proliferation of T-suppressor cells (41). In addition, the polysaccharide capsule inhibits phagocytosis by macrophages (7). Kozel and Gotschlich (33) propose that the mechanism of inhibition is passive; the capsule prevents *C. neoformans* from being recognized by the macrophage. Similarly, we propose that the capsule prevents TNF-α induction in the macrophage (whether by passive or active means), thereby blocking the cascade of signals necessary for NOS induction.

C. neoformans is, therefore, one organism which fails to induce NOS in IFN-γ-primed murine macrophages, but it does not appear to be unique in this regard. Roach et al. (45) found that *Leishmania donovani*, an intracellular parasite, did not induce NOS in IFN-γ-primed resident peritoneal murine macrophages unless LPS was added to the cell culture. Accordingly, IFN-γ alone failed to induce leishmanicidal activity; LPS was an essential cofactor. Their results point to a species difference in the genus *Leishmania*. *L. major*, in contrast to *L. donovani*, has been found to induce NOS in IFN-γ-primed murine macrophages (23, 24, 36). The finding that *L. donovani* fails to induce IFN-γ-primed murine macrophages complements our observations regarding *C. neoformans* and may demonstrate a strategy employed by these organisms to evade detection and cytotoxicity by macrophages.

Because the inducible isoform of NOS (iNOS) is not readily detected in human macrophages treated with IFN-γ and a variety of TNF-α-inducing second signals, the relevance of these findings to human host defense mechanisms requires scrutiny. Two observations seem pertinent. First, until a reproducible human macrophage antifungal assay which exhibits the known characteristics of T cell-dependent macrophage activation is in place, the role of iNOS as a fungistat will continue to be speculative. At present no such assay exists. Indeed, our studies employing human pulmonary, peritoneal, and peripheral blood macrophages/monocytes revealed that human IFN-γ inhibited constitutive fungistatic activity (8, 9). Secondly, it has long been observed that mice possess an exquisite innate susceptibility to clinical isolates of *C. neoformans*. The findings reported here point to a striking defect in macrophage reactivity and hence may help to explain one aspect of a species-specific susceptibility to a common animal pathogen.

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REFERENCES

1. **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.
2. **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.
3. **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.
4. **Beutler, B., J. Mahoney, N. LeTrang, P. Pekala, and A. Cerami.** 1985. Purification of cachectin, a lipoprotein lipase-suppressing hormone secreted by endotoxin-induced RAW 264.7 cells. J. Exp. Med. **161:**984–995.
5. **Boockvar, K., D. L. Granger, R. M. Poston, M. Maybodi, M. K. Washington, J. B. Hibbs, and R. L. Kurlander.** 1994. Nitric oxide produced during murine listeriosis is protective. Infect. Immun. **62:**1089–1100.
6. **Bulmer, G. S., M. D. Sans, and C. M. Gunn.** 1967. *Cryptococcus neoformans*. I. Nonencapsulated mutants. J. Bacteriol. **94:**1475–1479.
7. **Bulmer, G. S., and J. R. Tacker.** 1975. Phagocytosis of *Cryptococcus neoformans* by alveolar macrophages. Infect. Immun. **11:**73–79.
8. **Cameron, M. L., and D. L. Granger.** 1994. Unpublished data.
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. **Cavaillon, J.-M., C. Fitting, and N. Haeffner-Cavaillon.** 1990. Recombinant C5a enhances interleukin 1 and tumor necrosis factor release by lipopolysaccharide-stimulated monocytes and macrophages. Eur. J. Immunol. **20:**253–257.
11. **Cenci, E., L. Romani, A. Mencacci, R. Spaccapelo, E. Schiaffella, P. Puccetti, and F. Bistoni.** 1993. Interleukin-4 and interleukin-10 inhibit nitric oxide-dependent macrophage killing of Candida albicans. Eur. J. Immunol. **23:**1034–1038.
12. **Ding, A., C. F. Nathan, J. Graycar, R. Derynck, D. J. Stuehr, and S. Srimal.** 1990. Macrophage deactivation factor and transforming growth factors-beta-1, -2, and -3 inhibit induction of macrophage nitrogen oxide synthesis by interferon-gamma. J. Immunol. **145:**940–944.
13. **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.
14. **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.
15. **Ferrante, A., R. E. M. Staugas, B. Rowan-Kelly, S. Bresatz, L. M. Kumaratilake, C. M. Rzepczyk, and G. R. Adolf.** 1990. Production of tumor necrosis factors alpha and beta by human mononuclear leukocytes stimulated with mitogens, bacteria, and malarial parasites. Infect. Immun. **58:**3996–4003.
16. **Fortier, A. H., T. Polsinelli, S. J. Green, and C. A. Nacy.** 1992. Activation of macrophages for destruction of *Francisella tularensis*: identification of cytokines, effector cells, and effector molecules. Infect. Immun. **60:**817–825.
17. **Granger, D. L., J. B. Hibbs, J. R. Perfect, and D. T. Durack.** 1988. Specific amino acid (L-arginine) requirement for the microbiostatic activity of murine macrophages. J. Clin. Invest. **81:**1129–1136.
18. **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 NG-monomethyl-L-arginine. J. Immunol. **146:**1294–1302.
19. **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.
20. **Granger, D. L., J. R. Perfect, and D. T. Durack.** 1985. Virulence of *Cryptococcus neoformans*: regulation of capsule synthesis by carbon dioxide. J. Clin. Invest. **76:**508–516.
21. **Granger, D. L., J. R. Perfect, and D. T. Durack.** 1986. Macrophage-mediated fungistasis: requirement for a macromolecular component in serum. J. Immunol. **137:**693–701.
22. **Granger, D. L., J. R. Perfect, and D. T. Durack.** 1986. Macrophage-mediated fungistasis in vitro: requirements for intracellular and extracellular cytotoxicity. J. Immunol. **136:**672–680.
23. **Green, S. J., R. M. Crawford, J. T. Hockmeyer, M. S. Meltzer, and C. A. Nacy.** 1990. Leishmania major amastigotes initiate the L-arginine-dependent killing mechanism of IFN-gamma stimulated macrophages by induction of

tumor necrosis factor-alpha. J. Immunol. **145:**4290–4297.
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. **Guthohrlein, G., and J. Knappe.** 1968. Modified determination of citrulline. Anal. Biochem. **26:**188–191.
26. **Haslberger, A., C. Romanin, and R. Koerber.** 1992. Membrane potential modulates release of tumor necrosis factor in lipopolysaccharide-stimulated mouse macrophages. Mol. Biol. Cell **3:**451–460.
27. **Hobbs, M. M., J. R. Perfect, D. L. Granger, and D. T. Durack.** 1990. Opsonic activity of cerebrospinal fluid in experimental cryptococcal meningitis. Infect. Immun. **58:**2115–2119.
28. **Jacobson, E. S., D. J. Ayers, A. C. Harrell, and C. C. Nicholas.** 1982. Genetic and phenotypic characterization of capsule mutants of *Cryptococcus neoformans*. J. Bacteriol. **150:**1292–1296.
29. **James, S. L., and J. Glaven.** 1989. Macrophage cytotoxicity against schistosomula of *Schistosoma mansoni* involved arginine-dependent production of reactive nitrogen intermediates. J. Immunol. **143:**4208–4212.
30. **Kovacs, E. J.** 1991. Control of IL-1 and TNF-alpha production at the level of second messenger pathways, p. 89–107. *In* E. S. Kimball (ed.), Cytokines and inflammation. CRC Press, Boca Raton, Fla.
31. **Kovacs, E. J., D. Radzioch, H. A. Young, and L. Varesio.** 1988. Differential inhibition of IL-1 and TNF-alpha mRNA expression by agents which block second messenger pathways in murine macrophages. J. Immunol. **141:**3101–3105.
32. **Kozel, T. R., and J. Cazin.** 1971. Nonencapsulated variant of *Cryptococcus neoformans*. I. Virulence studies and characterization of soluble polysaccharides. Infect. Immun. **3:**287–294.
33. **Kozel, T. R., and E. C. Gotschlich.** 1982. The capsule of *Cryptococcus neoformans* passively inhibits phagocytosis of the yeast by macrophages. J. Immunol. **129:**1675–1680.
34. **Kozel, T. R., M. A. Wilson, and J. W. Murphy.** 1991. Early events in initiation of alternative complement pathway activation by the capsule of *Cryptococcus neoformans*. Infect. Immun. **59:**3101–3110.
35. **Levitz, S. M., and D. J. DiBenedetto.** 1988. Differential stimulation of murine resident peritoneal cells by selectively opsonized encapsulated and acapsular *Cryptococcus neoformans*. Infect. Immun. **56:**2544–2551.
36. **Liew, F. Y., Y. Li, and S. Millott.** 1990. Tumor necrosis factor-alpha synergizes with IFN-gamma in mediating killing of *Leishmania major* through the induction of nitric oxide. J. Immunol. **145:**4306–4310.
37. **Lorsbach, R. B., and S. W. Russell.** 1992. A specific sequence of stimulation is required to induce synthesis of the antimicrobial molecule nitric oxide by mouse macrophages. Infect. Immun. **60:**2133–2135.
38. **Marletta, M. A., P. Yoon, R. Iyengar, C. D. Leaf, and J. S. Wishnok.** 1988. Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. Biochemistry **27:**8706–8711.
39. **McKinney, M. M., and A. Parkinson.** 1987. A simple, non-chromatographic procedure to purify immunoglobulins from serum and ascites fluid. J. Immunol. Methods **96:**271–278.
40. **Meltzer, M. S.** 1981. Macrophage activation for tumor cytotoxicity: characterization of priming and trigger signals during lymphokine activation. J. Immunol. **127:**179–183.
41. **Murphy, J. W., and J. W. Moorhead.** 1982. Regulation of cell-mediated immunity in cryptococcosis. I. Induction of specific afferent T suppressor cells by cryptococcal antigen. J. Immunol. **128:**276–283.
42. **Nakamura, L. T., B. A. Wu-Hsieh, and D. H. Howard.** 1994. Recombinant murine gamma interferon stimulates macrophages of the RAW cell line to inhibit intracellular growth of *Histoplasma capsulatum*. Infect. Immun. **62:**680–684.
43. **Oswald, I. P., R. T. Gazzinelli, A. Sher, and S. L. James.** 1992. IL-10 synergizes with IL-4 and transforming growth factor-beta to inhibit macrophage cytotoxic activity. J. Immunol. **148:**3578–3582.
44. **Renz, A., J.-H. Gong, A. Schmidt, M. Nain, and D. Gemsa.** 1988. Release of tumor necrosis factor alpha from macrophages: enhancement and suppression are dose-dependently regulated by prostaglandin E2 and cyclic nucleotides. J. Immunol. **141:**2388–2393.
45. **Roach, T. I. A., A. F. Kiderlen, and J. M. Blackwell.** 1991. Role of inorganic nitrogen oxides and tumor necrosis factor alpha in killing *Leishmania donovani* amastigotes in gamma interferon-lipopolysaccharide-activated macrophages from *Lsh*^{s} and *Lsh*^{r} congenic mouse strains. Infect. Immun. **59:**3935–3944.
46. **Ruco, L. P., and M. S. Meltzer.** 1978. Macrophage activation for tumor cytotoxicity: development of macrophage cytotoxic activity requires completion of a sequence of short-lived intermediary reactions. J. Immunol. **121:**2035–2042.
47. **Sanchez-Cantu, L., H. N. Rode, and N. V. Christou.** 1989. Endotoxin tolerance is associated with reduced secretion of tumor necrosis factor. Arch. Surg. **124:**1432–1436.
48. **Silva, J. S., D. R. Twardzik, and S. G. Reed.** 1991. Regulation of Trypanosoma cruzi infections in vitro and in vivo by transforming growth factor beta (TGF-beta). J. Exp. Med. **174:**539–545.
49. **Sommers, S. D., J. E. Weiel, T. A. Hamilton, and D. O. Adams.** 1986. Biochemical mechanisms of macrophage activation: phorbol esters and calcium ionophore act synergistically to prime macrophages for tumor cell destruction. J. Immunol. **136:**4199–4205.
50. **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.
51. **Stuehr, D. J., and M. A. Marletta.** 1987. Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-gamma. J. Immunol. **139:**518–525.
52. **Stuehr, D. J., and M. A. Marletta.** 1987. Synthesis of nitrite and nitrate in murine macrophage cell lines. Cancer Res. **47:**5590–5594.
53. **Vincendeau, P., and S. Daulouede.** 1991. Macrophage cytostatic effect on *Trypanosoma musculi* involves an L-arginine-dependent mechanism. J. Immunol. **146:**4338–4343.
54. **Wagner, D. A., V. R. Young, and S. R. Tannenbaum.** 1983. Mammalian nitrate biosynthesis: incorporation of 15NH3 into nitrate is enhanced by endotoxin treatment. Proc. Natl. Acad. Sci. USA **80:**4518–4521.