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Recombinant Murine Gamma Interferon Stimulates Macrophages of the RAW Cell Line To Inhibit Intracellular Growth of *Histoplasma capsulatum*

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Macrophages of the RAW 264.7 cell line, activated by pretreatment with recombinant murine gamma interferon, inhibit the intracellular growth of *Histoplasma capsulatum*. Growth inhibition occurred by a mechanism that was operative only when L-Arg metabolism was allowed to occur. When activated macrophages were cultured in the absence of L-Arg or in the presence of N^G-monomethyl-L-Arg, a competitive inhibitor of L-Arg metabolism, activation to the antihistoplasma growth-inhibitory state did not occur. An increase in levels of NO_2^-, an end product of L-Arg metabolism, was detected only after activation of RAW 264.7 cells to the growth-inhibitory state. In contrast, only baseline levels of NO_2^- were detected when L-Arg was excluded or when N^G-monomethyl-L-Arg was added to the culture medium. Nitric oxide (NO·), a reactive intermediate product of L-Arg metabolism, was implicated as the relevant antihistoplasma effector molecule. When *H. capsulatum* yeast cells were cultured for 24 to 28 h in a system designed to generate soluble NO·, a dose-dependent cytotoxic effect was observed.

The zoopathogenic fungus *Histoplasma capsulatum* is a facultative intracellular parasite of mononuclear phagocytes (27). Recombinant murine gamma interferon (rMuIFN-γ), as well as lymphokines prepared from immune splenocytes of concanavalin A-stimulated T-cell hybridomas, activates normal macrophages to inhibit the intracellular growth of *H. capsulatum* (25–27, 29–31). To study the molecular basis of such intracellular inhibition of fungal growth, murine macrophage cell lines could be used to generate larger volumes of cells.

The macrophage cell line $P388D_1$ has been employed to study phagosome-lysosome fusion, release of superoxide, and modification of acidification of phagolysosomes by macrophages inhabited by *H. capsulatum* (4–6). *H. capsulatum* grows within macrophages of the $P388D_1$ cell line with a generation time comparable to that with which it grows in normal resident peritoneal and red pulp splenic macrophages (13, 28). However, while rMuIFN-γ activates $P388D_1$ cells to express Ia antigens, it does not stimulate those macrophages to inhibit intracellular growth of *H. capsulatum* alone or in combination with lipopolysaccharide (LPS) (28). Peritoneal macrophages from normal mice of the DBA/2 strain (from which the $P388D_1$ cell line was derived) were stimulated by rMuIFN-γ to inhibit the fungus. Macrophages from the IC-21 cell line also could not be activated to fungistasis with rMuIFN-γ (28). Both peritoneal and splenic murine macrophages are activated to histoplasmostasis by interferon, and in that state they form inducible nitric oxide synthase (8, 9, 14, 17, 17a, 21, 22). Moreover, the gene for inducible nitric oxide synthase has been cloned and can be used to prepare valuable probes for message activity in macrophage populations (14, 17a). We set about to find a cell line of macrophages that would duplicate our experience with murine macrophages stimulated with rMuIFN-γ and LPS (14).

In the present report, we present data to show that macrophages from the RAW 264.7 (RAW) cell line can be activated by rMuIFN-γ to inhibit the intracellular growth of *H. capsulatum* and that arginine metabolism is required for this activation. Furthermore, soluble nitric oxide, a reactive intermediate product of arginine metabolism, was cytotoxic to *H. capsulatum* in an in vitro assay.

MATERIALS AND METHODS

Fungus. *H. capsulatum* 505 was used throughout the study. The fungus was maintained in yeast phase by weekly passage on blood-cysteine-glucose agar slants (24, 28).

Reagents. Macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection, Rockville, Md., and was maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical Co., St. Louis, Mo.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Logan, Utah). L-Arg-free DMEM and phenol red-free DMEM were prepared from medium kits (GIBCO, Grand Island, N.Y.). N^G-monomethyl-L-Arg (N^GMMLA) monoacetate was purchased from Calbiochem, San Diego, Calif. rMuIFN-γ was a gift from Genentech, South San Francisco, Calif.

Macrophage assays. The macrophage activation assay in peritoneal macrophages previously used for the study of the intracellular growth and fate of *H. capsulatum* was adapted to the work with RAW cells (10, 26, 29). Briefly, 8×10^4 washed, log-phase cells in 150 μl of DMEM–10% FBS were pipetted onto sterile, Formvar-coated coverslips in a 24-well cluster plate. After incubation for 2 to 4 h at 37°C, the coverslips were gently washed twice by addition and aspiration of 0.5 ml of warm, serum-free medium. Cells were then overlaid with 1 ml of DMEM–3% FBS, with or without rMuIFN-γ, and incubated overnight at 37°C. Monolayers were then washed with DMEM and covered with 8×10^4 viable *H. capsulatum* yeast cells in 200 μl of L-Arg-free DMEM–10% dialyzed FBS, with L-Arg or N^GMMLA added. After 1.5 to 2 h, free yeast cells were washed away by addition and aspiration of phosphate-buffered saline (PBS) three times, and monolayers were overlaid with 1 ml of

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appropriate medium. After 18 to 20 h at 37°C, the cells were washed gently, fixed with methanol, and stained with a periodic acid Schiff staining kit (EM Diagnostic Systems, Inc., Gibbstown, N.J.). Stained monolayers were examined microscopically, and activation of cells was determined as described elsewhere (26, 28–30).

Nitrite (NO_2^-) assay. RAW cells (1.5×10^6 in 0.5 ml of DMEM–10% FBS) were added to triplicate wells of a 24-well cluster plate and stimulated by incubation overnight with 10^4 U of rMuIFN-γ per ml. Monolayers were then infected with 1.5×10^6 *H. capsulatum* yeast cells as described above. After washing three times with PBS, cells were overlaid with phenol red-free, L-Arg-free DMEM–3% dialyzed FBS with rMuIFN-γ and L-Arg or N^GMMLA. After incubation for 18 to 20 h at 37°C, the nitrite concentration of filtered supernatants was determined by modifications to a colorimetric assay described previously (2, 7). 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), and the tubes were 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, Fair Lawn, N.J.]) was added to each tube. Samples were then vortexed and incubated in the dark, for 10 min, at room temperature. The nitrite concentrations of the samples were determined by comparing the optical density at 550 nm (OD_{550}) to a standard curve (1 to 200 μM) generated from freshly prepared solutions of $NaNO_2$ (Fluka Chemika-Bio-Chemika, Buchs, Switzerland).

Yeast cell viability assay. Sodium nitrite was twofold serially diluted in a microtiter plate in 100 μl of DMEM adjusted to pH 7.6 with $NaHCO_3$ or to pH 5.0 with sodium citrate (both from Sigma). Three million viable, log-phase *H. capsulatum* yeast cells in 100 μl of DMEM at the appropriate pH were then added to each well, and the plates were incubated at 37°C for 2 to 4 h or 24 to 28 h. After incubation, the plates were centrifuged (15 min at $900 \times g$), and 175 μl of medium was carefully aspirated from each well, without disturbing the yeast cells. Fresh DMEM, pH 7.6 (175 μl), was added, and the process was repeated for a total of three washes, after which the pH of all samples was 7.6. Following the final wash, cells were resuspended in medium containing 0.5 mg of tetrazolium salt MTT (Sigma) per ml, and the suspension was incubated for 4 h at 37°C. Yeast cells were lysed, and reduced dye was released by removal of 100 μl of medium and addition of 150 μl of isopropanol–0.5% HCl (Sigma). Cell wall ghosts were pelleted by centrifugation, and 200 μl of supernatant was transferred to a new microtiter plate. The OD_{540}s of all samples were determined in a Titertek (Multiskan MCC/340) plate reader (Flow Laboratories, Inc., McLean, Va.), and percent viabilities of triplicate cultures were calculated by the following equation: % Viability = $100 \times (OD_{540}$ with NO_2^-/ OD_{540} without $NO_2^-)$.

The tetrazolium salt MTT colorimetric assay of viability has been shown to be applicable to fungi (15). The CFU of viable *H. capsulatum* on GPA (25) corresponded to the percent viability indicated by the dye reduction test (data not shown).

TABLE 1. Activation of RAW cells by rMuIFN-γ

Row	rMuIFN-γ (U/ml)	No. of yeast cells/infected cell[a]	Growth[b]	% Reduction of growth[c]
1	0	9.4 ± 3.4	7.1	
2	10^3	3.0 ± 0.3	0.7	90
3	10^4	2.5 ± 0.3	0.2	97
4	10^5	2.4 ± 0.3	0.1	99

[a] The results are means ± standard errors of the means for three separate experiments. The mean ± standard error of the mean at zero time was 2.3 ± 0.6.
[b] Growth is given as the mean number of yeast cells per RAW cell at time 17 minus the mean number of yeast cells per RAW cell at time zero (2.3 ± 0.6).
[c] Data are calculated by the following formula: [1 − (growth within RAW cells in rMuIFN-γ containing media/growth within RAW cells in control medium)] × 100.

RESULTS

Activation of macrophage cell line RAW by rMuIFN-γ. RAW is a monocyte macrophage cell line established from a virus-induced ascites tumor (20). L-Arg metabolism in such macrophages is up-regulated by stimulation with IFN-γ and LPS (22). The data shown in Table 1 indicate that *H. capsulatum* grows well in RAW cells (Table 1, row 1). But unlike $P388D_1$ macrophages (28), RAW cells inhibited the growth of *H. capsulatum* following pretreatment with 10^3, 10^4, or 10^5 U of rMuIFN-γ per ml and incubation in complete medium (Table 1, rows 2 to 4). Hence, RAW cells mimic the growth-inhibitory activity observed in peritoneal macrophages (25–27, 29–31) and appear to be a suitable model for in vitro studies.

RAW cell requirement of L-Arg for resistance to intracellular growth of *H. capsulatum*. Having established that RAW cells can be activated to inhibit the intracellular growth of *H. capsulatum*, we were interested in determining whether the growth-inhibitory state required the presence of L-Arg in culture media. To address this question, we examined the growth of *H. capsulatum* in activated RAW cells after culture in medium without L-Arg or with 50 μM added N^GMMLA. Table 2 shows the composite results of four separate experiments performed under identical conditions. When RAW cells were activated by pretreatment with 10^4 U of rMuIFN-γ per ml and incubated in medium with 0.6 mM L-Arg, there was a significant decrease in yeast cell growth (Table 2, row 2) compared with that in nonactivated controls (Table 2, row 1).

TABLE 2. RAW cell resistance to *H. capsulatum* requires L-Arg

Row	rMuIFN-γ (10^4 U)	L-Arg (0.6 mM)	N^GMMLA (50 μM)	No. of yeast cells/infected macrophage[a]	Growth[b]	% Reduction of growth[c]
1	−	+	−	5.8 ± 0.8	4.0	
2	+	+	−	2.7 ± 0.7	0.9	78
3	+	−	−	7.9 ± 0.5	6.1	0
4	+	−	+	7.7 ± 1.9	5.9	0

[a] The results are means ± standard errors of the means. The mean ± standard error of the mean at zero time was 1.8 ± 0.2.
[b] Growth was calculated as explained in footnote *b* to Table 1.
[c] Results were calculated as explained in footnote *c* to Table 1.

TABLE 3. Correlation of RAW cell resistance with *H. capsulatum* and NO_2^- production

Row	rMuIFN-γ (10^4 U)	0.6 mM (L-Arg)	N^GMMLA (50 μM)	No. yeast cells/infected macrophage[a]	Growth[b]	% Reduction of growth[c]	NO_2^- (μM)[d]
1	−	+	−	5.99	4.43	0	1.7 ± 2.4
2	+	+	−	1.83	0.27	94	28.0 ± 0.4
3	+	−	−	7.79	6.23	0	5.2 ± 1.5
4	+	−	+	7.46	5.90	0	7.9 ± 1.3
5	+	+	−				26.3 ± 4.3[e]

[a] Results are the means ± standard errors of the means for a representative experiment. The mean ± standard error of the mean at zero time was 1.56. The incubation period was 20 h.
[b] Growth was calculated as explained in footnote *b* to Table 1.
[c] Results were calculated as explained in footnote *c* to Table 1.
[d] Means ± standard errors of the means for triplicate determinations in a single representative experiment, which was repeated three times.
[e] These values are from uninfected RAW cells.

However, when RAW cells were exposed to rMuIFN-γ and cultured in the absence of L-Arg (Table 2, row 3) or in medium with 50 μM N^GMMLA (Table 2, row 4), the macrophages were not activated to inhibit *H. capsulatum*. Consequently, it is apparent that RAW cells require L-Arg metabolism to inhibit the intracellular growth of *H. capsulatum*.

Correlation of resistance to intracellular growth of *H. capsulatum* and NO_2^- production by rMuIFN-γ-activated RAW cells. Nitric oxide (NO·) has received considerable recent attention as one of the effector molecules of antitumor cell and antimicrobial activity (8, 9, 11, 16, 17, 23). Metabolism of L-Arg by activated macrophages generates NO· and other toxic nitrogen-containing intermediate products which may induce loss of cellular iron and inactivate a number of enzymes which are important to DNA replication and mitochondrial function (3, 8, 21). The reactive intermediates are short-lived; however, up-regulation of L-Arg metabolism can be detected by increases in the concentration of the metabolic end products NO_2^- and NO_3^- in culture supernatants (18, 21, 23).

Since the growth of *H. capsulatum* in activated RAW cells was not inhibited when L-Arg was excluded from or N^GMMLA was added to culture media (Table 2), our data suggest that the mechanism of resistance to yeast cell growth may involve reactive intermediate products of L-Arg metabolism. Table 3 gives the results of a representative experiment which compared the intracellular growth of *H. capsulatum* and the production of NO_2^- by RAW cells cultured under the same conditions. rMuIFN-γ-activated RAW cells generated 28 μM NO_2^- and inhibited yeast cell growth by 94% (Table 3, row 2) compared with nonactivated RAW cells (Table 3, row 1), which generated only background levels of NO_2^- (Table 3, row 1). Activated RAW cells which were cultured in the absence of L-Arg (Table 3, row 3) or with 50 μM N^GMMLA (Table 3, row 4) did not inhibit the growth of *H. capsulatum* and generated only low levels of NO_2^- (5.2 and 7.9 μM, respectively). Therefore, we conclude that growth inhibition does correlate with NO_2^- production in culture supernatants, suggesting that L-Arg metabolism is required for RAW cell resistance to intracellular *H. capsulatum* growth. Moreover, up-regulation of L-Arg metabolism is a consequence of rMuIFN-γ activation rather than infection per se, since activated, noninfected RAW cells generated levels of NO_2^- (Table 3, row 5) that were comparable to those of infected cells cultured under similar conditions (Table 3, row 2).

In vitro effect of NO· on *H. capsulatum*. The data suggest that rMuIFN-γ-activated macrophages inhibit the intracellular growth of *H. capsulatum* by a mechanism which requires L-Arg metabolism. Therefore, we were interested in determining the direct effect of NO· on *H. capsulatum*. To examine this question, we cultured suspensions of *H. capsulatum* yeast cells under conditions which were designed to generate soluble NO·. In acidic medium (pH 5.0), NO_2^- is reduced to NO·, while NO_2^- at pH 7.6 is not significantly affected (1, 19). When we cultured *H. capsulatum* for 24 or 28 h in medium at pH 5.0 (without added NO_2^-), a decrease in viability relative to that of yeast cells cultured at pH 7.6 was observed in two of three experiments (15 and 23%, respectively). However, when increasing concentrations of NO_2^- were added to *H. capsulatum* cultured at pH 5.0, there was a marked decline in viability after 24 or 28 h (Fig. 1). As determined by a dye reduction assay, viability was approximately 50% in the range of 100 μM added NO_2^- and was near 0 at slightly more than 1 mM added NO_2^-. In contrast, the viability of *H. capsulatum* cultured at pH 7.6 remained more than 90%, even when the NO_2^- concentration was increased to 5 mM. Thus, it is clear that soluble NO· is directly cytotoxic to *H. capsulatum* in suspension culture.

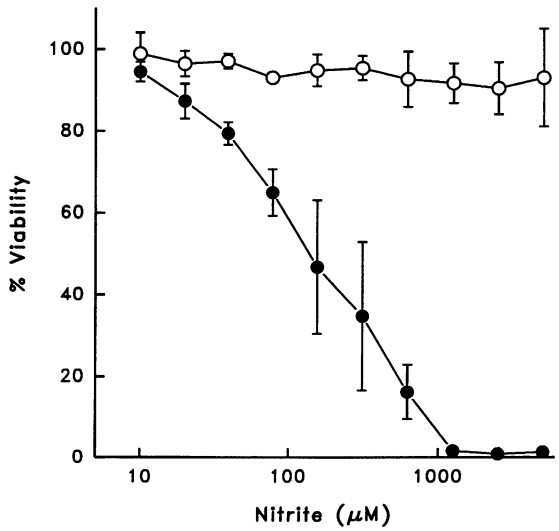

FIG. 1. Killing of *H. capsulatum* in a NO·-generating medium. Yeast cells (3×10^6) were incubated in DMEM at pH 7.6 (open circles) or pH 5.0 (filled circles), with increasing amounts of $NaNO_2$ (NO_2^-). After 24 or 28 h at 37°C, viability was determined by a dye reduction (MTT) assay. Values are the means of triplicate determinations from three separate experiments, and bars represent standard errors of the means.

DISCUSSION

In most human infections with *H. capsulatum* and in sublethal experimental infections, recovery occurs as intracellular growth is suppressed and the tissues are cleared of the fungus (10, 27). Previous work in our laboratory demonstrated that murine peritoneal macrophages are activated to an antihistoplasma state by infection or by in vitro pretreatment with lymphokine-containing supernatants (25–27, 29–31). The supernatants were not directly cytotoxic or growth inhibitory to *H. capsulatum*, but pretreatment rendered macrophages resistant to the intracellular replication of yeast cells. Subsequently, rMuIFN-γ was identified as the key element within the lymphokine-containing supernatants which was responsible for activating macrophages to the growth-inhibitory state. Polyclonal rabbit antibody to IFN-γ neutralized the stimulatory activity of lymphokine-containing supernatants, and rMuIFN-γ alone could replace that activity (27, 30, 31).

The events which occur subsequent to IFN-γ activation are still not well defined (27). In the present study, we have focused on the role of L-Arg metabolism and its reactive, nitrogen-containing intermediate products in murine macrophage resistance to *H. capsulatum*. Here, we present evidence that L-Arg metabolism is required for the in vitro growth-inhibitory state of activated macrophages. When L-Arg was replaced in culture media by N^GMMLA, a nonmetabolized L-Arg analog, the rMuIFN-γ-stimulated growth-inhibitory effect was almost completely abolished. The dependence of antimicrobial action by interferon-stimulated macrophages on arginine metabolism has been noted by others (1, 21). However, in contrast to results with *Leishmania major*, the growth of *H. capsulatum* was inhibited but the organism itself was not killed by the macrophages treated with rMuIFN-γ in the presence of L-Arg (17). This result corresponds to the inhibition rather than killing of *Cryptococcus neoformans* by nitrogen oxides (1).

In an earlier study, we had wished to identify a macrophage cell line that would serve as an appropriate model for the peritoneal macrophage response to *H. capsulatum* infection. Macrophage cell lines $P388D_1$ and IC-21 were easily infected and supported the intracellular growth of *H. capsulatum*. However, pretreatment with rMuIFN-γ did not alter the course of in vitro infection in either of these lines (28). In the present study, we show that the macrophage cell line RAW mimics the response of peritoneal macrophages to *H. capsulatum*. RAW cells can also be infected in vitro and support the intracellular growth of yeast cells. However, unlike $P388D_1$ or IC-21, pretreatment of RAW cells with rMuIFN-γ activates them to the growth-inhibitory state. Thus, it appears that RAW cells are a good model for the study of the resistance of murine peritoneal macrophages to intracellular growth of *H. capsulatum*. Indeed, when rMuIFN-γ-activated RAW cells were infected with *H. capsulatum* and cultured in medium in the absence of L-Arg or with 50 μM added N^GMMLA, the growth-inhibitory state was completely abrogated. These results are also consistent with our studies of the L-Arg requirement for peritoneal macrophages (19a).

Since our experiments demonstrated a requirement for the presence of L-Arg in culture media, we next examined L-Arg metabolism as indicated by the generation of NO_2^-, a metabolic end product. Our results showed that increased levels of NO_2^- were generated only when macrophages were stimulated with rMuIFN-γ and cultured in media containing L-Arg. When rMuIFN-γ-activated macrophages were cultured in the absence of L-Arg or with 50 μM added N^GMMLA, only low levels of NO_2^- were detected (5.2 ± 1.5 and 7.9 ± 1.3 μM, respectively). These levels of NO_2^- were above the background level (1.5 ± 2.1 μM), possibly because of residual levels of intracellular L-Arg not removed by preculture washes. However, NO_2^- production was clearly suppressed compared with that of macrophages activated with rMuIFN-γ and cultured in medium with L-Arg (28.0 ± 0.4 μM). Thus, our results show that the rMuIFN-γ-stimulated *H. capsulatum*-resistant state is correlated with L-Arg metabolism, a result noted in a large number of systems (1, 3, 8, 9, 16–19, 21–23). When macrophages were activated to inhibit intracellular fungal growth, L-Arg metabolism was up-regulated, whereas when L-Arg metabolism was inhibited, growth inhibition was not observed. In addition, we determined that maintenance of the growth-inhibitory state required continuous L-Arg metabolism. When complete medium (containing 0.6 mM L-Arg) was replaced after 18 to 20 h in culture with medium containing 50 μM N^GMMLA, growth of yeast cells in macrophages accelerated in the subsequent 24 h. When identical cultures were replenished with medium containing L-Arg, yeast cell growth was still inhibited (data not shown).

Nitric oxide (NO·), a reactive intermediate product of L-Arg metabolism, has been identified as an important macrophage effector molecule in a number of antitumor and antimicrobial systems (8, 9, 11, 16, 17, 23). To assess directly the effect of NO· on *H. capsulatum*, we cultured yeast cells in suspension under conditions designed to generate NO· in solution. In acidic medium (pH 5.0), added NO_2^- is reduced to NO· (1, 19). Under these conditions, *H. capsulatum* viability declined very rapidly, in a dose-dependent manner. Fifty percent viability was observed with approximately 100 μM added NO_2^-, and few live yeast cells were seen at 1 mM added NO_2^-. In medium adjusted to pH 7.6, *H. capsulatum* viability was fairly stable when NO_2^- was added, even at concentrations as high as 5 mM. These findings reveal that *H. capsulatum* is killed in a NO·-generating medium. The effect is concentration dependent. A previous study in our laboratory suggested that rMuIFN-γ-activated macrophages express only growth-inhibitory antihistoplasma activity. Germ tube formation was noted in 86% of the yeast cells recovered from macrophages after 44.5 h in culture (30). The results described in the present report indicate that *H. capsulatum* is killed in a NO·-generating medium. These findings, i.e., of fungistasis in macrophages and fungicidal effects of a NO·-generating medium, are not necessarily in conflict, for the following reasons. (i) The cytotoxic effect was clearly dose dependent. It is unlikely that the levels of NO· attained in the macrophage assay could be approached by those used in the yeast cell viability assay. (ii) The macrophage assay cannot be used to study the fate of *H. capsulatum* beyond 48 h, since untreated controls are overcome by replicating yeast cells (30, 31). However, there is a time-dependent effect, in addition to the dose-dependent effect noted in Fig. 1, since yeast cell viability declined over time, even at low concentrations (data not shown). At intracellular levels of NO·, yeast cell death may increase significantly at some point beyond the useful time limits of the macrophage assay. (iii) In a recent study, others have reported that NO·, generated in a similar system, was cytostatic for *C. neoformans* for as long as 4 h. After that time, yeast cell viability declined, until complete sterilization of cultures was accomplished at 24 h (1). (iv) Clearly, there must be some mechanism for killing intracellular *H. capsulatum*, since our earlier studies showed that the number of viable yeast cells that can be recovered from the spleens of infected animals peaks at 5 to 7 days postinfection and declines thereafter. After 30 days, mouse tissues are essentially free of yeast (25).

NO· or other nitrogen-containing products of L-Arg metab-

olism in activated macrophages were observed to induce loss of cellular iron and inhibit mitochondrial function and DNA replication in tumor target cells (3, 23, 24). It was speculated that such metabolic changes would enhance resistance to intracellular pathogens (1, 8, 17, 19). More recently, it has been proposed that formation of complexes of NO· with iron-sulfur prosthetic groups inhibits target enzymes (for a review, see reference 3). Consistent with these ideas, it was reported that treatment of murine peritoneal macrophages with deferoxamine, an intracellular iron chelator, inhibited the intracellular growth of *H. capsulatum*. Treatment with holotransferrin suppressed the growth-inhibitory effects of deferoxamine and rMuIFN-γ activation (12). The precise role of NO· in the resistance of murine macrophages to *H. capsulatum* infection, however, remains unelucidated. Resolution of this question should be the focus of future studies.

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REFERENCES

1. **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.
2. **Bartholomew, B.** 1984. A rapid method for the assay of nitrate in urine using the nitrate reductase enzyme of *Escherichia coli*. J. Chem. Toxicol. **22:**541–543.
3. **Drapier, J.-C., and J. B. Hibbs, Jr.** 1988. Differentiation of murine macrophages to express nonspecific cytotoxicity for tumor cells results in L-arginine-dependent inhibition of mitochondrial iron-sulfur enzymes in the macrophage effector cells. J. Immunol. **140:**2829–2838.
4. **Eissenberg, L. G., and W. E. Goldman.** 1987. *Histoplasma capsulatum* fails to trigger release of superoxide from macrophages. Infect. Immun. **55:**29–34.
5. **Eissenberg, L. G., W. E. Goldman, and P. H. Schlessinger.** 1993. *Histoplasma capsulatum* modulates the acidification of phagolysosomes. J. Exp. Med. **177:**1605–1611.
6. **Eissenberg, L. G., P. H. Schlessinger, and W. E. Goldman.** 1988. Phagosome-lysosome fusion in $P388D_1$ macrophages infected with *Histoplasma capsulatum*. J. Leukocyte Biol. **43:**483–491.
7. **Elliott, R. J., and A. G. Porter.** 1971. A rapid cadmium reduction method for the determination of nitrate in bacon and curing brines. Analyst **96:**522–527.
8. **Hibbs, J. B., Jr.** 1990. Synthesis of nitric oxide from L-arginine: a recently discovered pathway induced by cytokines with antitumour and antimicrobial activity. Res. Immunol. **142:**565–569.
9. **Hibbs, J. B., Jr., R. R. Taintor, Z. Vavrin, and E. M. Rachlin.** 1988. Nitric oxide: a cytotoxic activated macrophage effector molecule. Biochem. Biophys. Res. Commun. **157:**87–94.
10. **Howard, D. H., V. Otto, and R. K. Gupta.** 1971. Lymphocyte-mediated cellular immunity in histoplasmosis. Infect. Immun. **4:**605–610.
11. **Kilbourn, R. G., and P. Belloni.** 1990. Endothelial cell production of nitrogen oxides in response to interferon-γ in combination with tumor necrosis factor, interleukin-1, or endotoxin. J. Natl. Cancer Inst. **82:**772–776.
12. **Lane, T. A., B. A. Wu-Hsieh, and D. H. Howard.** 1991. Iron limitation and the gamma interferon-mediated antihistoplasma state of murine macrophages. Infect. Immun. **59:**2274–2278.
13. **Lane, T. A., B. A. Wu-Hsieh, and D. H. Howard.** 1993. Gamma interferon cooperates with lipopolysaccharide to activate mouse splenic macrophages to an anti-histoplasma state. Infect. Immun. **61:**1468–1473.
14. **Lane, T. E., B. A. Wu-Hsieh, and D. H. Howard.** Submitted for publication.
15. **Levitz, S. M., and R. D. Diamond.** 1985. A rapid colorimetric assay of fungal viability with the tetrazolium salt MTT. J. Infect. Dis. **152:**938–945.
16. **Liew, F. Y., and F. E. G. Cox.** 1991. Nonspecific defense mechanism: the role of nitric oxide. Immunol. Today **12:**A17–A21.
17. **Liew, F. Y., S. Millott, C. Parkinson, R. M. J. Palmer, and S. Moncada.** 1990. Macrophage killing of *Leishmania major* parasite *in vivo* is mediated by nitric oxide from L-arginine. J. Immunol. **144:**4794–4797.

17a. **Lowenstein, C. J., C. S. Glatt, D. S. Bredt, and S. H. Synder.** 1992. Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme. Proc. Natl. Acad. Sci. USA **89:**6711–6715.

18. **Marletta, M. A., P. S. 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. Biochem. J. **27:**8706–8711.
19. **Mauël, J., A. Ransijn, and Y. Buchmuller-Rouiller.** 1991. Killing of Leishmania parasites in activated murine macrophages is based on an L-arginine-dependent process that produces nitrogen derivatives. J. Leukocyte Biol. **49:**73–80.

19a. **Nakamura, L. T., B. A. Wu-Hsieh, and D. H. Howard.** Unpublished data.

20. **Raschke, W. C., S. Baird, P. Ralph, and I. Nakoinz.** 1978. Functional macrophage cell lines transformed by Abelson leukemia virus. Cell **15:**261–267.
21. **Stuehr, D. J., and M. A. Marletta.** 1987. Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-γ. J. Immunol. **139:**518–525.
22. **Stuehr, D. J., and M. A. Marletta.** 1987. Synthesis of nitrite and nitrate in murine macrophage cell lines. Cancer Res. **47:**5590–5594.
23. **Stuehr, D. J., and C. F. Nathan.** 1989. Nitric oxide: a macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. J. Exp. Med. **169:**1543–1555.
24. **Wharton, M., D. L. Granger, and D. T. Durack.** 1988. Mitochondrial iron loss from leukemia cells injured by macrophages. J. Immunol. **141:**1311–1317.
25. **Wu-Hsieh, B.** 1989. Relative susceptibilities of inbred mouse strains C57BL/6 and A/J to infection with *Histoplasma capsulatum*. Infect. Immun. **57:**3788–3792.
26. **Wu-Hsieh, B., and D. H. Howard.** 1984. Inhibition of growth of *Histoplasma capsulatum* by lymphokine-stimulated macrophages. J. Immunol. **132:**2593–2597.
27. **Wu-Hsieh, B., and D. H. Howard.** 1989. Histoplasmosis, p. 199–225. *In* R. A. Cox (ed.), Immunology of the fungal diseases. CRC Press, Inc., Boca Raton, Fla.
28. **Wu-Hsieh, B., and D. H. Howard.** 1989. Macrophage cell lines $P388D_1$ and IC-21 stimulated with gamma interferon fail to inhibit the intracellular growth of *Histoplasma capsulatum*. Infect. Immun. **57:**2903–2905.
29. **Wu-Hsieh, B., A. Zlotnick, and D. H. Howard.** 1984. T-cell hybridoma-produced lymphokine that activates macrophages to suppress intracellular growth of *Histoplasma capsulatum*. Infect. Immun. **43:**380–385.
30. **Wu-Hsieh, B. A., and D. H. Howard.** 1987. Inhibition of the intracellular growth of *Histoplasma capsulatum* by recombinant murine gamma interferon. Infect. Immun. **55:**1014–1016.
31. **Wu-Hsieh, B. A., and D. H. Howard.** 1990. Gamma interferon and experimental murine histoplasmosis, p. 133–146. *In* E. M. Ayoub, G. H. Cassell, W. C. Branche, Jr., and T. J. Henry (ed.), Microbial determinants of virulence and host response. American Society for Microbiology, Washington, D.C.