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Differential Susceptibility of Yeast and Hyphal Forms of *Candida albicans* to Macrophage-Derived Nitrogen-Containing Compounds

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***Candida albicans* is a dimorphic fungus capable of transition from the yeast form (Y-*Candida*) to the hyphal form (H-*Candida*). Both Y-*Candida* and H-*Candida* are known to be growth inhibited by murine macrophages (Mϕ) in vitro. In the present report, we demonstrate that Mϕ exposed to interferon gamma (IFN-γ) plus lipopolysaccharide (LPS) show enhanced anti-Y-*Candida* and anti-H-*Candida* activities. To further investigate the phenomenon, Y-*Candida* and H-*Candida* were assessed for susceptibilities to Mϕ-derived supernatants. Only the growth of H-*Candida*, and not that of Y-*Candida*, is impaired by cell-free supernatants from Mϕ treated with IFN-γ plus LPS. In contrast, no H-*Candida* growth inhibition occurs when supernatants from Mϕ exposed to IFN-γ plus LPS in the presence of N^G-monomethyl-L-arginine, an inhibitor of nitric oxide (NO) synthesis, are employed. Finally, supernatants from Mϕ incubated with sodium nitroprusside, an NO-generating agent, also show anti-H-*Candida* activity. In conclusion, these results indicate that H-*Candida* but not Y-*Candida* is susceptible to extracellular antifungal mechanisms employed by Mϕ, which likely involve stable nitrogen-containing compounds.**

The opportunistic pathogen *Candida albicans* is a serious agent of infection in immunocompromised hosts (23). The dimorphic property of *C. albicans*, which under certain circumstances undergoes a transition from the yeast form (Y-*Candida*) to the hyphal form (H-*Candida*), is generally considered a virulence factor. In fact, during the early stages of infection, the phagocytic clearance of *C. albicans* enables the host to remove and destroy most of the Y-*Candida*, while microorganisms that escape phagocytosis rapidly convert into H-*Candida* and invade the host, producing granulomas and causing tissue damage (23).

Most of the studies on the anti-*Candida* activity of macrophages (Mϕ) have dealt with Y-*Candida*. However, there is evidence that Mϕ (27), as well as other natural effector cells (3, 11), can also affect H-*Candida*. We have recently demonstrated that Mϕ cell lines of different anatomical origins are proficient effector cells not only against Y-*Candida* but also against H-*Candida* microorganisms (7). While many studies have elucidated the mechanisms through which anti-Y-*Candida* activity is exerted (5, 9), little is known about anti-H-*Candida* activity. Likely because of its large size, H-*Candida* is not ingested by phagocytic cells, which therefore must act through extracellular mechanisms in order to affect fungal viability and growth.

Nitric oxide (NO) has recently been identified as a potent effector molecule released by phagocytes and responsible for many of the adverse metabolic changes that are observed in damaged target cells (19, 21, 22). Murine Mϕ synthesize NO upon appropriate stimulation, i.e., after exposure to interferon gamma (IFN-γ) plus either microbial products, such as lipopolysaccharide (LPS) or muramyl-dipeptide, or cytokines, such as tumor necrosis factor alpha or tumor necrosis factor beta (12, 13). Because NO is a gaseous, unstable molecule, not only its production per se but also the form in which it is packaged, transported, and targeted may determine its toxic potential (28, 29).

In this study we investigated the mechanisms through which Mϕ exert anti-*Candida* activity. We found that the L-arginine-dependent mechanism is crucial for Mϕ-mediated anti-H-*Candida* activity but not anti-Y-*Candida* activity. The potential contribution of stable nitrogen-containing compounds, as tools for buffering the concentration of free NO (28, 29), is discussed.

MATERIALS AND METHODS

Reagents. Recombinant IFN-γ was purchased from Genentech Inc., South San Francisco, Calif. LPS from *Escherichia coli* (serotype O128:B12) and sodium nitroprusside (SNP) were obtained from Sigma Chemical Co. (St. Louis, Mo.). N^G-Monomethyl-L-arginine (NMMA) was purchased from Calbiochem-Behring Corp. (La Jolla, Calif.).

Mϕ. The ANA-1 Mϕ, derived by immortalization of bone marrow cells from C57BL/6 mice with a recombinant retrovirus carrying the v-*raf* and v-*myc* oncogenes (4), were employed. ANA-1 Mϕ were cultured in RPMI 1640 medium supplemented with glutamine (4 mM), gentamicin (50 μg/ml), and 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, Utah) (complete medium).

***C. albicans*.** *C. albicans* CA-6, serotype A, which was used throughout this study, was isolated from a clinical specimen. It was grown at 28°C with mild agitation in low-glucose Winge medium as previously described (20). Under these conditions, the organism grew as an essentially pure Y-*Candida* population. To obtain H-*Candida*, pure Y-*Candida* was harvested from Winge medium, washed twice in saline, resuspended in complete medium, dispensed in 96-well flat-bottom microtiter plates as specified below, and incubated at 37°C in 5% CO_2. More than 98% of the microorganisms showed the hyphal form after 3 h of incubation, as detailed elsewhere (27).

Mϕ cultures and anti-*Candida* assay. Mϕ (2×10^5/0.1 ml per well) were plated in 96-well flat-bottom microtiter plates (Costar, Cambridge, Mass.) and incubated for 18 h at 37°C in 5% CO_2 in the presence or absence of IFN-γ (100 U/ml) plus LPS (10 ng/ml). Mϕ cultures or cell-free supernatants were then assessed for anti-*Candida* activity by a colorimetric assay, as previously described in detail (17) but with minor changes. Briefly, Mϕ cultures were infected with

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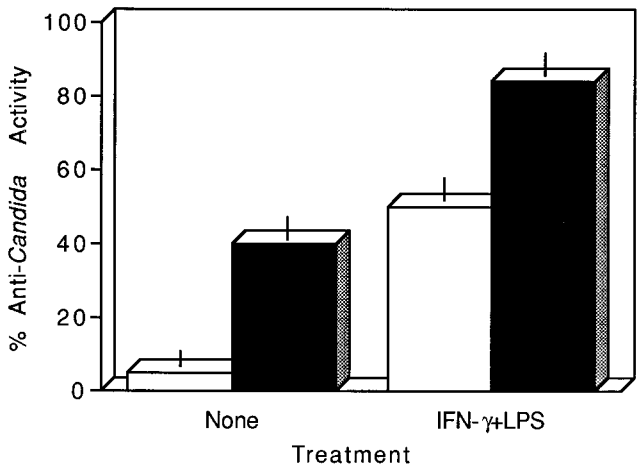

FIG. 1. Effect of treatment with IFN-γ plus LPS on anti-Y-*Candida* and anti-H-*Candida* activities of ANA-1 Mϕ. ANA-1 Mϕ were incubated in the absence or presence of IFN-γ (100 U/ml) plus LPS (10 ng/ml) for 18 h. Mϕ were then exposed to Y-*Candida* (■) and H-*Candida* (□) for 3 h and assayed for anti-*Candida* activity as detailed in Materials and Methods. Data represent the means ± standard errors of the mean of values from five separate experiments. The value from each experiment is the mean of triplicate determinations.

Y-*Candida* or added to H-*Candida*, at an effector-to-target ratio of 1:1, while different dilutions of supernatants (100 μl) were added to Y-*Candida* or H-*Candida* (5×10^3/0.1 ml per well). After 3 h (for Mϕ plus *C. albicans*) or 18 h (for cell-free supernatants plus *C. albicans*) of incubation at 37°C in 5% CO_2, 20 μl of 10% Triton X-100 in distilled water was added to lyse the Mϕ. After three washes with 0.2 ml of distilled water, 0.1 ml of RPMI 1640 without phenol red containing 0.5 mg of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) per ml was added to the wells, and the plates were incubated for an additional 4 h at 37°C. After that time, the plates were centrifuged, supernatants were discarded, and the pellets were resuspended in 0.1 ml of dimethyl sulfoxide. The A_{540} and A_{690} of each well were determined by using an automated microplate reader (Titertek Multiscan; Flow Laboratories, McLean, Va.). A well containing only dimethyl sulfoxide was used as a blank. Control wells, containing Y-*Candida*, H-*Candida*, or Mϕ alone, were included in each experiment. Each sample was assessed in triplicate. The percentage of Y-*Candida* or H-*Candida* growth inhibition was calculated by using the formula % anti-*Candida* activity = 1 − [(optical density of test wells/optical density of control wells)] × 100.

Nitrite determination. The nitrite (NO_2^-) concentration in Mϕ culture supernatants was used as a relative measure of NO synthesis (12) and was assayed in culture supernatants by a standard Griess reaction adapted to microplates (15). The Griess reagent was prepared by mixing equal volumes of sulfanilamide (1.5% in 1 M HCl) and naphthylethylene diamine dihydrochloride (0.15% in H_2O). A volume of 100 μl of reagent was mixed with 100 μl of test supernatants and incubated for 30 min in the dark. The A_{540} of the chromophore formed was measured in an automated plate reader. Nitrite was quantitated by using $NaNO_2$ as a standard. Each sample was tested in triplicate.

RESULTS

Effect of IFN-γ plus LPS on anti-Y-*Candida* and anti-H-*Candida* activities of ANA-1 Mϕ. We have previously demonstrated that ANA-1 Mϕ are proficient effector cells against both Y-*Candida* and H-*Candida*, although to different extents (7). In order to further characterize this phenomenon, ANA-1 Mϕ were treated with IFN-γ (100 U/ml) plus LPS (10 ng/ml) prior to assessment for anti-Y-*Candida* and anti-H-*Candida* activities. We found that such treatment was effective in consistently enhancing both anti-Y-*Candida* and anti-H-*Candida* activities when an effector-to-target ratio of either 1:1 (Fig. 1) or 5:1 (data not shown) was employed. When phagocytosis was evaluated in parallel cultures, we observed Y-*Candida* inside the Mϕ, while H-*Candida* was consistently found in the outer space (data not shown).

Anti-*Candida* activity of supernatants from control or IFN-γ-plus-LPS-treated ANA-1 Mϕ. In order to establish the potential involvement of soluble factors in the above-described phenomenon, 18-h-old cell-free supernatants from control ANA-1 Mϕ or from ANA-1 Mϕ treated with IFN-γ plus LPS were evaluated for anti-*Candida* activity. As depicted in Fig. 2, supernatants from untreated control Mϕ did not exert anti-*Candida* activity against either H-*Candida* or Y-*Candida*. Surprisingly, high levels of anti-H-*Candida* activity were exerted by supernatants from ANA-1 Mϕ treated with IFN-γ plus LPS. Conversely, such supernatants were totally ineffective against Y-*Candida* at all dilutions assessed (Fig. 2).

Effect of NMMA and SNP on anti-H-*Candida* activity of ANA-1 Mϕ supernatants. Since it is known that Mϕ produce NO in response to IFN-γ plus LPS (12), experiments were performed to ascertain the relationship between NO_2^- levels, which were indicative of NO production, and anti-H-*Candida* activity in our experimental model. Table 1 shows the concomitant presence of high levels of NO_2^- and anti-H-*Candida* activity in the supernatants from ANA-1 Mϕ treated with IFN-γ plus LPS. Moreover, the addition of NMMA to IFN-γ and LPS at doses that did not affect cell viability, as determined by trypan blue dye exclusion (data not shown), abolished both NO_2^- production and anti-H-*Candida* activity. Finally, supernatants from ANA-1 Mϕ treated with SNP, an NO-generating agent (8), exhibited levels of NO_2^- and anti-H-*Candida* activity comparable to those observed in supernatants from Mϕ treated with IFN-γ plus LPS (Table 1). In contrast, none of these supernatants were effective in inhibiting Y-*Candida* growth (data not shown). Neither NMMA nor SNP exerted direct toxic effects against *C. albicans* at the doses employed, as assessed by MTT staining (data not shown).

DISCUSSION

While little is known about the mechanisms through which anti-H-*Candida* activity is exerted by Mϕ, extensive studies have focused on anti-Y-*Candida* activity, a phenomenon attributable mainly to an intracellular cascade of events. Among the mechanisms mediating such activity, a key role has been ascribed to cationic proteins because of their direct candidacidal activity (25), while oxygen-mediated mechanisms are less important for Mϕ than for other phagocytic cells (2). The presence of ions in the medium and their availability inside the cells are also crucial for anti-Y-*Candida* activity, as documented by studies on murine microglial Mϕ (5) or human alveolar Mϕ (30). In the present study, we obtained evidence that anti-Y-*Candida* activity, as well as anti-H-*Candida* activity, is susceptible to modulation by Mϕ exposure to IFN-γ plus LPS, with the optimal times and doses (data not shown) being similar to those known to be effective in modulating other Mϕ functions, such as antitumor activity (31). Interestingly, while direct Mϕ-mediated antifungal activity against both *Candida* morphological forms is detectable, Mϕ supernatants act only against H-*Candida*, provided that Mϕ have been exposed to IFN-γ plus LPS. These results, together with the knowledge that Y-*Candida*, but not H-*Candida*, is rapidly ingested by Mϕ (6), strongly support the possibility that an exclusively intracellular killing occurs against the former target, while extracellular mechanisms must be employed against H-*Candida*.

Increasing evidence underlines the role played by NO in Mϕ-mediated cytotoxicity. For example, NO production is considered to be a crucial event in Mϕ killing of *Toxoplasma gondii* (1), *Leishmania major* (16), *Mycobacterium bovis* (14), and *Ehrlichia risticii* (24). In our model, NO production and anti-H-*Candida* activity are concomitantly present in superna-

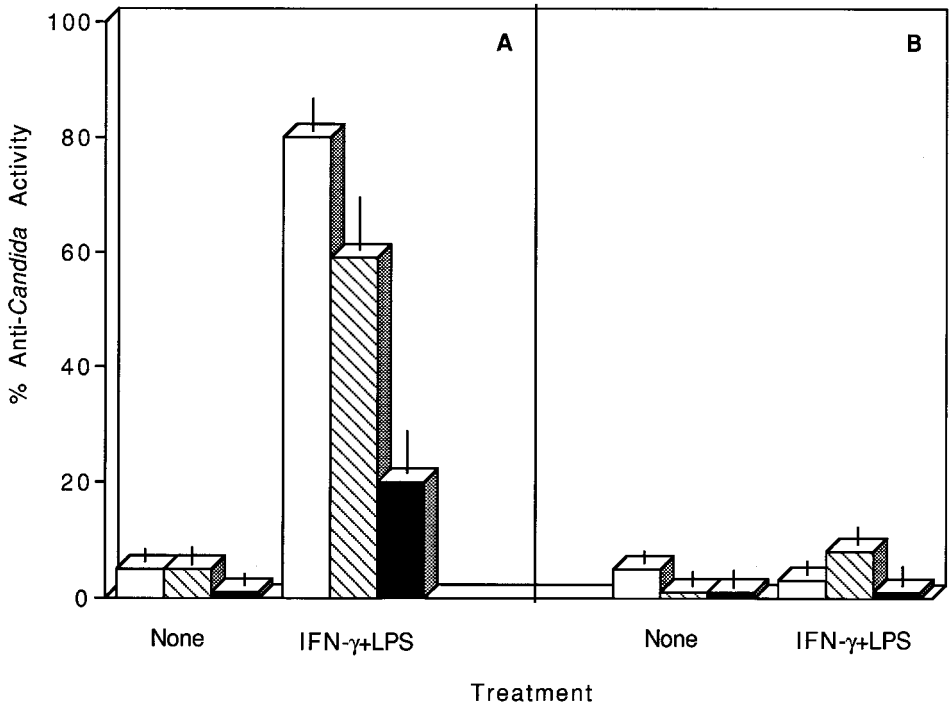

FIG. 2. Anti-H-*Candida* and anti-Y-*Candida* activities of supernatants from ANA-1 Mϕ treated with IFN-γ plus LPS. ANA-1 Mϕ were incubated in the absence or presence of IFN-γ (100 U/ml) plus LPS (10 ng/ml) for 18 h. Cell-free supernatants were then harvested, diluted at 1:2 (□), 1:64 (▨), and 1:128 (■), and then assayed for anti-H-*Candida* (A) and anti-Y-*Candida* (B) activities as described in Materials and Methods. Data represent the means ± standard errors of the mean values from four separate experiments. The value from each experiment is the mean of triplicate determinations.

tants from Mϕ exposed to IFN-γ plus LPS and both are sensitive to NMMA addition, implying that such Mϕ-mediated anti-H-*Candida* activity is related to the production of nitrogen-containing effector molecules. Not merely NO, because of its short life, but likely also stable nitrogen intermediates may provide an important contribution to the above-described phenomenon. According to Stamler et al. (28, 29), the generation of nitrogen-containing compounds may, in fact, be viewed as a means through which transport, lifetime, and targeting properties of NO and its various redox forms can be tailored to evoke specific biological responses, including Mϕ-mediated antimicrobial activity. The employment of SNP further supports the involvement of such compounds in the exertion of anti-H-*Candida* activity by ANA-1 Mϕ. Similarly to what was reported by Park and Rikihisa (24), our results demonstrate that SNP, an NO-generating agent (8), is effective in inducing antimicrobial activity in murine Mϕ. However, SNP per se does not have anti-*Candida* effects, thus suggesting that *C. albicans*, similarly to *E. risticii* (24) but differently from *Leishmania* spp. (18), is sensitive to rather complex effector systems involving the formation of nitrogen-containing compounds, such as nitrosothiols and nitrosoproteins. In this respect, our hypothesis, which is in line with a recent report showing that nitrosothiols have antiplasmodial activity (26), is further supported by the demonstration that an acidified nitrite solution (pH ranging from 7 to 4.5) does not affect H-*Candida* viability (data not shown). In the present paper, we provide formal proof that Y-*Candida* is not susceptible to Mϕ-derived supernatants, for either control Mϕ or Mϕ exposed to IFN-γ plus LPS. This finding implies that Y-*Candida* is totally refractory to extracellular cytotoxic systems, including NO, whose participation in the exertion of intracellular anti-Y-*Candida* activity has recently been proposed (10).

TABLE 1. NO_2^- production and anti-H-*Candida* activity of supernatants from ANA-1 Mϕ exposed to various treatments[a]

Treatment	NO_2^- production (μM)	Anti-H-*Candida* activity (% growth inhibition)
None	ND[b]	4.3 ± 0.5
IFN-γ + LPS	20.8 ± 2.7	70.6 ± 4.2
IFN-γ + LPS + NMMA	0.9 ± 0.2	5.8 ± 0.6
SNP	17.8 ± 1.5	63.8 ± 2.8

[a] ANA-1 Mϕ (2×10^6/ml) were incubated with complete medium in the absence or presence of IFN-γ (100 U/ml) plus LPS (10 ng/ml) or SNP (200 μM) for 18 h. When used, NMMA was added at 100 μM during treatment. Supernatants were then collected and assayed for NO_2^- production or anti-H-*Candida* activity as detailed in Materials and Methods. Data represent the means ± standard errors of the mean of values from four separate experiments. The value from each experiment is the mean of triplicate determinations.

[b] ND, not detectable.

In conclusion, our results give further insight about the differences between the two morphological forms of *C. albicans*, demonstrating that H-*Candida*, unlike Y-*Candida*, is damaged by Mϕ treated with IFN-γ plus LPS, through extracellular mechanisms involving the production of nitrogen-containing compounds.

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