**Brief Definitive Report**

Human Astrocytes Inhibit Cryptococcus Neoformans Growth by a Nitric Oxide-mediated Mechanism

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**Summary**

Cryptococcus neoformans is an opportunistic fungus that causes life-threatening meningoencephalitis in 5–10% of patients with acquired immune deficiency syndrome. Cryptococcal meningoencephalitis is characterized by a lymphohistiocytic infiltrate, accumulation of encapsulated forms of *C. neoformans*, and varying degrees of glial reaction. Little is known about the contribution of endogenous central nervous system cells to the pathogenesis of cryptococcal infections. In this study, we investigated the role of astrocytes as potential effector cells against *C. neoformans*. Primary cultures of human fetal astrocytes, activated with interleukin 1β plus interferon γ inhibited the growth of *C. neoformans*. The inhibition of *C. neoformans* growth was paralleled by production of nitrite, and reversed by the inhibitors of nitric oxide (NO-) synthase, NG-methyl-arginine and NC-nitro-arginine methyl ester. The results suggest a novel function for human astrocytes in host defence and provide a precedent for the use of NO- as an antimicrobial effector molecule by human cells.

**Materials and Methods**

**Astrocyte Culture.** Astrocyte cultures were prepared from second trimester human fetal abortuses, as previously described (19). The purity of astrocytes was achieved by repeated trypsinization of mixed cultures and final cultures consisted of >99% glial fibrillary acidic protein-positive cells. Astrocytes seeded in 96-well, flat-bottom tissue culture plates at 10^4 cells per well in DMEM/10% heat-inactivated FCS were grown to reach confluence at 37°C in 5% CO₂ (estimated final cell density was 20,000 cells per well).

**Cell Stimulation and r-[³H]leucine Incorporation.** Cultures were then stimulated with 200 U/ml each of recombinant human IL-1β (gift of Dr. C. Reynolds, National Cancer Institute, Frederick, MD) and IFN-γ (Genzyme Corp., Cambridge, MA), or grown with medium alone (control). 24 h later, *C. neoformans* (strain 24067, 365 J. Exp. Med. © The Rockefeller University Press 0022-1007/94/07/0365/05 $2.00

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CNS tissue is characterized by an extensive neuroglial network; astrocytes are ubiquitous in the brain (14, 15). In the subpial and perivascular zone, a specialized barrier formed by astrocytes (glia limitans) separates CNS parenchyma from subarachnoid space (14, 15) (see Fig. 1). The glia limitans is composed in part by basement membrane produced by astrocytes and permeable to diffusible molecules such as NO- or cytokines. Astrocytes are immunocompetent cells that can express immune molecules, present antigen, produce cytokines, and express activities of NO- synthase (NOS) (14, 16–19). Therefore we entertained the possibility that astrocytes could mediate antifungal activity by virtue of their capacity to produce soluble effector molecules, including NO-.
serotype D, from the American Type Culture Collection, Rockville, MD) cells at varying numbers were added resulting in approximate E/T ratios of 20:1, 40:1, and 80:1, and the cultures were further incubated for 24 h at 37°C. Astrocyte cultures were pulsed with 1 μCi of t-[3H]leucine (Amersham, Arlington Heights, IL; sp act, 150 Ci/mmol) per well 24 h after the challenge with C. neoformans. The cultures were incubated for an additional 24 h and C. neoformans-incorporated [3H]leucine was determined by measurement of cpm using an automated cell harvester and liquid scintillation counter. This method was adapted from previously published protocols (20) with minor modifications. There was a good correlation between cpm and the number of C. neoformans added, even with the low yeast numbers used (data not shown). The background cpm values in astrocyte cultures without C. neoformans were <300 (cpm in cytokine activated cultures were less than or equal to control astrocyte cultures), and these values were subtracted from the cpm values of experimental wells.

**CFU Assay.** Human fetal astrocytes were prepared and stimulated as described above, except that 24 h after the addition of fungi, the incubation was terminated and the number of viable fungi was determined by CFU assay. A companion set of cultures was prepared in which N⁰-methyl-endo-arginine (NMMA) or N⁰-nitro-

argonine methyl ester (NAME) (200 μM final concentrations; Sigma Chemical Co., St. Louis, MO) were added along with cytokines. Cytokine- and/or drug-treated cultures were challenged with C. neoformans 24 h later at the E/T ratio of 20:1, 40:1, and 80:1. For the CFU assay, supernatants and the cell lysates, prepared by the addition of distilled water, were combined, and serial dilutions of organisms in each well were plated on Sabouraud dextrose agar. The colonies were counted after 48 h of incubation at 30°C.

**Results**

Two methods were used to study the effect of astrocytes on C. neoformans growth, determination of CFUs and [3H]-leucine incorporation (20). CFU determination measures the number of viable yeast cells after 24-h coincubation with astrocytes. [3H]leucine incorporation, on the other hand, reflects growth of yeast surviving 48-h coincubation with astrocytes (20). Both methodologies showed that IL-1β plus IFN-γ activated astrocytes inhibited fungal growth. Inhibition of fungal growth by cytokine-activated astrocytes was demonstrated in six separate experiments. Fig. 2 illustrates results obtained using [3H]leucine incorporation as an indicator of fungal growth. At effector (astrocyte) to target (cryptococcus) ratios of 20:1–80:1, there was a 10–70% reduction in [3H]leucine incorporation for C. neoformans cells in the presence of cytokine-activated astrocytes relative to control, nonactivated astrocytes. The effect was greatest at the higher E/T ratios. The differences in fungal growth between wells containing activated and nonactivated astrocytes could be readily detected visually by microscopic examination of the culture at 24 and 48 h. As shown in Fig. 3, in the presence of

![Figure 1](https://example.com/f1.png)

**Figure 1.** A section from a human case of cryptococcal meningitis stained for glial fibrillary acidic protein (GFAP), an astrocyte-specific protein, showing proximity of GFAP-positive subpial astrocytes and C. neoformans (arrows). Hematoxylin-eosin counterstain (Nomarski optics). Astrocyte foot processes abut the subarachnoid space that contains inflammatory cells and C. neoformans.

![Figure 2](https://example.com/f2.png)

**Figure 2.** Activated human astrocytes limit the proliferation of C. neoformans as measured by [3H]leucine incorporation. Astrocytes plated at 20,000 cells per well in 96-well microtiter wells were grown with medium only (control) or with 200 U/ml rIL-1β and IFN-γ. 24 h later, C. neoformans at various numbers (or indicated E/T ratios) were added to cultures in four replicate wells and further grown for 48 h before harvest. t-[3H]leucine at 1 μCi per well was present for the last 24 h of culture. Counts per minute was calculated for each well as described in Materials and Methods. Values shown are the mean and standard deviation of four replicate wells. Data represent one of four similar experiments. (*) Significant differences (p <0.05) compared with controls (Student’s t test).
Figure 3. Phase contrast microscopy of *C. neoformans* and human astrocyte in coculture. (a) *C. neoformans* grow over control astrocyte monolayer in clumps or dispersed single organisms. Highly refractile, variably sized budding yeast are present in multiple planes of focus (arrowhead). (b) Marked reduction in *C. neoformans* growth is observed in cytokine-treated astrocyte cultures (arrowhead). After IL-1β plus IFN-γ treatment, astrocytes develop processes (17).

![Figure 3](image_url)

Figure 4. l-arginine analogs inhibit the cytokine-mediated human astrocyte anticytotoxic activity. Astrocytes were prepared and stimulated as described in the legend to Fig. 2, except this time parallel sets of cytokine-

of "resting astrocytes" *C. neoformans* grew extracellularly; no yeast cells were apparently internalized. In contrast, fungal growth was significantly reduced in cytokine-activated astrocyte cultures. Fig. 4 shows a 24-h CFU assay done at the same E/T ratios as in Fig. 2. There was a 52–64% inhibition of *C. neoformans* CFU in the presence of cytokine-activated astrocytes relative to nonactivated astrocytes.

Measurement of nitrite production in parallel astrocyte cultures revealed nitrite levels of 10 ± 1.0 μM in astrocyte cultures stimulated with cytokines compared with controls.

stimulated astrocytes were also treated with 200 μM of NMMA or NAME. After 24 h of coculture with *C. neoformans* at various E/T ratios, cultures were terminated and CFUs were determined as described in Materials and Methods. Values shown are the mean and standard deviation of four replicate wells. Data represent one of two similar experiments. (*) Denote significant differences (p < 0.005) compared with controls (Student’s t test).
that had nitrite levels of 1.6 ± 1.0 μM (lower limit of sensitivity for assay). To demonstrate more directly the involvement of NO--mediated mechanisms in the antifungal effect, the l-arginine analogues, NMMA and NAME, were studied for their ability to inhibit the anticytotoxic activity of cytokine-stimulated astrocyte cultures. Fig. 4 shows that the addition of either inhibitor at a concentration of 200 μM completely inhibited the anticytotoxic activity of cytokine-activated astrocyte cultures. Nitrite levels measured in parallel wells demonstrated an 85 and 70% inhibition of nitrite production by NMMA and NAME, respectively, similar to the degree of inhibition reported by us (17) and others (21) at this concentration of inhibitors. Addition of IL-1β and IFN-γ alone or in combination without astrocytes, did not exert antifungal activity (data not shown).

Discussion

Two lines of evidence implicate NO· as the effector molecule in the astrocyte mediated inhibition of C. neoformans growth shown in this study: (a) nitrite, a byproduct of NO· synthase activity, was present only in cytokine-activated astrocytes cultures and its presence was associated with fungal growth inhibition; and (b) the l-arginine analogues NMMA and NAME completely abolished the antifungal effect of cytokine-activated astrocytes.

The magnitude of in vitro antifungal effects observed for cytokine-treated astrocytes is similar to that reported for other effector cells, such as macrophages (9), lymphocytes (7), and NK cells (11). The E/T ratios at which astrocytes were shown to exert anticytotoxic activity were also comparable with E/T ratios at which peripheral immune cells have been shown to exhibit anticytotoxic activity (7, 9, 11).

NO· and its derivatives have been shown to be important mediators of antifungal activity by murine macrophages (12, 22) and to directly inhibit C. neoformans in vitro (23). In contrast to mice, human mononuclear phagocytes, including microglia, have no convincing inducible NOS activity (17, 21), making it unlikely that NO· is an antimicrobial molecule used by human phagocytic cells. On the other hand, evidence presented here that activated astrocytes inhibit C. neoformans growth in part at least through NO--mediated mechanism opens the possibility that similar antimicrobial mechanisms may be operant in vivo. A complex interactive network may exist among the various brain cell types to provide a coordinated local defense to infection. For example, microglia are efficient phagocytic cells that produce IL-1β (18), which is a potent inducer of nitrite synthesis by astrocytes (17). IFN-γ, a T cell cytokine, in turn, provides an additional signal for nitrite production by astrocytes (17).

In human infection, preliminary data suggest that the number of cryptococci in brain tissue is inversely correlated with the number of infiltrating inflammatory cells, including lymphocytes (Lee, S. C., unpublished observations). Cryptococcal meningoencephalitis in patients with AIDS often involves the accumulation of yeast in the brain with little inflammation or glial activation (2). These cases may reflect a lack of proper signals for astrocyte activation.

The results of this study demonstrate that activated astrocytes can inhibit C. neoformans growth in vitro and strongly implicate NO· as an antifungal effector molecule in humans. The close proximity of astrocytes and C. neoformans cells in human cryptococcal meningoencephalitis suggests a novel role of activated astrocytes as antimicrobial effector cells. Therapeutic strategies directed at enhancing astrocyte activation may be useful in the therapy of human cryptococcosis.

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