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Expression of Inducible Nitric Oxide Synthase in Rat Pulmonary *Cryptococcus neoformans* Granulomas

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Rats, like humans, have extremely effective immune mechanisms for controlling pulmonary* Cryptococcus neoformans *infection. The mechanism(s) responsible for efficient immunity in rat experimental infection is unknown. Recently, induction of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) have been implicated as an important microbicidal mechanism by which activated macrophages effect cytotoxicity against microbes. In this report, we investigated the expression of iNOS in rat pulmonary cryptococcosis. Localization and regulation of NO production was studied by immunohistochemistry for iNOS in conjunction with immunohistochemistry for cell markers, cytokines, and cryptococcal capsular polysaccharide. iNOS immunoreactivity was detected in macrophages, neutrophils, vascular endothelium, and respiratory epithelium. Double-immunolabeling studies revealed that the most prominent iNOS immunoreactivity was localized to epithelioid macrophages (CD11b/c$^+$) within granulomas; CD4$^+$ and CD8$^+$ T cells were numerous around granulomas but did not express iNOS. iNOS immunoreactivity was detected in a selective population of epithelioid macrophages within some granulomas but not others. iNOS$^-$ granulomas were identical to iNOS$^+$ granulomas with respect to morphology and immunohistochemical profiles. Macrophage iNOS immunoreactivity was detected 1 week after infection in one out of four rats and was strongly expressed in all rats at 2 weeks (in up to 50% of the granulomas) but declined considerably by 25 days. iNOS expression coincided with granuloma formation and preceded a decrease in lung fungal burden, suggesting an anticryptococcal role for NO. By double labeling, cytokines that have been shown to promote (interferon-γ, granulocyte/macrophage colony-stimulating factor) and inhibit (transforming growth factor-β) macrophage iNOS expression were detected around iNOS$^+$ granulomas. iNOS immunoreactivity was expressed in selected neutrophils (1 and 2 weeks) and endothelial cells (1 and 2 weeks and 25 days) in the inflamed lung. Airway iNOS immunoreactivity was limited to the luminal border of rare bronchiolar epithelial cells. iNOS immunoreactivity was not detected in uninfected rats. The present study provides the first evidence for association of iNOS expression with protective cellular responses to cryptococcal infection* in vivo. *(Am J Pathol 1996, 148:1275–1282)

Cryptococcus neoformans is the causative agent of cryptococcal meningoencephalitis, a major opportunistic infection in patients with acquired immune deficiency syndrome. Specific and nonspecific cellular immunity (natural killer cells, macrophages, lymphocytes, and neutrophils) are generally acknowledged to be responsible for effective immune defense.[1] This view is supported by the association of cryptococcal meningoencephalitis with illnesses resulting in impaired cellular immunity[2] and mouse experiments showing that depletion of either CD4 or CD8 cells exacerbates cryptococcal infection.[3,4]

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Table 1. *Monoclonal and Polyclonal Antibodies Used in This Study*

Antibody directed against	Species/isotype	Source*	Dilution	Cell type reactive
iNOS	Murine IgG2a	Transduction Laboratories	1:200	Primarily macrophages
CD3	Murine IgG3	Pharmingen	1:200	T cells
CD4	Murine IgG2a	Pharmingen	1:200	T helper cells
CD8	Murine IgG1	Pharmingen	1:100	T suppressor cells
CD11b/c(OX-42)	Murine IgG2a	Bioproducts for Science	1:100	Macrophages
GM-CSF	Rabbit IgG	Sigma	1:500	Rat/mouse GM-CSF
TGF-β	Murine IgG1	Genzyme	1:100	TGF-β1, -2, and -3
IFN-γ	Murine IgG1	Biosource	1:200	Mouse IFN-γ
Cryptococcal polysaccharide	Murine IgG3, IgG1	Mukherjee et al†	1:1000	*C. neoformans*

*For detailed information, see Materials and Methods.
†See reference 26.

The respiratory system is believed to be the initial focus of infection in cryptococcal disease. To study the pathogenesis of cryptococcal infection, we developed a rat model of pulmonary cryptococcosis.[5] In this model, endotracheal infection with *C. neoformans* produced self-limited pulmonary infection with minimal extrapulmonary dissemination and remarkably low levels of cryptococcal polysaccharide antigenemia. Furthermore, we demonstrated that granuloma formation preceded the clearing of *C. neoformans* organisms in the lung, suggesting a role for cell-mediated immunity in protective pulmonary responses.

Effective anti-fungal mechanisms require activation of macrophages via cytokines secreted by activated lymphocytes and macrophages. Macrophage antifungal mechanisms include reactive oxygen and nitrogen intermediates and fungicidal cationic proteins. Nitric oxide (NO) has been implicated as a final pathway by which cytokine- or endotoxin-activated inflammatory cells can mediate cytotoxic injury against microbes or the host.[6–8] NO is produced by NO synthase (NOS). In murine models, NO has been demonstrated to be important in the defense against several microbial species, including *Mycobacterium tuberculosis*.[9] *In vitro*, NO or its derivatives are fungistatic or fungicidal depending on concentration.[10] Chemically generated NO[10] and NO derived from rodent macrophages[11] and microglial cell line,[12] and human astrocytes[13] have been demonstrated to be fungistatic. The direct role of NO in animal or human cryptococcal infection has not been established.

At least three isoforms of NOS have been cloned. Activation of inducible NOS (iNOS) results in large amounts of sustained NO release and has been implicated in host defense as well as host injury.[6,7] Although many cell types are capable of expressing iNOS *in vitro* after proper stimulation, their expression of iNOS *in vivo* is less certain.

This study is a continuation of previous studies of rat pulmonary cryptococcal infection and attempts to better define the mechanisms of fungal clearance in the lung. In this model we examined by immunocytochemistry 1) iNOS expression in the pulmonary tissue, 2) cellular localization of iNOS expression, and 3) the extent of iNOS expression with respect to granuloma formation, fungal number, and cytokine expression.

Materials and Methods

Infection

Male Fischer rats weighing approximately 200 g were obtained from the National Cancer Institute (Frederick, MD) and infected by endotracheal infection with *C. neoformans* American Type Culture Collection (Rockville, MD) strain 24067 as described.[5] Uninfected rats were used as controls.

Pathology

Formalin-fixed, paraffin-embedded tissue at 1, 2, and 4 weeks after infection were examined with hematoxylin and eosin, periodic acid Shiff, and mucicarmine for evaluation of histopathology and fungal distribution.[5] Lung tissue from four rats per time point was examined. Lung fungal burden was determined by culture of organ homogenates on Sabouraud's agar. The lung colony-forming units and details of histopathology for the rats studied have been published.[5]

Antibodies

The antibodies used for immunocytochemistry are listed in Table 1. A monoclonal antibody (MAb) ob-

tained from Transduction Laboratories (Lexington, KY) that recognizes a 130-kd iNOS protein from cytokine-activated murine macrophages (not shown) was used for iNOS immunocytochemistry. T cell marker studies were performed with mouse anti-rat CD4, CD8, and CD3 (Pharmingen, San Diego, CA) MAbs. A mouse anti-rat CD11b/c (OX-42) MAb, used to label macrophages, was obtained from Bioproducts for Science (Indianapolis, IN). For cytokine immunocytochemistry, antibodies specific to mouse/rat interferon (IFN)-γ (Biosource, Camarillo, CA), transforming growth factor (TGF)-β (which reacts with TGF-β1, -2, and -3; Genzyme; Cambridge, MA), and granulocyte/macrophage colony-stimulating factor (GM-CSF; Sigma Chemical Co., St. Louis, MO) were used. For capsular polysaccharide antigen immunohistochemistry, MAbs to *C. neoformans* glucuronoxylomannan (3E5 or 2H1) were used.[13] Irrelevant isotype controls were a murine IgG2a MAb to smooth-muscle-specfic antigen (Dako Corp., Carpinteria, CA) and a murine IgG1 MAb to human macrophage antigen (Dako). Cryostat sections were used for cell marker studies and cytokine immunocytochemistry. Both cryostat and paraffin-embedded sections were used for iNOS immunocytochemistry (see below) and double-label immunocytochemistry (see below).

Immunocytochemistry

Cryostat sections (4 μm) were cut from OCT-embedded lung tissue. Sections were fixed in ice-cold acetone or methanol for 10 minutes and air dried. Endogenous peroxidase was blocked by incubation in 0.3% H_2O_2/phosphate-buffered saline (PBS) for 30 minutes. Nonspecific antibody binding was blocked with 10% normal goat serum. Primary antibodies (see Table 1 for dilutions) were applied to tissue sections for 16 hours at 4°C in 10% normal goat serum in PBS. Secondary antibodies were isotype-specific peroxidase- or alkaline-phosphatase-conjugated goat antibodies (Southern Biotechnology, Birmingham, AL), which were applied for 2 hours at room temperature. Color was developed with diaminobenzidine, or nitroblue tetrazolium in 5-bromo-4-chloro-3-indolyl phosphate buffer containing levamisole (Sigma). Some sections were counterstained with hematoxylin. After dehydration in alcohol, slides were placed in xylene and mounted with Permount (Sigma). Paraffin-embedded sections were processed identically as for cryostat sections, except for initial deparaffinizing steps.[5] For iNOS immunocytochemistry, paraffin sections were microwaved in distilled water or antigen retrieval solution (Biogenex, San Ramon, CA) according to the manufacturer's instructions to enhance antigen detection.[14] Staining controls included 1) reaction with secondary antibody alone. 2) reaction with irrelevant isotype-matched primary antibodies, and 3) staining of uninfected animals (see Results). For cytokine staining, specificity of the staining was controlled by titrating antibody concentrations until irrelevant antibodies or normal rabbit serum did not show reactivity.

Double-Label Immunocytochemistry

Double-label staining for iNOS, cell markers, cytokines, or cryptococcal polysaccharide was done sequentially. Primary antibodies for all markers (except iNOS) were first applied and detected with peroxidase-conjugated secondary antibodies and diaminobenzidine (brown). Application of anti-iNOS antibody followed, and antibody binding was detected by alkaline-phosphatase-conjugated secondary antibody and nitroblue tetrazolium (blue).

Results

iNOS Is Expressed in C. neoformans*-Infected Lung in Epithelioid Macrophages within Granulomas*

iNOS staining of cryostat and paraffin-embedded sections yielded comparable results in the extent and distribution of immunoreactivity (Figure 1). However, paraffin sections showed better preservation of morphology (Figure 1). Specificity of iNOS staining was confirmed by absence of staining in control slides (see Materials and Methods). iNOS expression was detected in lung tissue as early as 7 days after infection with *C. neoformans* and was temporally associated with early granuloma formation (Figure 1, A–C). iNOS expression was localized to inflamed regions of the lung and was not present in uninvolved areas. Uninfected rats did not express iNOS immunoreactivity. The majority of iNOS immunoreactivity localized to subpopulations of epithelioid macrophages within granulomas that were surrounded by $CD8^+$ and $CD4^+$ lymphocytes (Figure 1D). Double labeling revealed iNOS-immunoreactive cells within granulomas to be CD11b/c^+, indicative of macrophages (Figure 1E). However, not all macrophages within a particular granuloma were immunoreactive for iNOS (Figure 1E). Furthermore, not all granulomas within a given lung area were immunoreactive for iNOS (Figure 1E). iNOS immunoreactivity was not detected in lymphocytes. Most iNOS-reactive granulomas had intracellular *C. neoformans* or-

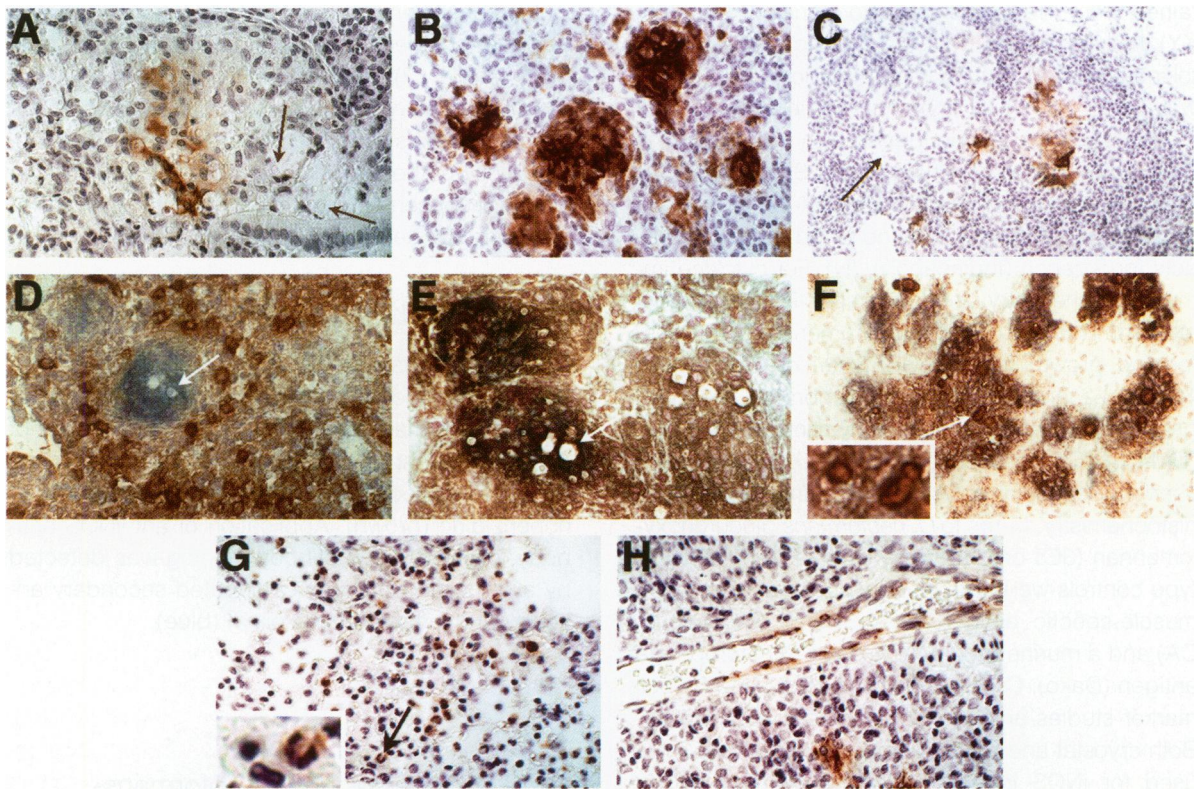

Figure 1. *iNOS immunoreactivity in* C. neoformans*-infected rat lung.* **A**, **B**, *and* **C:** *iNOS immunohistochemistry of paraffin-embedded lung tissue from rats at 1 week* (**A**), *2 weeks* (**B**), *and 25 days* (**C**) *after infection with* C. neoformans. **A:** *iNOS immuoreactivity is localized to occasional epithelioid cells in an immature peribronchial granuloma.* C. neoformans *organisms* (**arrows**) *are present in the region. Magnification,* ×*550.* **B:** *At 2 weeks after infection, iNOS expression within granulomas is maximal and can be found in approximately 50% of granulomas. iNOS immunoreactivity is also noted in occasional isolated macrophages.* ×*550.* **C:** *At day 25, iNOS immunoreactivity is substantially decreased and is detected focally in some granulomas but not in others. The* **arrow** *points to a region of granuloma that is not iNOS immunoreactive.* ×*134.* **D**, **E**, *and* **F:** *Double immunohistochemistry for iNOS (nitroblue tetrazolium, blue) and CD8* (**D**), *CD11b/c* (**E**), *and cryptococcal capsular polysaccharide* (**F**). *The second chromogen in all is diaminobenzidine (brown). Cryostat sections are of 2-week lung.* **D:** *Both* $CD8^+$ *and* $CD4^+$ *cells (not shown) are abundant and surround* $iNOS^+$ *granulomas containing* C. neoformans (**arrow**). ×*275.* **E:** *Double labeling demonstrates that iNOS immunoreactivity is present in a subset of* $CD11b/c^+$ *cells in granulomas. Two granulomas to the left are double labeled, whereas the third granuloma to the right is single labeled (brown only), thus* $iNOS^-$. *The* **arrow** *points to a* C. neoformans *organism.* ×*550.* **F:** *A cluster of* $iNOS^+$ *granulomas are shown to contain* C. neoformans*-bearing cryptococcal polysaccharide-positive capsules as well as non-organism-associated polysaccharide antigen (diffuse brown staining) within the epithelioid cells of granulomas. The* **arrow** *points to a budding* C. neoformans *organism.* ×*275. The* **inset** *shows organisms at higher magnification.* **G** *and* **H:** *Paraffin sections from 2-week lung tissue immunostained for iNOS demonstrating* $iNOS^+$ *neutrophils* (**arrow**) *in inflammatory exudates.* ×*550. The* **inset** *shows a neutrophil at higher magnfication* (**G**) *and in endothelial cells of a vessel surrounded by inflammatory cells* (**H**). ×*550. In* **H**, *iNOS immunoreactivity is also detected in some perivascular inflammatory cells. Note punctate perinuclear iNOS immunoreactivity in endothelial cells.*

ganisms that were demonstrated by combined histochemistry and inmunocytochemistry for mucin and iNOS (not shown) or by combined immunocytochemistry for cryptococcal polysaccharide and iNOS (Figure 1F). Staining for cryptococcal polysaccharide demonstrated *C. neoformans* organisms and non-*C. neoformans*-associated polysaccharide inside granulomas (Figure 1F).

iNOS Expression Correlates with Granuloma Formation

Maximal iNOS expression occurred 2 weeks after infection, which coincided with the height of granuloma formation in rat lungs. Semiquantitative analysis of iNOS immunoreactivity is summarized in Table 2 (see also Figure 1). Colony-forming unit data showed no increase in the number of *C. neoformans* organisms between 1 and 2 weeks, which was followed by a 1.6 log decrease by day 25, after the peak of iNOS expression (Figure 1C).

INOS Is Expressed in Selected Neutrophils and Endothelial Cells in C. neoformans*-Infected Lung*

In addition to epithelioid histiocytes, iNOS immunoreactivity was detected in infiltrating polymorphonuclear cells, isolated macrophages, vascular endo-

Table 2. *Semiquantitative Analysis of iNOS Immunoreactivity in* C. neoformans*-Infected Rat Lung*

	Fungal burden*	Granuloma formation	iNOS immunoreactivity† in Epithelioid macrophages	Neutrophils	Endothelial cells
1 Week	6.8 ± 0.5	+	±	±	±
2 Weeks	6.8 ± 0.4	++++	++++	+	+
3 Weeks	5.2 ± 0.1	++	++	−	++

*Fungal burden is expressed in average log 10 colony-forming units ± 1 SD. Data are from previoulsy published work.[13]
†Extent of iNOS immunoreactivity was assessed in an arbitrary scale from none (−) to maximal (++++).

thelial cells, and respiratory epithelial cells (Figure 1). Overall, iNOS staining in these cells was less extensive than in the epithelioid cells of granulomas. Clusters of iNOS-immunoreactive polymorphonuclear cells were observed at 1 and 2 weeks in necrotic lung areas (Figure 1G) and in the luminal exudates within the bronchioles. Polymorphonuclear cells were not observed in 25-day lungs. Isolated iNOS-immunoreactive macrophages were seen both adjacent to (Figure 1B) and remote from (not shown) iNOS-reactive granulomas. The staining intensity of single macrophages was less than that in epithelioid cells in granulomas. Endothelial cell iNOS immunoreactivity was detected in vessels of all sizes but was most prominent in medium and large muscular vessels. In endothelial cells, the staining was localized to discrete perinuclear regions (Figure 1H) rather than cytosol. iNOS immunoreactivity in endothelial cells varied between individual rats but showed an overall increase reaching a maximum at 25 days. Most $iNOS^+$ vessels were found within the inflamed regions. iNOS immunoreactivity in epithelial cells was distinctly rare and limited to the apical surface of a few bronchial epithelial cells (not shown).

IFN-γ, GM-CSF, and TGF-β Are Expressed in Cryptococcal Granulomas

As cytokines regulate induction and suppression of iNOS expression, we analyzed the expression of several cytokines in *C. neoformans*-infected rat lung by immunocytochemistry. Lung tissue from uninfected rats or uninvolved regions of *C. neoformans*-infected lungs did not show cytokine immunoreactivity (not shown). At 2 weeks after infection, immunoreactivity for all three cytokines, IFN-γ, GM-CSF, and TGF-β were expressed and localized to discrete cells (Figure 2). The number of immunoreactive cells was small, and they localized both within and outside the $iNOS^+$ granulomas (Figure 2). Double-labeling studies with cell-type-specific markers and cytokine antibodies have not been performed. Several anti-tumor-necrosis-factor-α antibodies have been found not to be optimal for immunocytochemistry on rat tissue.

Discussion

In rat pulmonary cryptococcosis, robust iNOS immunoreactivity was expressed in mature granulomas and localized primarily to epithelioid histiocytes. The results indicated that immune-activated macrophages in *C. neoformans*-infected tissue express iNOS. iNOS immunoreactivity to a smaller degree was also detected in neutrophils, non-granuloma-associated macrophages, and vascular endothelial cells and in rare bronchiolar epithelial cells. These findings are consistent with studies that have reported iNOS immunoreactivity in the alveolar macrophages and airway epithelium of endotoxin-treated rats[15] and in macrophages of mice infected with *Leishmania major*.[16]

iNOS was expressed only in a subpopulation of macrophages, neutrophils, and granulomas. At 2 weeks, there were many granulomas in which iNOS immunoreactivity was not detected. There was no apparent differences between $iNOS^+$ and $iNOS^-$ granulomas with respect to cell composition, number of *C. neoformans* organisms, and capsular polysaccharide immunoreactivity. These findings are consistent with local regulation of iNOS expression. The signals for iNOS expression in rat pulmonary cryptococcosis are not known. The murine macrophage iNOS expression is typically induced by proinflammatory cytokines such as IFN-γ.[6] TGF-β has been shown to exert profound negative regulatory effect on iNOS expression in murine macrophages.[17] We have demonstrated that IFN-γ, GM-CSF, and TGF-β are expressed simultaneously during the time of maximal granuloma formation (Figure 1). Coordinate expression of cytokines that up-regulate or down-regulate NO production may provide a means of protecting host tissue from the damaging effects of NO.

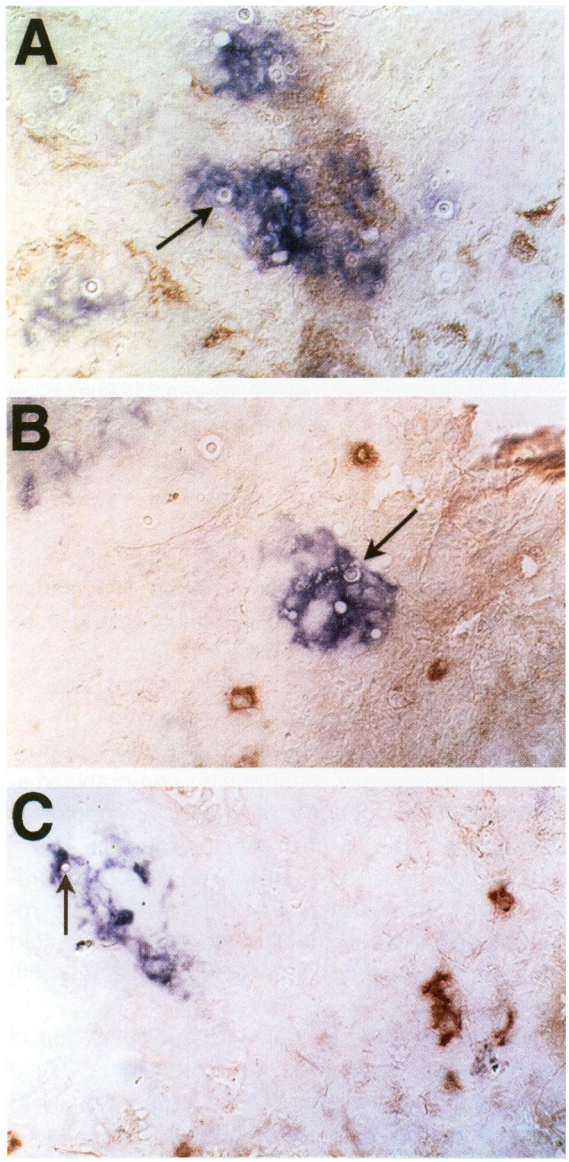

Figure 2. *Double immunocytochemistry for iNOS and cytokines. At 2 weeks after infection, double immunohistochemistry for iNOS (nitroblue tetrazolium, blue) and TGF-β* (**A**), *IFN-γ* (**B**), *and GM-CSF* (**C**; *all diaminobenzidine, brown) shows scattered cytokine-positive cells adjacent to iNOS$^+$ granulomas. Studies were performed on frozen sections.* **Arrows** *point to* C. neoformans *organisms. Magnification, ×275.*

Bacterial products such as endotoxin can also induce iNOS expression in rodent macrophages *in vivo* and *in vitro*. In cryptococcosis, it is likely that inflammatory cytokines that are secreted by activated lymphocytes and macrophages (rather than *C. neoformans* organisms themselves[18]) are responsible for iNOS regulation. *C. neoformans* or cryptococcal soluble polysaccharide (which are primarily intracellular in granulomas by 2 weeks) may contribute to the expression of iNOS, via interacting with opsonins and activating macrophage Fc receptors. Immune complexes can enhance IFN-γ-mediated NO production in the murine macrophage-like cell line J774.[19]

The role of NO in the immune response to *Cryptococcus neoformans* infection has not been clearly defined. Alspaugh and Granger[10] have demonstrated that chemically generated NO is either fungistatic or fungicidal for *C. neoformans in vitro*, depending on concentration. Similarly, we have demonstrated that cultured human astrocytes can exert extracellular stasis of *C. neoformans* by a NO-mediated mechanism.[13] These *in vitro* data suggest that NO or related reactive nitrogen species may be effector molecules against *C. neoformans in vivo*. The expression of iNOS by epithelioid macrophages within granulomas as well as the co-localization of iNOS immunoreactivity and *C. neoformans* inside granulomas is consistent with a role for NO in fungistasis/killing. It was significant that neutrophils that are found in large aggregates in *C. neoformans*-infected lungs from day 3 to day 14 also showed iNOS expression. The demonstration of iNOS in neutrophils suggests that NO is used by polymorphonuclear cells as an antimicrobial molecule in this model. In keeping with our findings, studies in mice have shown exacerbation of infection after inhibition of NO production in infection secondary to *Mycobacterium tuberculosis*, *Listeria monocytogenes, Leishmania major,* malaria, and *Francisella tularemia*.[9,20–22] Demonstration of a direct antimicrobial role for NO in this model awaits additional experiments with iNOS inhibitors or with genetically engineered, iNOS-deficient animals.

In addition to exerting cytotoxicity toward microbes, NO may have other immunoregulatory roles in pulmonary cryptococcosis. We have found that the peak of iNOS expression was followed by resolution of granulomas and reduction in inflammatory cells.[5] NO has been shown to mediate the suppressive effect of activated macrophages on the mitogen- or antigen- stimulated proliferation of lymphocytes *in vitro*,[23,24] suggesting a role for NO in down-regulating inflammation. Furthermore, the localization of iNOS expression to vascular endothelial cells and the temporal pattern of endothelial iNOS expression (maximal iNOS expression on day 25 after infection, despite resolving inflammation) suggests that NO may also be involved in the regulation of blood flow to the lungs in response to hypoxemia induced by infection.

The implications of our findings for human cryptococcosis is uncertain as it has been difficult to demonstrate the presence of a high-output-mode NO system in human macrophages.[6,25] Nevertheless,

our findings provide the first evidence associating iNOS expression with a protective cellular response to cryptococcal infection *in vivo*.

Note Added in Proof

Another group has recently demonstrated iNOS expression in association with pulmonary clearance of *C. neoformans* in mice.[27]

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