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# Production of Tumor Necrosis Factor Alpha in Human Leukocytes Stimulated by *Cryptococcus neoformans*

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**Tumor necrosis factor alpha (TNF-α) is a key mediator of inflammation and may promote human immunodeficiency virus replication in latently infected cells. Since cryptococcosis often is associated with aberrations in the host inflammatory response and occurs preferentially in persons with AIDS, we defined the conditions under which human leukocytes produce TNF-α when stimulated by *Cryptococcus neoformans*. Peripheral blood mononuclear cells (PBMC) produced comparable amounts of TNF-α following stimulation with *C. neoformans* and lipopolysaccharide. Detectable TNF-α release in response to *C. neoformans* occurred only when fungi with small-sized capsules were used and complement-sufficient serum was added. Fractionation of PBMC established that monocytes were the predominant source of TNF-α. TNF-α gene expression and release occurred significantly later in PBMC stimulated with *C. neoformans* than in PBMC stimulated with LPS. *C. neoformans* was also a potent inducer of TNF-α from freshly isolated bronchoalveolar macrophages (BAM). Upon in vitro culture, BAM and monocytes bound greater numbers of fungal cells, yet their capacity to produce TNF-α following cryptococcal stimulation declined by 74 to 100%. However, this decline was reversed if the BAM and monocytes were cultured with gamma interferon. These data establish that *C. neoformans* can potently stimulate TNF-α release from human leukocytes. However, several variables profoundly affected the amount of TNF-α released, including the type of leukocyte and its state of activation, the size of the cryptococcal capsule, and the availability of opsonins.**

The opportunistic fungus *Cryptococcus neoformans* has a marked propensity to cause infections in persons with impaired cell-mediated immunity, especially those with AIDS (5, 17). Exposure typically occurs as a consequence of inhalation of airborne fungi which, in susceptible individuals, may proliferate in the lungs and disseminate hematogenously (17). Although advances in diagnosis and treatment have improved the prognosis for patients with cryptococcosis, morbidity and mortality remain appreciable. Greater understanding of the immunology of cryptococcosis may lead to improved therapeutic approaches, including the use of agents that alter the inflammatory response.

Leukocytes synthesize and secrete a variety of multifunctional cytokines in response to diverse soluble and particulate stimulation. One such cytokine, tumor necrosis factor alpha (TNF-α), has a broad spectrum of immunoregulatory, metabolic, and inflammatory activities (14, 34, 35). TNF-α promotes inflammation, possibly through the induction of cell adhesion molecules, neutrophil and macrophage chemotactic factors, acute-phase proteins, and the generation of other proinflammatory cytokines such as interleukin-1 and interleukin-6. Although TNF-α is derived mainly from monocytes and macrophages, other cell types including lymphocytes and neutrophils also secrete TNF-α in response to certain stimuli (1, 8, 38). When produced in appropriate quantities, proinflammatory cytokines play a beneficial role as mediators of host resistance to infectious agents. However, overproduction can lead to local and systemic toxicity including fever, cachexia, sepsis syndrome, and death. TNF-α induces human immunodeficiency virus (HIV) replication in latently infected cells, and it has been postulated that the release of TNF-α in vivo could accelerate HIV progression (11, 16, 28, 30). Administration of anti-TNF antibody is deleterious in a mouse model of cryptococcosis (7). Given the potential relevance of TNF-α in patients with cryptococcosis, we examined the ability of *C. neoformans* to stimulate gene expression and release of TNF-α from human mononuclear leukocytes.

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## MATERIALS AND METHODS

**Materials.** All reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.) unless stated otherwise. All experiments were performed under conditions carefully designed to minimize endotoxin contamination. RPMI 1640 and phosphate-buffered saline (PBS) were obtained from Biowhittaker, Inc. (Walkersville, Md.) and contained less than 0.005 endotoxin U/ml. Lipopolysaccharide (LPS) was from *E. coli* O111:B4. The LPS antagonist *Rhodobacter sphaeroides* lipid A was prepared as described previously (12, 32) and was the gift of Nilo Qureshi (Middleton VA Hospital, Madison, Wis). All suspensions of LPS and *R. sphaeroides* lipid A were prepared in pyrogen-free PBS (Biowhittaker, Inc.) as 0.5- to 1-mg/ml stocks and were stored at −20°C. Immediately prior to use, the stock suspensions were thawed and subjected to 2 min of sonication in a water-bath sonicator (Laboratory Supplies, Inc., Hicksville, N.Y.). Pooled human serum (PHS) was obtained by combining sera from at least 10 healthy donors under conditions preserving complement activity. Heat-inactivated PHS (ΔH-PHS) was obtained by incubation of PHS at 56°C for 30 min. All glassware and plasticware not prepackaged were autoclaved and baked at 300°C for 4 h or 125°C overnight, respectively, to destroy contaminating LPS.

**Antibodies, cytokines and plasmids.** Recombinant TNF-α

and gamma interferon (IFN-γ) were gifts of Genentech, Inc. (South San Francisco, Calif.). A monoclonal antibody against human TNF-α was the gift of Miles, Inc. (West Haven, Conn.). Polyclonal anti-TNF-α was purchased from Genzyme (Boston, Mass.). Directly conjugated anti-CD14 and anti-CD20 monoclonal antibodies were purchased from Caltag Laboratories (South San Francisco, Calif.). The full-length cDNA plasmid for TNF-α was the gift of Leo Lina (Cetus Corp., Emeryville, Calif.) and was prepared as a 0.6-kb *Hin*dIII-*Kpn* fragment for Northern or slot blot hybridization of extracted RNA.

***C. neoformans.*** Serotype A strain 145 (20, 24) was used for all studies presented here. Results similar to those presented here were obtained in representative experiments with serotype D strain B3501 (data not shown). Previous investigations have demonstrated that bicarbonate and physiological pH favor capsule production (13). In order to study the effect of capsule size on *C. neoformans*-induced TNF-α release, three different media and growth conditions were used to propagate the yeast cells: (i) RPMI 1640 with 24 mM bicarbonate (pH 7.2) at 37°C in air containing 5% $CO_2$; (ii) RPMI 1640 without bicarbonate (pH 6.0) at 37°C in air not supplemented with $CO_2$; and (iii) Sabouraud dextrose agar at 30°C in air not supplemented with $CO_2$. Under such conditions, capsule thickness averaged 5.1 μm, 1.2 μm, and 0.7 μm, respectively, as measured with a light microscope equipped with a calibrated ocular micrometer following negative staining with India ink. Unless stated otherwise, all experiments utilized *C. neoformans* grown in RPMI 1640 without bicarbonate (pH 6.0) at 37°C in air not supplemented with $CO_2$. *C. neoformans* was harvested after 4 days of growth, washed at least five times in PBS, heat-killed at 50°C for 30 min, and stored at 4°C until use. Heat-killed organisms were utilized to minimize the potential problem of protease production by live organisms breaking down secreted TNF-α. Moreover, over the course of an 18-h experiment, growth of *C. neoformans* inside phagocytes could lyse the cells. Prior to use, organisms were washed an additional five times in endotoxin-free media.

**Isolation of leukocyte populations.** Leukocyte populations were purified as in previous studies by standard techniques (19, 20). Peripheral human blood was obtained from healthy volunteers by venipuncture. For each set of experiments, no donor was used more than once. Blood was anticoagulated with 10 U of pyrogen-free heparin per ml (Elkins-Sinn, Inc., Cherry Hill, N.J.) and was centrifuged at 500 × *g* for 15 min, and the leukocyte-rich buffy coat was harvested. Polymorphonuclear leukocytes and peripheral blood mononuclear cells (PBMC) were collected from the pellet and interface, respectively, following centrifugation of the buffy coat over a gradient of Ficoll-Hypaque. Nonadherent PBMC were obtained by sequential adherence to plastic petri dishes and nylon wool columns. In our experience, nonadherent PBMC contain undetectable numbers of monocytes and B cells as measured by flow microfluorimetry with anti-CD14 and anti-CD19 monoclonal antibodies, respectively (19, 20). In some experiments, PBMC were depleted of T cells and some NK cells because of their capacity to rosette neuraminidase-treated sheep erythrocytes. T cells and some NK cells express CD2 and rosette sheep erythrocytes, whereas other cell types including monocytes do not (19). Bronchoalveolar macrophages (BAM) were obtained by lavaging the lungs of healthy volunteers with a total of 240 ml of normal saline, exactly as described previously (37). Lavaged cells averaged over 90% macrophages.

**TNF-α release.** Leukocytes were mixed with *C. neoformans* in either 1.5-ml polypropylene tubes or 96-well polypropylene plates (Costar Corporation, Cambridge, Mass.) at 37°C in humidified air supplemented with 5% $CO_2$. Polypropylene was selected to minimize activation of monocytes and BAM as a result of adherence to the surface of the reaction vessel. All incubations were in RPMI 1640 containing 5% PHS except where 5% ΔH-PHS is specified. For negative controls, *C. neoformans* was omitted, whereas for positive controls, 100 ng of LPS per ml was substituted for *C. neoformans*. In agreement with published data (15), preliminary experiments in our laboratory demonstrated that this concentration of LPS elicited near-maximal TNF-α release. At defined intervals, supernatants were removed and stored at −70°C until analysis for immunoreactive TNF-α by "sandwich" enzyme-linked immunosorbent assay (ELISA), using the antibodies listed above, as described in full elsewhere (22). The ELISA was sensitive over a range of 100 to 3,000 pg of TNF-α per ml of supernatant. To facilitate comparisons between experiments, results are expressed as nanograms of TNF-α released per $10^6$ cells.

**Isolation and quantitation of TNF-α mRNA.** Total cellular RNA was extracted from $10^7$ cells per sample by using an RNA isolation kit (Tri-reagent; Medical Research Center, Cincinnati, Ohio) per the manufacturer's instructions. For each sample point, 10 μg of RNA was dissolved in 100 μl of deionized formamide, mixed with 100 μl of spotting buffer, and applied to a presoaked Zeta-probe membrane through a slot blot apparatus (Bio-Rad Laboratories, Richmond, Calif.) under gentle suction. Alternatively, RNA was separated in a 1.2% agarose-formaldehyde gel and transferred to nylon membranes (Schleicher & Scheull, Keene, N.H.). mRNA was hybridized to TNF-α cDNA which had been $^{32}$P-labeled by random priming with the Klenow fragment of DNA polymerase I (Boehringer Mannheim, Indianapolis, Ind.) and [α-$^{32}$P]dCTP (26, 29). Hybridized probe was visualized by autoradiography and quantitated by densitometry (Personal Densitometer equipped with ImageQuant software; Molecular Dynamics, Sunnyvale, Calif.). Data are expressed as the increase in optical density over background values.

**Binding assay.** Following removal of the supernatants for assay of TNF-α secretion, cells were incubated with 0.1% Fungiqual A (also known as Uvitex 2B and diaethanol; Specialty Chemicals for Medical Diagnostics, Kandern, Germany) for 30 min, washed to remove unbound *C. neoformans* and free diaethanol, and fixed in 1% buffered formaldehyde. Under these conditions, both intracellular and extracellular *C. neoformans* stain with diaethanol. By using an inverted microscope equipped with epifluorescence, at least 100 leukocytes per well were scored for the presence of cell-associated (bound and internalized) *C. neoformans*. Results are expressed as a binding index, which represents the mean number of cell-associated *C. neoformans* per 100 leukocytes (21).

**Statistics.** Means and standard errors (SE) were compared by using the two-tailed, two-sample *t* test. For experiments in which multiple comparisons were made, adjustments for significance were made by using Bonferroni's correction.

## RESULTS

**Effect of growth conditions and opsonization.** Initial experiments examined the capacity of *C. neoformans* grown under three different conditions to stimulate TNF-α release from PBMC (Fig. 1). *C. neoformans* grown in RPMI 1640 plus supplemental $CO_2$ at physiologic pH (conditions promoting large-sized capsules) did not stimulate significant TNF-α release. In contrast, *C. neoformans* grown in either RPMI (pH 6.0) without bicarbonate and supplemental $CO_2$ or Sabouraud dextrose agar (conditions promoting small-sized capsules) stimulated TNF-α release, provided that PHS was present during stimulation. Concentrations of TNF-α stimulated by

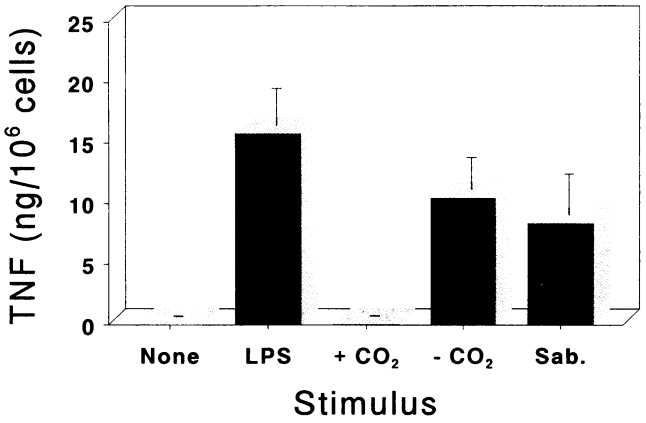


FIG. 1. TNF-α release from PBMC stimulated by *C. neoformans* grown under various growth conditions. PBMC ($10^6$) were incubated in RPMI 1640 plus 10% PHS for 18 h in the presence of no stimulus (None), 100 ng of LPS per ml (LPS), *C. neoformans* ($10^7$) grown in RPMI + $CO_2$ at pH 7.2 (+$CO_2$), RPMI without $CO_2$ at pH 6.0 (−$CO_2$), or Sabouraud dextrose agar (Sab.). Results are means + SE of four separate experiments, each performed in triplicate. *P* was $<10^{-4}$ when *C. neoformans* grown in the presence of $CO_2$ was compared with any other stimulus.

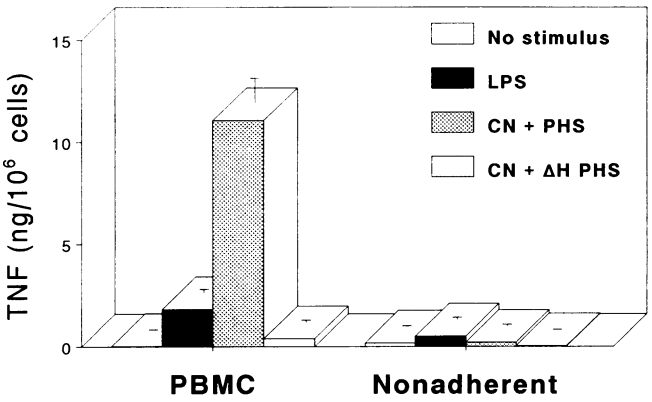


FIG. 2. TNF-α release from PBMC depleted of adherent cells. PBMC were stimulated either without further fractionation (PBMC) or after depletion of cells adherent to plastic and nylon wool (Nonadherent). Leukocytes ($5 \times 10^5$) were assayed for TNF-α release following an 18-h incubation with no stimulus, 100 ng of LPS per ml, or *C. neoformans* ($5 \times 10^6$) in the presence of either 10% PHS (CN + PHS) or 10% ΔH-PHS (CN + ΔH-PHS). Results are means ± SE of three separate experiments, each performed in triplicate. *P* was $<10^{-4}$ when PBMC and nonadherent PBMC stimulated with either LPS or PHS-opsonized *C. neoformans* were compared.

small-capsule *C. neoformans* were comparable to those produced by stimulation with 100 ng of LPS per ml. The failure of the large-capsuled organisms to stimulate TNF-α release may have been at least partly secondary to diminished recognition, since the binding index was considerably lower when monocytes were challenged with fungi containing large, compared with small, capsules (mean ± SE binding index of 32.3 ± 2.9 versus 206.0 ± 25.7, respectively; $P = 10^{-4}$).

The addition of 1 μg of the endotoxin antagonist *R. sphaeroides* lipid A per ml to the mixture of mononuclear cells and *C. neoformans* did not affect the subsequent release of TNF-α. In contrast, *R. sphaeroides* lipid A inhibited the response to up to 100 ng of LPS per ml by greater than 90% (data not shown). Heat-inactivation of PHS resulted in loss of detectable TNF-α release in response to *C. neoformans*, regardless of the conditions used to grow the organisms. In contrast, heat-inactivation of PHS did not affect LPS-stimulated cytokine secretion (data not shown). C5a has been reported by some (but not all) investigators to stimulate TNF-α release from monocytes (27). We studied whether supernatants derived from incubating *C. neoformans* in PHS under conditions previously shown to generate C5a (10) would stimulate TNF-α secretion from PBMC. However, such supernatants failed to stimulate detectable TNF-α release (data not shown).

**Subpopulations of PBMC responsible for TNF-α release.** PBMC comprise a mixture of leukocyte subpopulations, including T lymphocytes, NK cells, B cells, monocytes, and basophils. We sought to determine which subpopulations of leukocytes were responsible for the responses we observed to *C. neoformans*. We used two leukocyte fractionation techniques—rosetting to sheep erythrocytes and adherence to nylon wool and plastic—to determine which cell types are predominantly responsible for TNF-α release stimulated by *C. neoformans*.

First, PBMC were compared before and after depletion of monocytes and B lymphocytes by adherence to tissue culture plastic and nylon wool. TNF-α secretion stimulated by LPS and PHS-opsonized *C. neoformans* was almost completely abolished when PBMC were depleted of adherent cells (Fig. 2). Second, PBMC were fractionated into populations enriched for monocytes and T lymphocytes on the basis of their ability to rosette sheep erythrocytes (Fig. 3). TNF-α release in response to *C. neoformans* was observed almost exclusively in the monocyte-rich (interface) fraction. Reducing the ratio of *C. neoformans* to leukocytes from 10:1 down to 1:1 and 1:10 resulted in stepwise reductions in the amount of TNF-α released. TNF-α was undetectable when ΔH-PHS was substituted for PHS, suggesting that engagement of complement receptors was necessary for monocyte activation. When LPS

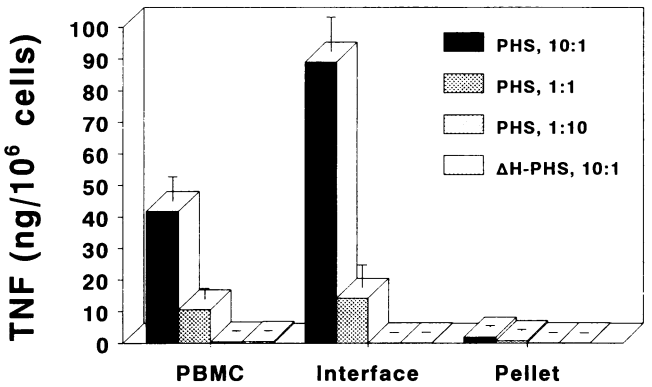


FIG. 3. TNF-α release from PBMC separated on the basis of ability to rosette sheep erythrocytes. PBMC were stimulated either without further fractionation (PBMC) or subsequent to fractionation into cell populations that formed rosettes (Pellet) or did not form rosettes (Interface) with sheep erythrocytes. Leukocytes were assayed for TNF-α release following an 18-h incubation with *C. neoformans*. Fungi were opsonized with either PHS or ΔH-PHS and were used at a 10:1, 1:1, or 1:10 ratio of fungi to cells. Results are means + SE of two to four separate experiments, each performed in triplicate. *P* was $<10^{-4}$ for cells in the pellet compared with those in the PBMC or interface groups when cells were stimulated by PHS-opsonized *C. neoformans* at a 10:1 or 1:1 ratio. TNF-α release was undetectable if leukocytes were left unstimulated (not shown).

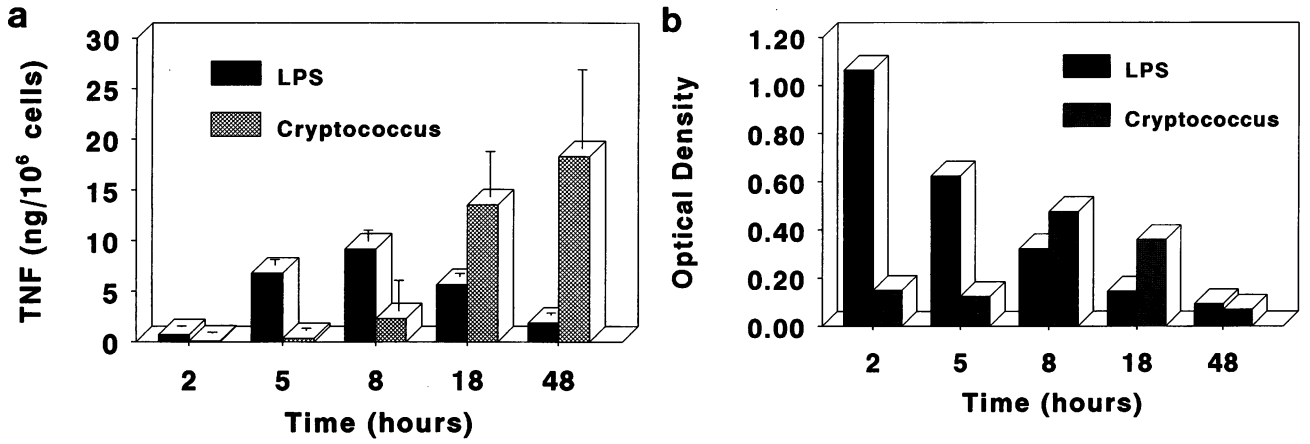


FIG. 4. Time course of TNF-α gene expression and release by PBMC stimulated with LPS or *C. neoformans*. PBMC were challenged for variable times with either 100 ng of LPS per ml or *C. neoformans* (at a 10:1 fungus-to-cell ratio) in the presence of 10% PHS. (a) TNF-α release. Data represent means + SE of three experiments each performed in triplicate. *P* was $\leq 10^{-4}$ when LPS and *C. neoformans* were compared at all time points except 18 h. TNF-α release was not detectable from unstimulated cells or from cells stimulated with *C. neoformans* in the presence of ΔH-PHS (not shown). (b) TNF-α gene expression. Data are from a representative experiment (of a total of three).

was used as a stimulus, TNF-α release was eight times higher in the monocyte-rich interface than in the pellet (data not shown).

**Kinetics of TNF-α gene expression and release.** We next sought to determine if the kinetics of *C. neoformans*-induced TNF-α expression resembled those of bacterial LPS. PBMC were stimulated with either LPS or serum-opsonized *C. neoformans*, and at defined time points (2, 5, 8, 18, and 48 h) TNF-α gene expression and release were determined (Fig. 4). While both LPS and *C. neoformans* stimulated TNF-α release, the kinetics of cellular activation were different. Detectable levels of TNF-α were seen within 2 h of LPS stimulation, and near-maximal levels were achieved by 5 h. In contrast, TNF-α release in response to *C. neoformans* was first detectable 5 h after stimulation and reached near-peak levels at 18 h. Moreover, while TNF-α levels in the supernatants began to decline after 18 h in the LPS group—presumably as a result of cellular consumption and/or destruction by proteases—levels continued to rise, even at 48 h, in supernatants from leukocytes stimulated with *C. neoformans*. The kinetics of TNF-α gene expression paralleled TNF-α release (Fig. 4b). Maximum TNF-α message was seen at 2 and 8 h when PBMC were stimulated with LPS and *C. neoformans*, respectively.

**Effect of culture with and without IFN-γ.** Blood monocytes differentiate into macrophage-like cells upon culture in vitro. We next studied the effect of monocyte differentiation upon TNF-α release in response to LPS and *C. neoformans* (Table 1). Monocytes were cultured for 0, 1, 2, or 6 days and were then challenged for 18 h with serum-opsonized *C. neoformans* or LPS. Surprisingly, *C. neoformans*-induced TNF-α release declined by greater than 96% (from 24.5 ng per $10^6$ cells to less than 1 ng per $10^6$ cells) after just 1 day in culture. Moreover, after 6 days in culture, TNF-α levels were undetectable. This decline was not due to failure of cultured monocytes to recognize *C. neoformans*, because monocytes cultured for 6 days bound greater than twice as many fungi as did freshly isolated cells (Table 1). When 500 U of IFN-γ per ml was added during the final 2 days of the 6-day culture, the cultured and fresh monocytes released comparable amounts of TNF-α. Consistent with results reported from other laboratories (4), compared with freshly isolated cells, cultured monocytes released less TNF-α in response to LPS stimulation. However, as with *C. neoformans*, TNF-α release induced by LPS greatly increased when the cultured monocytes were incubated with IFN-γ.

**TNF-α release from BAM and polymorphonuclear leukocytes.** BAM are thought to be the initial phagocytes responsible for recognition of *C. neoformans* following inhalation (17). We examined the conditions under which human BAM released TNF-α when stimulated by *C. neoformans* (Table 2). BAM were tested either the day of isolation or following 2 days of culture with or without IFN-γ. *C. neoformans* was a potent inducer of TNF-α from freshly isolated BAM. Compared with BAM that were freshly isolated, when BAM cultured for 2 days without IFN-γ were stimulated with PHS-opsonized *C. neoformans*, they exhibited a 285% increase in the binding index yet a 74% decrease in the amount of TNF-α released. However, similar to the situation with cultured monocytes, IFN-γ restored the capacity of cultured BAM to release TNF-α in response to cryptococcal stimulation. Results following LPS stimulation were qualitatively similar to those seen following *C. neoformans* stimulation, although the amount of TNF-α released was greater.

TABLE 1. Monocyte binding and TNF-α release stimulated by *C. neoformans*: effect of culture with and without IFN-γ

| Days in culture[a] | IFN-γ[b] | Binding index[c] | TNF-α[d] release induced by: | |
|---|---|---|---|---|
| | | | *C. neoformans* | LPS |
| 0 | − | 80 ± 14 | 24.58 ± 4.23 | 76.85 ± 26.93 |
| 1 | − | 55 ± 6 | 0.96 ± 0.59 | 19.71 ± 3.90 |
| 2 | − | 71 ± 8 | 0.97 ± 0.34 | 23.60 ± 12.19 |
| 6 | − | 213 ± 25 | <0.4 | 37.87 ± 10.4 |
| 6 | + | 358 ± 20 | 20.83 ± 5.88 | 102.57 ± 21.31 |

[a] Days monocytes ($1.5 \times 10^5$ per well) were cultured before being stimulated for 18 h with either PHS-opsonized *C. neoformans* ($1.5 \times 10^6$ per well) or 100 ng of LPS per ml.

[b] 500 U of IFN-γ per ml for the final 48 h of culture.

[c] Number of *C. neoformans* organisms bound per 100 monocytes (means ± SE of four separate experiments each performed in triplicate.

[d] Nanograms of immunoreactive TNF-α per $10^6$ cells. TNF-α release was below the limit of detection (<0.4 ng/$10^6$ cells) in unstimulated cells or cells stimulated with *C. neoformans* that was opsonized with ΔH-PHS (data not shown).

TABLE 2. BAM binding and TNF-α release stimulated by *C. neoformans*: effect of culture with and without IFN-γ

| Days in culture[a] | IFN-γ[b] | Binding index[c] | TNF-α[d] release induced by: | |
|---|---|---|---|---|
| | | | *C. neoformans* | LPS |
| 0 | − | 137 ± 19 | 61.7 ± 13.0 | 243.5 ± 49.1 |
| 2 | − | 391 ± 75 | 16.0 ± 1.1 | 58.3 ± 8.6 |
| 2 | + | 296 ± 36 | 50.7 ± 11.2 | 311.3 ± 65.0 |

[a] Days BAM ($1.5 \times 10^5$ per well) were cultured before being stimulated for 18 h with either PHS-opsonized *C. neoformans* ($1.5 \times 10^6$ per well) or 100 ng of LPS per ml.

[b] 500 U of IFN-γ per ml for the entire culture period.

[c] Number of *C. neoformans* bound per 100 BAM (means ± SE of three separate experiments each performed in triplicate). TNF-α release in unstimulated cells was less than 2% of the value seen in LPS-stimulated cells (data not shown).

[d] Nanograms of immunoreactive TNF-α per $10^6$ cells.

Neutrophils from three different donors failed to release detectable amounts of TNF-α following 18-h stimulation with a 10-fold excess of *C. neoformans* opsonized with PHS or with 100 ng of TNF-α per ml (data not shown).

## DISCUSSION

The data presented here establish that *C. neoformans* stimulates TNF-α release from some (but not all) populations of human leukocytes. However, TNF-α release was seen only under defined conditions, which included the presence of an intact complement system and use of fungi possessing small-sized capsules. Such conditions may have clinical relevance. Encapsulated *C. neoformans* is a potent stimulator of the complement system and, in the presence of overwhelming cryptococcosis, complement depletion may occur (23). Although capsule size on environmental isolates of *C. neoformans* is small, once the fungi are inhaled, capsule size tends to rapidly increase (13). Thus, the in vivo presence of complement depletion and/or large capsules may alter the host inflammatory response by diminishing TNF-α release.

The monocyte is thought to be the predominant cell type in human blood responsible for TNF-α release following LPS stimulation (8). However, other cell types including T cells, NK cells, and neutrophils, under defined conditions, can be stimulated to produce TNF-α (1, 8, 38). Since human peripheral blood monocytes, T lymphocytes, NK cells, and neutrophils all bind to and exert antimicrobial activity against *C. neoformans* (18–20), we studied which of these cell types produced TNF-α following cryptococcal stimulation. The data from experiments utilizing two purification schemes, one based on nonadherence to plastic and nylon wool and the other based on sheep erythrocyte rosetting, strongly suggest that monocytes are the predominant cell type secreting TNF-α in response to cryptococcal stimulation. It is unknown whether the small amounts of TNF-α detected in the monocyte-depleted fractions were generated by lymphocytes or by occasional contaminating monocytes. TNF-α concentrations were below the limits of detection when neutrophils were stimulated with either *C. neoformans* or LPS. Other investigators have shown that *Candida albicans* and *Saccharomyces cerevisiae* induce the release of small amounts of TNF-α from neutrophils (1, 38).

Our finding that *C. neoformans* stimulates abundant TNF-α release from monocytes and BAM suggests that the generation of TNF-α during the course of a cryptococcal infection could accelerate the progression of AIDS by promoting HIV replication in chronically infected cells (6, 11, 16, 28). HIV-positive PBMC and BAM stimulated with LPS produce TNF-α in amounts at least comparable to those produced by uninfected control cells (25, 36). The large fungal burden—sometimes with relatively small-sized capsules (2)—observed in AIDS patients with cryptococcosis should favor TNF-α production. The occurrence of fever in the majority of AIDS patients with cryptococcosis (5) provides indirect evidence for the release of TNF-α and/or other pyrogenic cytokines. These observations suggest that a therapeutic approach that needs to be critically examined, both in vitro and in vivo, is the utility of inhibitors of TNF-α biosynthesis, such as pentoxifylline (30), to interfere with HIV disease progression in patients with cryptococcosis. Recently, Pettoello-Mantovani et al. demonstrated another potential mechanism by which *C. neoformans* may potentiate HIV infection: the presence of cryptococcal capsular polysaccharide significantly increased production of p24 antigen by HIV-1-infected cells (31).

Both peripheral blood monocytes and BAM were potent producers of TNF-α when stimulated with PHS-opsonized *C. neoformans*. However, following in vitro culture, the capacity of monocytes and BAM to release TNF-α in response to cryptococcal stimulation dramatically decreased. The addition of IFN-γ to the cultures restored this capacity. IFN-γ is released by $CD4^+$ lymphocytes activated during the course of a cell-mediated immune response (9). Although the particular predisposition of individuals with impaired cell-mediated immunity to cryptococcosis is undoubtably multifactorial, diminished local IFN-γ production at sites of tissue infection may contribute to defective host defenses by decreasing the capacity of tissue macrophages to release TNF-α. In agreement with Burchett et al. (4), we too found that the capacity of monocytes to secrete TNF-α in response to LPS decreased in culture but was markedly augmented by IFN-γ. Reiner et al. demonstrated that TNF-α production by fresh monocytes stimulated by *Leishmania donovani* substantially increased when the monocytes were preincubated with IFN-γ (33).

The amount of TNF-α released by PBMC in response to *C. neoformans* was roughly comparable to that seen with LPS. Although TNF-α is thought to be the major cytokine mediating septic shock, a septic shock-like syndrome is extremely rare in patients with cryptococcosis. This is not surprising inasmuch as *C. neoformans* grows considerably more slowly than gram-negative organisms and is not known to release a potent soluble inducer of TNF-α (such as is the situation with gram-negative bacteria releasing LPS). Moreover, TNF-α gene expression and release occurred notably faster in response to LPS than to *C. neoformans*. Thus, rather than a sudden release of massive quantities of TNF-α, such as is thought to occur in patients with gram-negative sepsis, patients with cryptococcosis appear more likely to have sustained release of moderate quantities of TNF-α.

Future studies are needed to determine the mechanisms responsible for the delayed monocyte TNF-α gene expression and release in response to cryptococcal stimulation. Several cytokines (e.g., transforming growth factor β) are known to induce TNF-α (3). Thus, rather than directly stimulating TNF-α release, *C. neoformans* could be inducing other cytokines which then turn on TNF-α production.

Several lines of evidence, taken together, essentially eliminate the possibility that results observed were secondary to endotoxin contamination of yeast cells. First, TNF-α release in response to *C. neoformans* was not seen if the PHS was heat inactivated. In contrast, heat-inactivation of serum did not affect TNF-α release induced by LPS. Second, the kinetics of TNF-α gene expression and release were different for LPS and *C. neoformans*. Third, the addition of the LPS antagonist *R. sphaeroides* lipid A inhibited TNF-α induction in response to

LPS but not to *C. neoformans*. Finally, meticulous attention was paid to keeping endotoxin out of the experimental system, including extensively washing the fungal cells, prior to use, in buffer containing very low amounts of endotoxin.

Thus, the data presented here establish that *C. neoformans* can stimulate TNF-α release from human leukocytes. However, several variables profoundly affected the amount of TNF-α released, including the type of leukocyte and its state of activation, the size of the cryptococcal capsule, and the availability of opsonins. The clinical relevance of our findings remains speculative. As is the case with other infectious diseases, TNF-α production in cryptococcosis is analogous to a two-edged sword. When it is produced in moderate amounts, its proinflammatory properties are likely to benefit the host. However, overproduction may contribute to an inflammatory response that is damaging to the host and, in AIDS patients, may result in accelerated HIV replication.

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