Cryptococcus neoformans is a predominantly saprophytic yeast that can cause serious infections, mostly in individuals with compromised cellular immunity. Cryptococcosis is the fourth most common cause of mortality in patients with AIDS (7,16). Currently, 4% of patients with AIDS in the United Kingdom (18), 5 to 10% of AIDS patients in the United States (10,13), and an increasing percentage in the developing countries are known to have developed cryptococcosis (34,42).

The yeast, occurring as four serotypes, is surrounded by a capsule primarily composed of glucuronoxylomannan (GXM) (9) and composed, to a lesser extent, of galactoxylomannan (GalXM) and mannanproteins (MP).

Approximately 80% of the clinical isolates in the United States are of serotype A (21). Infection follows inhalation from environmental sources of poorly encapsulated yeasts, which are normally ingested and killed by pulmonary host defenses (4,40). Increased capsule production and replication within the lung may lead to dissemination to other tissues, frequently resulting in fatal meningoencephalitis. It is likely that the capsule acts as a virulence factor by inhibiting opsonophagocytosis (19,20,30), although the specific mechanisms for this effect are not entirely clear.

Cytokines are low-molecular-weight proteins involved in a variety of specific and nonspecific responses to infection (6). Some of these mediators, including tumor necrosis factor alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin 1 (IL-1), may play a role in the pathogenesis of cryptococcosis (8,23,41). TNF-α can exert both beneficial and detrimental effects in AIDS patients infected with C. neoformans. Release of TNF-α and GM-CSF by macrophages upon interaction with acapsular C. neoformans can stimulate, by an autocrine mechanism, increased phagocytosis of encapsulated yeasts (8,12). A beneficial role of TNF-α is also suggested by observations that TNF-α blockade can accelerate the progression of cryptococcosis in a murine model (11). In AIDS patients, however, TNF-α production may lead to increased replication of human immunodeficiency virus in latently infected cells and progression of the disease (17,33). Little information is available on the identities of cryptococcal components responsible for cytokine release. Poorly encapsulated or acapsular cells of C. neoformans are potent inducers of TNF-α from freshly isolated human monocytes and alveolar macrophages in the presence of complement-sufficient serum (24). The presence of a large capsule or the addition of purified GXM down-regulates the production of TNF-α (41). Better understanding of the mechanisms whereby C. neoformans induces TNF-α production may lead to improved therapeutic approaches using specific antibodies or modifiers of the inflammatory response. Indeed, conventional chemotherapy is only partially successful in AIDS patients, and infection can be controlled only with continuous suppressive therapy (2,5).

This study was undertaken to identify cryptococcal components responsible for stimulating TNF-α release. Specifically, a number of capsular and cell wall products, including GXM, GalXM, MP, and cell wall β(1-3) glucan were tested for their ability to stimulate human leukocytes to release TNF-α.
MATERIALS AND METHODS

Cryptococcal strains. Encapsulated C. neoformans A 9759, serotype A, was kindly provided by E. Reiss, Centers for Disease Control, Atlanta, Ga. The acapsular strain CAP 67 was provided by E. Jacobson, Richmond, Va.

Purified cryptococcal components. Insoluble C. neoformans cell wall glucan was a kind gift of E. Reiss. The other components were obtained from culture supernatants of strains A 9759 (GXM) and CAP 67 (GalXM and MP). Late-log-phase cryptococcal cultures were obtained after incubation at 30°C under agitation for 4 days in a chemically defined, entirely dialyzable medium (3). After the removal of yeast cells by tangential filtration through 0.2-μm-pore-size cassettes (Pellicon System; Millipore, Rome, Italy), culture supernatants were concentrated 20-fold by tangential filtration with 10,000-Dalton cassettes and dialyzed against phosphate-buffered saline (PBS) (0.01 M phosphate, 0.15 M NaCl; pH 7.2). GXM was obtained from culture supernatants of serotype A 9759 by gel filtration chromatography. Before gel filtration, concentrated supernatants were heat inactivated (56°C for 30 min) and supplemented with 25 μg of phenylmethylsulfonyl fluoride (Gibco BRL) per ml to inactive cytoenzymes possibly present in these preparations.

GalXM and MP were obtained from CAP 67 supernatants by affinity chromatography on concanavalin A (ConA) Sepharose 4B (Pharmacia), exactly as described previously (14). Briefly, GalXM was recovered in the elution fractions from the ConA-Sepharose column, whereas MP was eluted from the latter with a 0 to 0.3 M gradient of α-methyl-D-mannopyranoside. Both GalXM and MP were dialyzed against water and lyophilized.

The purity of GXM, GalXM, and MP was confirmed by PAGE analysis (29) of overloaded gels. GXM and GalXM preparations contained <1% proteins (Bio-Rad protein assay; Bio-Rad Laboratories, Richmond, Va.). The lack of carbohydrate contamination in GXM and GalXM preparations was demonstrated by monosaccharide constituent analysis using anion-exchange high-performance liquid chromatography and a pulsed amperometric detector (27). In some experiments, the cytokine-inducing ability of purified components was compared with that of killed whole cryptococci. The latter were obtained from A 9759 or CAP 67 late log cultures, washed with water, heated at 80°C for 1 h, and kept frozen.

Detection of endotoxin. Cryptococcal components were tested for the presence of endotoxin with a Limulus amebocyte lysate assay kit (E-Toxate; Sigma). The levels of endotoxin were <5 pg/ml in all of the materials.

Whole-blood cultures. Samples of whole peripheral blood were obtained from healthy volunteers and mixed with equal volumes of RPMI 1640 medium (Life Technologies, Millan, Italy) containing heparin at 20 U/ml. One-milliliter volumes were dispensed to 24-well plates and incubated at 37°C for 24 h in 5% CO2 with the indicated amounts of cryptococcal constituents or heat-killed yeasts. MP, GXM, and GalXM were dissolved in PBS and filtered through 0.22-μm-pore-size filters (Lida Manufacturing Corp. Kenosha, Wis.) before the addition of leukocytes (PMN) were obtained from the interface and pellet, respectively. The purity of GXM, GalXM, and MP was confirmed by PAGE analysis (29) of overloaded gels. GXM and GalXM preparations contained <1% proteins (Bio-Rad protein assay; Bio-Rad Laboratories, Richmond, Va.). The lack of carbohydrate contamination in GXM and GalXM preparations was demonstrated by monosaccharide constituent analysis using anion-exchange high-performance liquid chromatography and a pulsed amperometric detector (27). In some experiments, the cytokine-inducing ability of purified components was compared with that of killed whole cryptococci. The latter were obtained from A 9759 or CAP 67 late log cultures, washed with water, heated at 80°C for 1 h, and kept frozen.

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- Isolated leukocyte cultures. Whole blood, obtained as described above, was sedimented in the presence of 5% dextran, and leukocyte-rich plasma was harvested. Leukocytes were washed (200 × g for 10 min) three times in RPMI 1640, resuspended, and centrifuged (400 × g for 30 min) over Ficoll-Hypaque gradients (38). Peripheral blood mononuclear cells (PBMM) and polymorphonuclear leukocytes (PMN) were obtained from the interface and pellet, respectively. Pelleted cells were >95% neutrophilic granulocytes by morphology. In further experiments, PMNN were separated into adherent and nonadherent populations by incubation in plastic tissue culture dishes for 2 h at 37°C in serum-free RPMI 1640. Adherent and nonadherent PMNN were >95% monocytes and lymphocytes, respectively, by morphology. To assess cytokine production, 2 × 105 separated or unseparated leukocytes were cultured for 18 h in RPMI 1640 with 10% autologous plasma in the presence of cryptococcal components. In some experiments, autologous plasma was omitted or heated at 56°C for 30 min to inactivate complement. In selected experiments, the viability of leukocyte populations at the end of culture was >90% both in the presence and absence of stimuli, as assessed by the trypan blue exclusion test.

RESULTS

TNF-α production in whole blood. GXM, GalXM, and MP, obtained from strain A 9759, as well as cell wall glucan, were added in increasing concentrations to whole-blood cultures, and the supernatants were tested for TNF-α (Fig. 1A). For
TNF-α AND CRYPTOCOCCAL COMPONENTS

FIG. 2. Time course of TNF-α production in cultures of whole blood stimulated with MP, GXM, GalXM, or heat-killed CAP 67 cells. Data are from a representative experiment of three separate experiments conducted with blood samples from different donors.

Comparative purposes, whole heat-killed cryptococci were also tested (Fig. 1B). TNF-α was not detected in unstimulated cultures. The acapsular CAP 67 strain induced significantly higher TNF-α secretion than the encapsulated A 9759 strain (Fig. 1B), confirming previous observations using leukocyte cultures (24). All of the cryptococcal components tested were capable of inducing TNF-α release. MP, however, were more potent in this activity than the other cryptococcal products or heat-killed cryptococci.

TNF-α levels induced by GalXM, GXM, or glucan were lower at all tested concentrations than those induced by MP ($P < 0.05$). As little as 0.5 μg of MP per ml was sufficient to produce slight but significant elevations of TNF-α release over the level for unstimulated controls ($P < 0.05$). In contrast, the minimal concentration required to induce significant stimulation was 50 μg/ml using GalXM, GXM, or glucan. Maximal TNF-α levels induced by MP were similar to those induced by S. enteritidis LPS, our positive control (Fig. 1). GXM induced slight, but significant, elevations in TNF-α concentrations ($P < 0.05$) over the level for unstimulated controls. TNF-α levels induced by GXM were significantly lower than those induced by GalXM ($P < 0.05$). In order to rule out contamination of cryptococcal preparations with endotoxin, the endotoxin-inactivating agent polymyxin B (25 μg/ml) was added to whole-blood cultures in selected experiments. The addition of polymyxin B resulted in 75% inhibition of LPS-induced TNF-α but did not affect stimulation of TNF-α release by any of the cryptococcal components (not shown).

**Time course of TNF-α production.** Next, we determined the kinetics of TNF-α production in whole blood in response to purified components and whole killed acapsular C. neoformans. Figure 2 shows that significant TNF-α production was already detectable at 4 h after the addition of all the tested agents. Maximal levels were reached at 4 to 8 h and slowly declined thereafter.

**Leukocyte types responsible for TNF-α release.** Cytokine-producing cells in human blood comprise granulocytes, monocytes, lymphocytes, and natural killer cells. In order to identify the cell types which were predominantly responsible for the cytokine responses observed in whole blood, leukocytes were centrifuged over Ficoll-Hypaque gradients and PBMMN were compared with PMN for their ability to produce TNF-α. Unseparated leukocytes, PBMMN, and PMN were cultured in the presence of 10% autologous plasma with optimal stimulating concentrations of cryptococcal components (Fig. 3A). Under these conditions, PMN released considerably less TNF-α than unseparated leukocytes or PBMMN did, in response to any of the tested agents.

Since these data indicated that the cell populations responsible for most of TNF-α production were contained in the PBMMN fraction, the latter were further separated by adherence to plastic. Figure 3B shows that most of the TNF-α-producing activity was found in the adherent cell population. These data suggested that monocytes are predominantly responsible for cytokine release in response to C. neoformans components, although the contribution of other cell types could not be excluded.

**Plasma factors affecting TNF-α release.** Since it was previously shown that TNF-α production in response to whole heat-killed C. neoformans occurs only in the presence of complement-sufficient plasma (24), it was of interest to determine if the same requirement applied to purified cryptococcal components. Therefore, monocytes were cultured without plasma and in the presence of either heat-inactivated (56°C for 30 min) or unheated autologous plasma (Fig. 4). With GXM or GalXM, significant TNF-α production was observed only in the presence of heat-labile plasma factors (Fig. 4B and C). However, neither heat-labile nor heat-stable plasma factors were abso-
ulations other than monocytes produce moderate amounts of TNF-α in response to cryptococcal components. Our data do not exclude that leukocyte populations and serum components are involved in phagocytosis-dependent and -independent activities of C. neoformans, but not those with a large capsule, can induce significant TNF-α production (24). Moreover, the addition of GXM inhibited cytokine induction by the acapsular yeasts, but not by LPS (41). It is possible that GXM inhibits TNF-α production by masking cell wall components (20, 37) with high TNF-α-inducing activities. It is tempting to speculate that such components are indeed MP. It is presently unclear, however, if MP are expressed on the cryptococcal cell wall in sufficient amounts to stimulate TNF-α production. Fluorescein-conjugated ConA was shown to bind to the acapsular CAP 67 strain. However, labeling with ferritin-conjugated anti-MP antibodies demonstrated that these constituents are predominantly localized to the inner two-thirds of cryptococcal cell walls, with low-level surface expression (39).

A recent study reported the presence of exposed carbohydrate residues, which are masked by the polysaccharide capsule in the encapsulated form, on the cell walls of acapsular organisms (12). These exposed residues are recognized by various members of the collectin family, including mannose-binding protein (36). Such surface components are believed to stimulate mononuclear phagocytes for increased yeast phagocytosis and TNF-α production (25). It will be of interest to assess if at least some of the collectin-binding receptors on the surface of acapsular C. neoformans are MP.

Previously, phagocytosis of acapsular C. neoformans was shown to induce the release of TNF-α from mononuclear cells (12). We have shown here that some soluble cryptococcal products can also induce TNF-α secretion. It is unlikely that phagocytosis of insoluble particles present in the MP, GXM, and GalXM preparations employed was responsible for the observed release of TNF-α. First, all of these preparations were freely soluble in aqueous solutions. Second, these solutions were passed through 0.22-μm-pore-size filters before being added to whole-blood or isolated leukocyte preparations. Therefore, apparently phagocytosis is not an obligatory event in cell activation mechanisms leading to TNF-α production. Experiments are under way to determine if similar receptors are involved in phagocytosis-dependent and -independent TNF-α release. The ability of soluble extracellular cryptococcal products to induce TNF-α production may be clinically relevant, since extracellular components are released in vivo during infection and may stimulate host cells at remote sites (16).

To study cytokine production, whole blood was initially concentrated with heat-inactivated plasma and plasma-free cultures. Concentrations of MP, since comparable levels of the cytokine were observed in the absence of plasma and in the presence of heat-inactivated or unheated plasma (Fig. 4A). With lower (10-μg/ml) MP concentrations, however, TNF-α release was significantly higher in the presence of unheated plasma, relative to both heat-inactivated plasma and plasma-free cultures (P < 0.05).

**DISCUSSION**

Previous studies have indicated that TNF-α has an important role in host defenses against cryptococcosis in both naive and immunized mice (1). C. neoformans-induced TNF-α production, however, may result in accelerated human immunodeficiency virus replication in AIDS patients (28). Therefore, better understanding of the mechanisms whereby cryptococci induce TNF-α production could be useful to devise strategies to slow down the progression of AIDS in patients infected with this fungus. Since surface components and extracellular products are likely to be responsible for the cytokine-inducing activities of C. neoformans, we focused on the ability of those constituents to stimulate TNF-α production in human blood.

The present data indicate that several such components can stimulate TNF-α. MP, however, were significantly more potent than other components in this activity. The minimal MP dose required to stimulate significant TNF-α production was 2 orders of magnitude lower than those of GXM, GalXM, and cell wall glucan. In addition, MP were significantly more potent than heat-killed yeasts in inducing the cytokine. These observations raise the possibility that MP are predominantly, although not exclusively, responsible for C. neoformans-induced TNF-α production. Additional in vivo studies using specific antibodies will be useful to test this hypothesis.

Our data indicate that GXM, the major capsular component, is a comparatively weak TNF-α inducer. These observations are in general agreement with those of a previous study showing that heat-killed, small-capsule or acapsular organisms, but not those with a large capsule, can induce significant TNF-α production (24). Moreover, the addition of GXM inhibited cytokine induction by the acapsular yeasts, but not by LPS (41). It is possible that GXM inhibits TNF-α production by masking cell wall components (20, 37) with high TNF-α-inducing activities. It is tempting to speculate that such components are indeed MP. It is presently unclear, however, if MP are expressed on the cryptococcal cell wall in sufficient amounts to stimulate TNF-α production. Fluorescein-conjugated ConA was shown to bind to the acapsular CAP 67 strain. However, labeling with ferritin-conjugated anti-MP antibodies demonstrated that these constituents are predominantly localized to the inner two-thirds of cryptococcal cell walls, with low-level surface expression (39).

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To study cytokine production, whole blood was initially used, since this is a highly sensitive system in which different cell populations and serum components are allowed to interact in near-physiological conditions (32). Since neutrophils, lymphocytes, and monocytes all interact with C. neoformans, it was of interest to determine which of these cell types produced TNF-α in response to cryptococcal components. Our data indicated that monocytes are likely to be the predominant cell type involved in TNF-α production in blood. This is in agreement with similar results obtained using whole heat-killed cryptococci (24). Our data do not exclude that leukocyte populations other than monocytes produce moderate amounts of

![Figure 4](image-url)

**FIG. 4.** TNF-α production from monocytes cultured without plasma or in the presence of heat-inactivated (50°C for 30 min) or unheated autologous plasma. Cells were incubated with the indicated concentrations of MP (A), GXM (B), and GalXM (C). Bars represent the means ± standard deviations of three different experiments conducted with blood samples from different donors.
TNF-α upon interaction with C. neoformans. In fact, in the present study, low-level, but significant, cytokine production was detected in PMN or lymphocyte cultures stimulated with cryptococcal components. It was previously shown that the presence of complement is an obligatory requirement for TNF-α induction by C. neoformans (34). Our data also indicate that heat-labile components such as complement are necessary for GXM- and GalXM-induced TNF-α production. With MP, this was true at low, but not high, stimulating doses. These data are consistent with the notion that GXM, GalXM, and MP all activate complement, which is, in turn, capable of inducing TNF-α production (14). Therefore, it appears that, with the exception of MP, cryptococcal components induce TNF-α production after interacting with heat-labile components and are incapable of directly stimulating monocytes for cytokine production. Experiments are under way to characterize the mechanisms of complement-independent cytokine induction by MP. Such mechanisms may be clinically relevant. Major foci of cryptococcal infection include serum-free sites such as the pulmonary alveoli and the central nervous system (22, 35). In addition, complement depletion, secondary to massive activations of complement or complement deficiency, may have an important role in meningitis (26).

In conclusion, our data indicate that cryptococcal MP are potent TNF-α inducers in human monocytes. Further studies will be needed to clarify the relevance of this finding to the pathogenesis of cryptococcosis.

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REFERENCES


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