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# *Cryptococcus neoformans* and Cryptococcal Glucuronoxylomannan, Galactoxylomannan, and Mannoprotein Induce Different Levels of Tumor Necrosis Factor Alpha in Human Peripheral Blood Mononuclear Cells

WENDY CHAKA,[1] ANDRÉ F. M. VERHEUL,[1] VARSHA V. VAISHNAV,[2] ROBERT CHERNIAK,[2] JELLE SCHARRINGA,[1] JAN VERHOEF,[1] HARM SNIPPE,[1] AND ILJA MOHANDAS HOEPELMAN[1,3]*

*Division of Vaccines, Eijkman-Winkler Institute for Microbiology, Infectious Diseases and Inflammation,[1] Division of Infectious Diseases, Department of Internal Medicine,[3] University Hospital Utrecht, Utrecht, The Netherlands, and Department of Chemistry, Georgia State University, Atlanta, Georgia[2]*

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**Tumor necrosis factor alpha (TNF-α) release by peripheral blood mononuclear cells (PBMC) during disseminated infection by *Cryptococcus neoformans* may initiate and amplify the immune response of the host, leading to elimination of the fungus. The ability to induce TNF-α in PBMC by four clinical strains of *C. neoformans*, a laboratory strain (NIH 37), and the purified cryptococcal components glucuronoxylomannan (GXM), galactoxylomannan (GalXM), and mannoproteins (MP1 and MP2) were investigated under different opsonic conditions. In the absence of serum, the levels of TNF-α induced by all strains and cryptococcal components were not above background levels. Normal human serum (NHS) enhanced TNF-α induction by whole cryptococci and the different cryptococcal components, with MP2 being the most potent TNF-α inducer. Inactivation of complement (HI NHS) almost abrogated the ability of whole cryptococci and the GXMs to induce TNF-α. In contrast, when MP1, MP2, and GalXM were incubated with HI NHS, 48, 71, and 44%, respectively, of the original TNF-α levels remained. MPs incubated with heat-inactivated immunoglobulin G (IgG)-depleted serum still induced 50% of the levels of TNF-α induced by components incubated with HI NHS. Both these sera contained the same very low levels of anti-MP IgG antibodies, indicating the opsonic effect of a heat-stable factor other than antibody. Two anti-CD14 monoclonal antibodies (60BCA and 3C10) inhibited the production of TNF-α induced by MP2. The results indicate that (i) induction of TNF-α by *C. neoformans* and GXMs strongly depends on complement, (ii) MP1 and MP2 induction of TNF-α is facilitated by a heat-stable serum factor other than Ig, and (iii) CD14 may be involved in the induction of TNF-α by MP2.**

The encapsulated yeast *Cryptococcus neoformans* is an opportunistic pathogen in patients with impaired cell-mediated immunity, especially those with AIDS (24, 33). The initial site of infection is the lung, where alveolar macrophages represent the first line of host defense. Host defense against *C. neoformans* is based on T-cell-mediated immunity and natural effectors cells such as monocytes, natural killer (NK) cells, and neutrophils (8, 25, 31).

Cytokines, such as interleukin-1 and tumor necrosis factor alpha (TNF-α) have been shown to contribute significantly to the pathogenesis of several infectious diseases and to the initiation and amplification of immune responses in general. TNF-α is a multifunctional, inflammatory cytokine produced primarily by mononuclear phagocytes (3, 46). It has autocrine and paracrine functions that are beneficial to the host. TNF-α stimulates the production of other cytokines, is involved in local induction of vascular endothelial cell adhesion molecules (both resulting in further phagocyte accumulation), and is implicated in overcoming the antiphagocytic properties of *C. neoformans* (8, 9). However, it has also been demonstrated that TNF-α induces human immunodeficiency virus (HIV) replication (15, 34). Furthermore, *C. neoformans* or its isolated glucuronoxylomannan (GXM) can enhance HIV replication in HIV-infected cells in vitro (32, 35). Therefore, induction of TNF-α by *C. neoformans* and identification of envelope components responsible for the TNF-α induction are worth exploring, because TNF-α induction might have implications for the pathogenesis of *C. neoformans* infections in AIDS patients and their subsequent treatment. Prevention or reduction of TNF-α levels induced during cryptococcal infection might prevent upregulation of HIV replication and might extend the life expectancy of these AIDS patients.

Studies of cytokine release as a result of interactions between host immune defenses and fungi have been conducted (1, 11, 25, 26, 29, 31). Limited reports on the induction of TNF-α by *C. neoformans* are available (26, 32, 42), and there is only one report on the identification of molecules affecting this process (43). The cell envelope of *C. neoformans* is composed mainly of carbohydrate (5–7, 18, 39, 40), a capsular polysaccharide (GXM), and two minor carbohydrate antigens, galactoxylomannan (GalXM) and mannoprotein (MP). GXM, which makes up 88% of the capsular mass, consists of mannose, xylose, glucuronic acid, and *O*-acetyl. GalXM and MP are composed of galactose, xylose, and mannose in different proportions. Based on the GXM, four serotypes of *C. neoformans* have been identified (serotypes A, B, C, and D). *C. neoformans* serotype A has been isolated from virtually 100% of AIDS patients with cryptococcosis (24).

* Corresponding author. Mailing address: Division of Infectious Diseases, Department of Internal Medicine, University Hospital Utrecht, P.O. Box 85500, 3508 GA Utrecht, The Netherlands. Phone: 31-30-2509111. Fax: 31-30-2518328. E-mail: I.M.Hoepelman@digd.azu.nl.

The present study assesses whether different strains of *C. neoformans* as well as purified cell envelope components of *C. neoformans* are able to induce TNF-α release by PBMC and investigates the type of opsonins required for this process. We report that five different strains of whole *C. neoformans* and purified cryptococcal cell envelope components are able to induce TNF-α release by PBMC. In addition, preliminary evidence is presented that the CD14 receptor may be involved in the release of TNF-α by mannoproteins.

## MATERIALS AND METHODS

**Cryptococcal strains and GXMs.** Two clinical isolates of *C. neoformans* were obtained from Utrecht University Hospital, Utrecht, The Netherlands. L926 was from a healthy patient with cryptococcemia without underlying disease, and N114 was from an AIDS patient with meningitis (both strains were serotype A). Two other clinical isolates, C16 and C32, were obtained from Parirenyatwa Hospital, Zimbabwe (serotype B/C). *C. neoformans* NIH 37, serotype A, obtained from the National Institutes of Health (Bethesda, Md.) was used as a reference in the study. The different strains were maintained on Sabouraud dextrose agar (Merck, Darmstadt, Germany) at 4°C. All strains tested were thinly encapsulated (<0.5 μm). Prior to the experiments, colonies were freshly inoculated into Sabouraud glucose liquid medium (Merck) and incubated for 18 h in a shaking water bath at 37°C. The cells were washed three times with Hanks' balanced salt solution containing 0.05% gelatin (pH 7.2), counted, heat killed for 30 min at 80°C, and resuspended in Hanks' balanced salt solution plus gelatin to a final concentration of $10^8$ ml$^{-1}$. Killing was checked by plating cryptococcal suspensions on Sabouraud dextrose agar. Five different GXMs were used: NIH 37 (serotype A), NCPF (serotype A), NIH 112 (serotype B), NIH 18 (serotype C), and B3502 (serotype D). GXMs NIH 37, NCPF, NIH 112, and NIH 18 were isolated and purified in our laboratory as previously described by Cherniak et al. (6). GXM B3502 was a gift from J. E. Bennett (National Institutes of Health). GXMs NCPF, NIH 37, NIH 112, and NIH 18 tested negative for endotoxin in the *Limulus* amoebocyte assay.

**Purification of cell envelope antigens other than GXM.** An acapsular mutant of *C. neoformans*, Cap67 (E. S. Jacobson, Medical College of Virginia), was cultured in a defined medium as described previously (5). The culture supernatant, containing GalXM and MP, was concentrated by ultrafiltration (spiral cartridge concentrator S1Y10; Amicon). The retentate was recovered by lyophilization after dialysis against water (10R; yield, 200 mg of retentate per liter). The initial fractionation of 10R was done by modification of the concanavalin A (ConA) affinity column chromatography procedure described previously (39). Briefly, 700 mg of 10R was dissolved in 40 ml of start buffer (0.5 M NaCl and 0.01 M Tris buffer [pH 7.2] containing 1 mM $MgCl_2$ and $CaCl_2$) and any insoluble material was removed by filtration (Milex-GS; pore size, 0.22 μm; Millipore). The filtrate was applied to a ConA-agarose (type VI C-7555; Sigma Chemical Co., St. Louis, Mo.) column (2.5 by 22 cm). The sample was recirculated through the column at a flow rate of 16 ml/h to ensure maximum binding. The effluent (GalXM) was reserved, and the column was washed with 300 ml of start buffer until the effluent was negative for carbohydrate by the phenol-sulfuric acid assay (12). Wash fractions containing carbohydrate were combined with the original effluent, and the negative fractions were discarded. The column was successively eluted with 0.2 M (432 ml) and 0.4 M (216 ml) methyl-α-D-mannopyranoside; the eluates were designated fraction A and fraction B, respectively. The three fractions, GalXM, fraction A and fraction B, were dialyzed, filtered (pore size, 0.22 μm), and recovered by lyophilization to give 315, 105, and 14 mg, respectively. In addition, the fractions were treated at 100°C for 5 min, resulting in denaturation and precipitation of contaminating ConA.

GalXM (1.0 g [GalXM fractions from other purifications were pooled] in 15 ml of 0.01 M Tris [pH 7.6]) was applied to a DEAE-cellulose column (DE 52, 4 by 25 cm; Whatman Chemical Separations Ltd.). The column was washed with 500 ml of start buffer (0.01 M Tris [pH 7.6]) or until the effluent was negative for carbohydrate. Bound GalXM was eluted with 2 liters of eluent with a linear gradient from 0.01 M Tris to 0.01 M Tris-1 M NaCl (pH 7.6) at a flow rate of 21 ml/h. The eluate was monitored for carbohydrate by the phenol-sulfuric acid assay (12), and the positive fractions were pooled, dialyzed, and lyophilized (630 mg). Fraction A (200 mg in 13 ml of start buffer [again, various 0.2 M methyl-α-D-mannopyranoside MP purifications were pooled]) was applied to a DEAE-cellulose column (DE 52, 2.5 by 21 cm; Whatman Chemical Separations Ltd.) The column was washed with 250 ml of start buffer or until the effluent was negative for carbohydrate. Bound fraction A was eluted with 1 liter of eluent with a linear gradient of 0.01 M Tris to 0.01 M Tris-1 M NaCl (pH 7.6) at a flow rate of 22 ml/h. The elution was monitored continuously at 205 nm and by the phenol-sulfuric acid assay (12). Two overlapping peaks designated MP1 and MP2 that absorbed at 205 nm and that were also positive for carbohydrate were obtained. Appropriate fractions were pooled, dialyzed, and lyophilized (MP1, 88 mg; MP2, 30 mg). The purified GalXM, MP1, and MP2 were used in these studies. GalXM has a molecular mass of 22.2 kDa and does not contain any detectable protein. MP1 contains 7% protein and 79% neutral sugar and has a molecular mass of 35.6 kDa. MP2 consists of 13.3% protein and 43% carbohydrate and has a molecular mass of 8.2 kDa. GalXM, MP1, and MP2 do not contain any contaminating ConA as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, amino acid analysis, and molecular mass determination (ConA has a molecular mass of 102 kDa). These components did not induce TNF-α in peripheral blood mononuclear cells (PBMC) when no serum was present, whereas PBMC incubated with a range of ConA concentrations (6.25 to 50 μg ml$^{-1}$) in the absence of serum gave high levels of TNF-α. This indicates that the MP1, MP2, and GalXM preparations did not contain contaminating ConA which was biologically active, because otherwise TNF-α induction would have been observed in the absence of serum (37). Heat treatment of ConA as for the components (100°C for 5 min) completely abrogated its ability to induce TNF-α (concentration range, 0.08 to 5 μg ml$^{-1}$) in the presence or absence of serum. Moreover, preincubation of MP2 with ConA diminished the levels of TNF-α induced by MP2 (data not shown). Therefore, none of the components contained any contaminating ConA.

**LPS.** Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 (Sigma) was dissolved in pyrogen-free water (1 mg ml$^{-1}$) and treated by ultrasonic treatment at 0°C. The pH was adjusted to 7.4 with triethylamine (3%), and the solution was stored at −20°C.

**Endotoxin contamination.** Experiments were carried out aseptically to avoid endotoxin contamination. Polymyxin B sulfate (Sigma) was added to all test mixtures at a final concentration of 10 μg ml$^{-1}$. This concentration of polymyxin B sulfate completely abolishes the stimulatory effects of endotoxin from *E. coli* O111:B4 at concentrations up to 100 ng ml$^{-1}$ (27).

**Serum source.** Sera from 30 healthy Dutch individuals were pooled and stored in small aliquots at −70°C (normal human serum [NHS]). In some experiments, NHS was heated at 56°C for 30 min to inactivate complement components (HI NHS). Immunoglobulin G (IgG)-depleted serum was obtained by passing NHS over a protein G-Sepharose 4 fast-flow column (Pharmacia, Uppsala, Sweden). The IgG contents of the IgG-depleted serum and NHS were <0.032 and 11 mg ml$^{-1}$, respectively, as determined by radial immunodiffusion in LC-Partigen plates (Behringwerke AG, Marburg, Germany). The C3-depleted serum used in the study was obtained from Sigma. This serum was depleted of C3 by immunoabsorption.

**Monoclonal antibodies.** Two monoclonal antibodies (MAb), 60BCA and 3C10 (American Type Culture Collection, Rockville, Md.) were used to study the involvement of CD14 in the release of TNF-α by PBMC in response to the cryptococcal MP2 fraction. MAb 60BCA and 3C10 are murine MAb of the IgG1 and IgG2b subclasses, respectively. Both recognize functional epitopes of human CD14. The isotype controls of the IgG1 and IgG2 subclasses were similarly of murine origin. All MAb were used at 10 μg ml$^{-1}$.

**Isolation and stimulation of PBMC.** Buffy coats were obtained from The Blood Bank, Utrecht. PBMC were isolated by centrifugation on a Ficoll-Paque density gradient (Pharmacia) as described by Boijum (4). The mononuclear cell layer was then washed and resuspended in RPMI 1640 (Gibco, Gaithersburg, Md.), containing 0.1% human serum albumin, to a final concentration of $10^7$ cells ml$^{-1}$. PBMC isolated were stimulated with either whole cryptococci or cell envelope components at the concentrations indicated. Control PBMC were incubated with RPMI 1640 without additions. Cryptococci and cell envelope components were incubated with NHS at 37°C for 30 and 60 min, respectively. In addition, whole cryptococci and cell envelope components were incubated with either HI NHS to investigate the effect of complement or IgG-depleted serum to investigate the effect of Ig. A total of $10^7$ *C. neoformans* cells were incubated with $10^6$ PBMC, and cell envelope components in the presence of serum were incubated with $10^6$ PBMC (final volume, 200 μl). Polymyxin B was added in all tests at a final concentration of 10 μg ml$^{-1}$. After being incubated, the samples were centrifuged, and the supernatants were collected and stored at −70°C prior to TNF assays.

**TNF ELISA.** TNF-α production in cell-free supernatants was determined by an enzyme-linked immunosorbent assay (ELISA). Immunoreactive TNF-α analysis was done by an ELISA developed in our laboratory. The MAb used in the ELISA was a purified mouse IgG1 against purified recombinant human TNF-α (MAb T11). For this experiment, 96-well polyvinyl plates (Costar, Cambridge, Mass.) were coated overnight at 4°C with MAb T11 in phosphate-buffered saline. The plates were washed three times with blocking buffer (double-distilled water, 0.05% Tween 20), blocked with 4% bovine serum albumin in blocking buffer for 1 h at 37°C, and washed three times again. Twofold serial dilutions in phosphate-buffered saline of reference recombinant TNF-α (Sigma) ranging from 2,000 to 15,625 pg ml$^{-1}$ were incubated in addition to the samples. The plates were washed three times, and a secondary biotinylated MAb (T4-biotin) was incubated for 1 h at 37°C. Excess MAb was washed off, streptavidin-peroxidase conjugate (Sigma) was added, and the mixture was incubated at 37°C for 1 h; then TMB peroxidase substrate was added, and the reaction was stopped with $H_2SO_4$. The absorbance at 450 nm was measured with a microplate reader (Bio-Rad, Tokyo, Japan). Sample concentrations were read from the dose-response curve obtained with reference recombinant TNF-α. The detection limit of the ELISA was 25 pg ml$^{-1}$; the detection limit was the lowest positive standard.

**ELISA for anticryptococcal MP antibodies.** Wells of microtiter plates (Titertek; Flow Laboratories) were coated overnight at 4°C with 10 μg of MP ml$^{-1}$ in 100 μl of 0.06 M sodium carbonate buffer (pH 9.6). The wells were washed three times with 0.01 M sodium phosphate buffer (pH 7.2) in 0.14 M NaCl and blocked

for 60 min with 200 μl of blocking buffer (4% bovine serum albumin and 0.05% Tween 20 in sodium phosphate buffer) per well. The plates were washed three times and incubated with serial 10-fold dilutions of NHS or IgG-depleted serum beginning with a 1:10 dilution. After a 1-h incubation at 37°C, the plates were washed three times, incubated with peroxidase-labelled secondary antibodies, washed, and finally incubated with 100 μl of TMB peroxidase substrate for 15 min. The reaction was stopped with 1 M $H_2SO_4$, and the absorbance at 450 nm was read with an ELISA reader.

**Statistical analysis.** Student's *t* test was used to assess the differences between means. A $P$ of $<0.05$ was considered statistically significant.

## RESULTS

**Stimulation of PBMC TNF-α release by *C. neoformans* strains.** PBMC isolated from blood of healthy human donors were stimulated with heat-killed *C. neoformans*, and the release of TNF-α was quantified. Polymyxin B (10 μg $ml^{-1}$) was added in all experiments to verify that the observed TNF-α release was due to *C. neoformans* rather than endotoxin contamination (27). In some experiments, LPS from *E. coli* O111:B4 (1 μg $ml^{-1}$) was used as a positive control. Preliminary investigations were performed to establish the working conditions. No significant differences in the TNF-α levels induced were observed when either live, heat-killed, or azide-killed organisms were used in experiments involving up to 6 h of stimulation. Therefore, heat-killed organisms were used to minimize the problem of phagocyte lysis due to *C. neoformans* growth, as well as the potential problem of protease production by live organisms, leading to breakdown of secreted TNF-α. The TNF-α levels induced depended on the percentage of NHS used, the cryptococcus/PBMC ratio used, and the time of stimulation of PBMC with *C. neoformans*. Although long incubation times (>3 h) enhanced the levels of TNF-α, the differences in TNF-α levels induced by different strains remained constant over longer incubations. After 3 h of incubation, a convenient time for our experiments, differences between test and control samples were observable. Thus, the conditions used in subsequent experiments were 10% NHS, a cryptococcus/PBMC ratio of 10:1, and 3 h of stimulation.

Direct stimulation of $10^6$ PBMC (by $10^7$ unopsonized *C. neoformans* cells resulted in the release of basal levels of TNF-α (<80 pg $ml^{-1}$). To investigate the influence of serum, *C. neoformans* strains were incubated with NHS. All strains tested were thinly encapsulated. PBMC released significantly higher TNF-α levels in the presence of NHS-opsonized *C. neoformans* than did PBMC alone ($P < 0.05$) (Fig. 1). Heat inactivation of serum (complement depletion) reduced this effect. The TNF-α levels released by PBMC stimulated with NHS-opsonized *C. neoformans* strains were comparable to the levels evoked by LPS (1 μg $ml^{-1}$) under the same conditions but were not significantly different among the five strains. The biological integrity of the TNF-α was confirmed by bioassay (data not shown).

**TNF-α induction of cryptococcal cell envelope components in PBMC.** Whole cryptococci were able to induce TNF-α production by PBMC. To investigate which envelope components of *C. neoformans* evoke TNF-α in PBMC, graded doses of purified GXM, GalXM, and MP constituents from an acapsular mutant were incubated with PBMC for 3 h and the supernatants were assayed for TNF-α. Incubation of PBMC with nonopsonized cell envelope components resulted only in the release of basal levels of TNF-α (<80 pg $ml^{-1}$). When PBMC were incubated with different concentrations of the cell envelope components in the presence of NHS, TNF-α was elicited by all components and dose-response curves were observed (Fig. 2A).

To establish which component was the most potent TNF-α inducer, we performed experiments in which PBMC were incubated with 25 μg of the various components in the presence of NHS (Fig. 2B). MP2 induced significantly higher levels of TNF-α than did MP1 ($P < 0.05$) and GalXM ($P < 0.01$). In contrast to GXM, the TNF-α induction by MP1, MP2, and GalXM was less dependent on complement (compare Fig. 2B and 3). Incubation of the components MP1, MP2, and GalXM with HI NHS resulted in 48, 71, and 44%, respectively, of the TNF-α values levels observed for NHS. These reductions were significant ($P < 0.01$ for MP1, $P < 0.05$ for MP2, and $P < 0.025$ for GalXM).

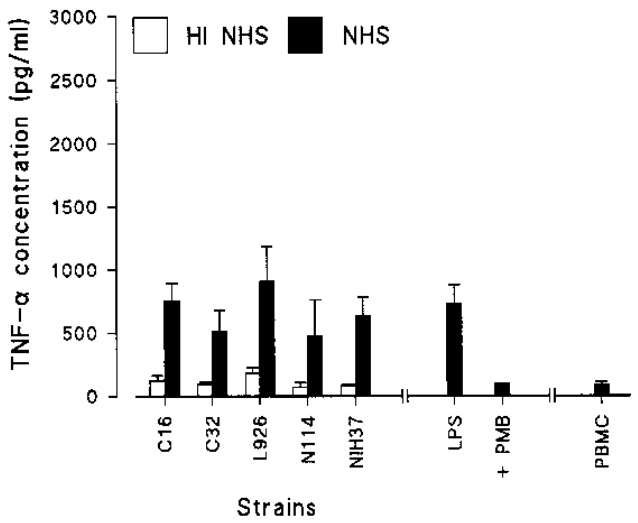


FIG. 1. TNF-α production measured by ELISA in supernatants of PBMC stimulated with different strains of *C. neoformans* and LPS from *E. coli* O111:B4 (1 μg $ml^{-1}$), in the presence of 10% HI NHS or 10% NHS. A total of $10^7$ *C. neoformans* cells were incubated with $10^6$ PBMC for 3 h in the presence of 10 μg of polymyxin $ml^{-1}$ (final concentration). The means ± standard errors of the mean (SEM) of three experiments performed with different donors are shown. NHS enhanced the response of PBMC to stimulation by the different strains. Heat inactivation of complement (56°C for 30 min) reduced this effect. Polymyxin B (PMB) (10 μg $ml^{-1}$) blocked LPS-induced TNF-α release to background levels.

To determine whether the ability of GXM to induce TNF-α depends on the cryptococcal serotype used, GXMs of the four known serotypes were tested (Fig. 3). GXM isolated from strain NIH 37 (serotype A) evoked significantly higher levels of TNF-α in PBMC than did GXM from serotype B (NIH 112), C (NIH 18), D (B3502), or another serotype A strain (NCPF) ($P < 0.01$). As with whole cryptococcal strains, the presence of an intact complement pathway is required for TNF-α induction. Heat inactivation of serum resulted in TNF-α levels not significantly different from the levels observed in nonstimulated PBMC (Fig. 3).

To confirm the influence of complement on the TNF-α induction by GXM, MP1, and MP2, experiments were performed with C3-depleted serum. The results were similar to those obtained with HI NHS: the TNF-α levels induced by the various GXMs were reduced to between 17.5 and 28% of the levels elicited when GXM was incubated with NHS, whereas for MP1 and MP2, 54 and 76% of the NHS-incubated levels still remained.

These results suggest that (i) a heat-labile factor, presumably complement, enhanced the TNF-α response of PBMC to whole cryptococci, GXMs, GalXM, MP1, and MP2, and (ii) a heat-stable factor is involved in mannoprotein-induced TNF-α response by PBMC.

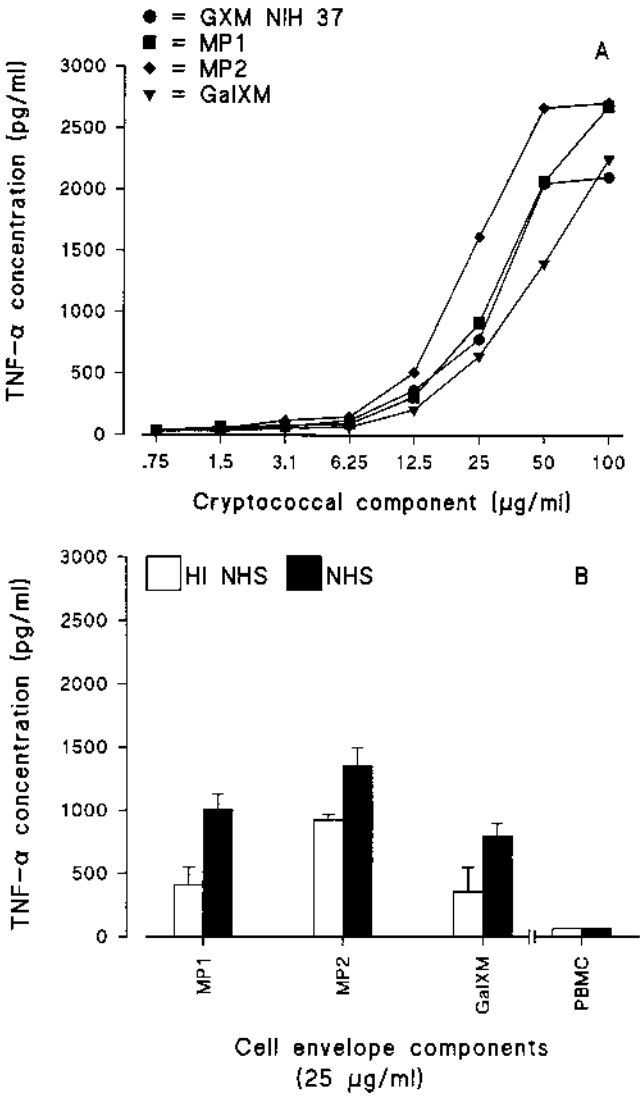


FIG. 2. (A) Dose-response curve of TNF-α production by $10^6$ PBMC stimulated with different concentrations of cell envelope components incubated with NHS. GXM NIH 37 is a capsular polysaccharide isolated from strain NIH 37 (serotype A); MP1, MP2, and GalXM were isolated from the acapsular mutant. Data are from a representative experiment. (B) MP2 induced significantly more TNF-α than did the other cell envelope components. Envelope components were incubated with either 10% NHS or 10% HI NHS. After incubation with PBMC for 3 h, supernatants were collected and TNF-α levels were determined by ELISA. Data are shown as means ± SEMs of six to eight experiments performed with various donors.

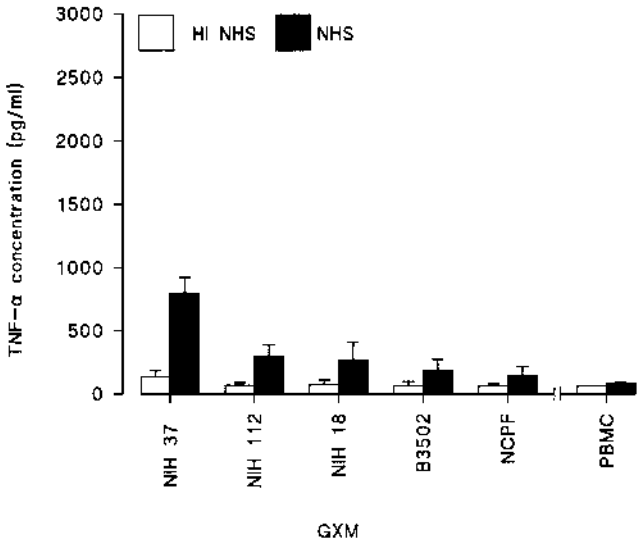


FIG. 3. TNF-α production by $10^6$ PBMC stimulated with capsular polysaccharide (25 μg/ml) of the four known serotypes of *C. neoformans*. GXMs were incubated with either NHS or HI NHS. After a 3-h incubation with PBMC, supernatants were collected and assayed for TNF-α levels by ELISA. The mean ± SEM of six experiments performed with different donors is shown. NIH 37 and NCPF are serotype A, NIH 112 is serotype B, NIH 18 is serotype C, and NIH B3502 is serotype D.

**Influence of HI IgG-depleted serum on TNF-α release by MP1 and MP2 in PBMC.** To investigate whether the heat-stable factor in the serum was antibody, we conducted experiments with HI IgG-depleted serum. MP1 and MP2 were incubated with either 5% NHS, 5% HI NHS, or 5% HI IgG-depleted serum. Only 5% serum was used due to the limited amounts of HI IgG-depleted serum available. This HI IgG-depleted serum contained less than 0.032 mg of antibody $ml^{-1}$ compared to 11 mg $ml^{-1}$ for NHS and HI NHS. Both NHS (and therefore also HI NHS) and HI IgG-depleted serum contained only low levels of anti-MP antibodies (NHS, IgG titer of ≤1:25 and IgM titer of ≤1:200; IgG-depleted serum, IgG and IgM titers of ≤1:25). As in the previous experiments, NHS was the most effective opsonizing agent, and heat inactivation reduced the TNF-α levels induced (Fig. 3B). Incubation with HI IgG-depleted serum, however, still resulted in TNF-α induction by MP1 and MP2 (about 50% of the HI NHS levels) (Fig. 4). This suggests that Ig is not the heat-stable serum factor responsible for the MP1- and MP2-induced TNF-α release in the presence of HI NHS.

**Effect of anti-CD14 MAb on MP2- or LPS-induced TNF-α release by PBMC.** LPS is known to interact with serum LPS-binding protein (LBP) and subsequently with the membrane-bound surface protein CD14 (46). To study if MP2 interacts with CD14, PBMC were preincubated in the presence of either of the two anti-CD14 MAb, 60BCA or 3C10 (10 μg $ml^{-1}$), for 30 min at 4°C. Then the PBMC were stimulated for 3 h with MP2 (25 μg $ml^{-1}$) or LPS (1 μg $ml^{-1}$), supernatants were collected, and TNF-α levels were determined (Fig. 5). The anti-CD14 MAb significantly blocked both MP2-induced and LPS-induced TNF-α release compared to the isotype control MAb (for LPS, 60BCA has $P < 0.025$ and 3C10 has $P < 0.05$; for MP2, both have $P < 0.001$). The two anti-CD14 MAb alone induced basal levels of TNF-α in PBMC (data not shown).

## DISCUSSION

Innate resistance mechanisms may play a role in the clearance of *C. neoformans* during infection. During primary pulmonary *C. neoformans* infections, natural effector cells (polymorphonuclear cells, monocytes, and NK cells) will move to the site of infection in the lungs, resulting in clearance of the microorganism (14, 25). Extensive research has been done on the mechanisms of the binding and phagocytosis of *C. neoformans* by PBMC, macrophages, and polymorphonuclear cells in vitro (see reference 22 for a review). These cells have been shown to kill the yeast (22), but many details of the mechanisms involved remain to be clarified.

Our data demonstrate that whole *C. neoformans* and its cell envelope components GXM, GalXM, and MP stimulate TNF-α release by PBMC. However, differences were observed

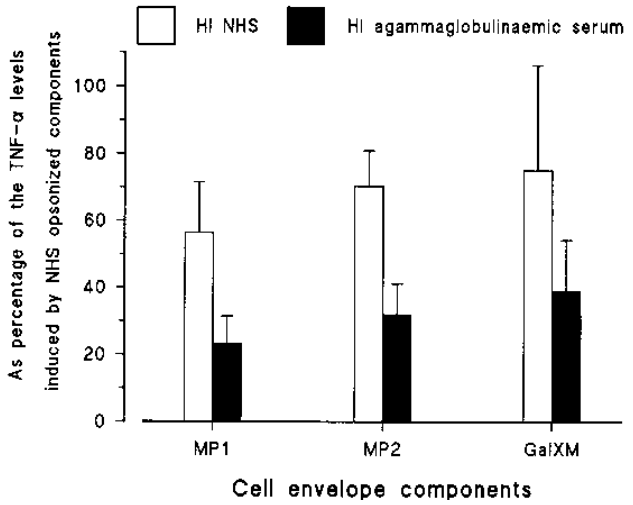


FIG. 4. Incubation of cryptococcal envelope components with heat-inactivated IgG-depleted serum promotes TNF-α release. MP1 and MP2 (25 μg $ml^{-1}$) were each incubated with NHS, HI NHS, or HI IgG-depleted serum. After a 3-h incubation with PBMC, supernatants were collected and assayed for TNF-α content. Results are expressed as percentages of the TNF-α levels induced by the components opsonized by NHS and are shown as the means ± SEMs of three or four experiments performed with different donors. In the supernatants of PBMC in combination with only serum, the following TNF-α levels were detected: 112 ± 4 pg $ml^{-1}$ (NHS), 88 ± 4 pg $ml^{-1}$ (HI-NHS), and 44 ± 4 pg $ml^{-1}$ (HI-agamma serum). This was 10% or less of TNF-α levels induced by the opsonized components.

between their ability to stimulate PBMC and the type of opsonin required. All strains of intact *C. neoformans* tested induced similar levels of TNF-α in PBMC when opsonized by complement-sufficient serum. These results are consistent with the finding of Levitz et al. (26), who demonstrated TNF-α induction by *C. neoformans* (serotypes A and D) in PBMC in the presence of NHS. These authors also showed that the size of the capsule of a strain influences TNF-α induction: high TNF-α release is caused by fungi possessing thin capsules. The observed homogeneity of the TNF-α levels induced by all strains tested might therefore be a consequence of the similarity in encapsulation of these strains, since all strains were thinly encapsulated. After a 3-h incubation, we observed lower TNF-α levels than those observed by Levitz et al. (26) at 18 h.

For several pathogens like *Candida albicans*, *Staphylococcus epidermidis*, and *Mycobacterium tuberculosis*, cell envelope components play a significant role in cytokine induction (1, 2, 13, 15, 19, 27, 38, 42, 47). To our knowledge, this is the first report on the involvement of cell envelope components of *C. neoformans* other than the capsular polysaccharide in the induction of TNF-α (43). We tested GXMs (25 μg $ml^{-1}$) of the four known serotypes for their capacity to induce TNF-α in PBMC. GXM isolated from strain NIH 37 (serotype A) evoked significantly higher TNF-α levels than did the GXMs isolated from the other strains (Fig. 3; $P < 0.01$). This difference cannot be attributed to the difference in serotype, since GXM isolated from strain NCPF was also serotype A. A possible explanation for the different abilities of serotype A GXMs to induce TNF-α in PBMC is difficult to give but might be due to the observed heterogeneity within serotype A GXM (40). However, to elucidate a potential relationship between "certain types" of serotype A GXM and the level of TNF-α induction, a large number of serotype A GXMs extensively characterized for their microheterogeneity have to be tested.

The capacity of whole cryptococci and the isolated GXMs to induce TNF-α is strongly dependent on the presence of complement. Incubation of cryptococci or GXM with HI NHS instead of NHS abrogated their ability to induce TNF-α of PBMC (Fig. 1 and 3), and incubation of GXMs with C3-depleted serum gave the same results. The capsule of *C. neoformans* activates the alternative pathway of human complement, presumably resulting in vast C3b deposition on the organism (10, 16, 23). This would be in line with other observations indicating that cryptococcosis is associated with the depletion of complement (C3) activity (33). The TNF response probably does not depend on C5a, since it has been shown by others that the incubation of the supernatants of *C. neoformans* with human serum does not increase the release of TNF-α (24).

In contrast to our results, Vecchiarelli et al. (43) reported that substantial levels of TNF-α were observed only after 18 h, whereas we detected high levels of TNF-α after only 3 h (Fig. 3). This difference between our group and the results of Vecchiarelli et al. can be ascribed to the different PBMC/cryptococcus ratios used for stimulation. We used a PBMC/cryptococcus ratio of 1:10, whereas Vecchiarelli et al. performed the experiments with a 1:1 ratio. In preliminary experiments, we detected no TNF-α after 3 h with a PBMC/cryptococcus ratio of 1:1 (data not shown). In addition, Levitz et al. demonstrated that at a PBMC/cryptococcus ratio of 1:1, only minimal levels of TNF-α are detected after 18 h of stimulation, and that at a PBMC/cryptococcus ratio of 10:1, no TNF-α levels were measured above background level (26). This suggests that the PBMC/cryptococcus ratio used is critical for TNF-α induction and that small decreases in this ratio have a profound influence on the TNF-α levels induced.

Vecchiarelli et al. showed in the same study that the amount of TNF-α production by monocytes induced either by acapsular *C. neoformans* or LPS could be suppressed by purified GXM (43). In contrast, we demonstrated that GXM itself can induce TNF-α in PBMC (Fig. 3). Although their report suggests that GXM downregulates TNF-α induction by an unknown mechanism on the cellular level, their observation might be explained by the inhibition of phagocytosis of unencapsulated strains as a result of the presence of low concentrations of free GXM. Kozel and coworkers demonstrated that

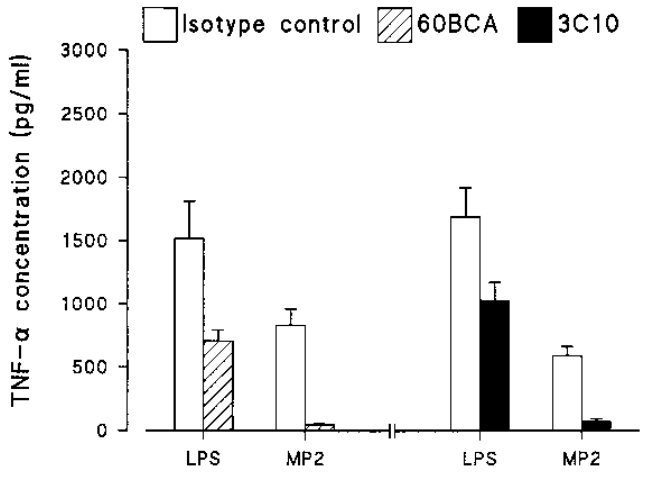


FIG. 5. Influence of blocking CD14 receptors on monocytes on TNF-α induction by MP2 (25 μg $ml^{-1}$) and LPS (1 μg $ml^{-1}$). MAb 60BCA and 3C10 inhibited MP2-induced TNF-α release by PBMC (Fig. 4) in the presence of 10% NHS. Data represent means ± SEMs of four to six experiments as determined by ELISA.

GXM concentrations as low as 0.5 μg/ml have a profound influence on the phagocytosis of nonencapsulated cryptococci (a decrease of 80%) (21, 28). In addition, they showed that the lower the PBMC/cryptococcus ratio, the stronger is the inhibition of phagocytosis of unencapsulated cryptococci by a certain concentration of GXM (21, 28). This inhibition is not based on competition between free GXM and cryptococci for opsonizing antibodies, but GXM directly interferes with Fc-mediated phagocytosis (28). The mechanism for the inhibition of LPS-induced TNF-α by GXM is unknown, but in our opinion, direct interference in the uptake of LPS by monocytes by a process similar to that described for the unencapsulated cryptococci cannot be excluded.

In their experiments, Vecchiarelli et al. used concentrations of GXM as high as 500 μg/ml, and therefore one might expect TNF-α induction by free GXM itself. However, GXM binds to the nonencapsulated cryptococci so that the actual concentrations of free GXM present will be (much) lower (21). Furthermore, GXMs differ in their ability to induce TNF-α in PBMC, with some of them evoking TNF-α levels just above background levels (± 200 pg/ml [Fig. 3]). In addition, Vecchiarelli used the L-cell assay for measuring TNF-α, which detects only the bioactive form, whereas we used an ELISA system (which detects total TNF-α).

The cell envelope components GalXM, MP1, and MP2 induced high levels of TNF-α in PBMC, with MP2 being the most potent inducer, when these components were incubated with NHS. In contrast to whole cryptococci and the isolated GXMs, the ability to induce TNF-α was much less dependent on the presence of an intact complement system. While heat inactivation of NHS gave a TNF-α release by PBMC (on stimulation by *C. neoformans* or GXMs) just above the level of unstimulated PBMC, 40 to 70% of the original TNF-α remained when MP1, MP2, or GalXM was used (Fig. 1 to 3). This suggests that a heat-stable factor is involved in enhancing TNF-α release by PBMC in response to these cell envelope components. Antibodies against MP have been found in the sera of normal individuals (17, 20, 36), and IgG-containing immune complexes can cross-link Fc receptors on the surface of monocytes and induce TNF-α secretion (25). However, only very low levels of IgG antibodies and somewhat higher levels of IgM antibodies against mannoproteins were detected in the NHS and HI NHS pool. Heat-inactivated IgG-depleted serum, which contained the same amount of anti-MP IgG antibodies as the HI NHS pool, induced about half of the TNF-α as that evoked by the components that were opsonized with HI NHS (Fig. 4). Currently there are no known receptors on human mononuclear cells capable of interacting with IgM antibodies (41). Our results indicate that the heat-stable factor in MP1- and MP2-enhanced TNF-α release by PBMC is not Ig.

The mannoproteins of *C. neoformans* are immunogenic in rabbits and are involved in delayed-type hypersensitivity responses observed with a mixture of antigens obtained from culture filtrates (30). Our study demonstrates that one of the mannoprotein fractions is the most potent inducer of TNF-α in PBMC. In this regard, cryptococcal mannoproteins might be comparable to the well-studied mannoprotein of *Candida albicans*. Candidal MP has been shown to induce TNF-α in experiments with mouse splenic macrophages (36). In addition, these components act as recall antigens in lymphoproliferation and the production of gamma interferon (1, 19). Therefore, cryptococcal mannoproteins might possess similar properties and, as a consequence, play an important role in the host defense against *C. neoformans* infection.

MP2 and LPS both stimulated PBMC to release TNF-α in the presence of NHS. Since the TNF-α response to LPS involves, among others, LBP-mediated binding to CD14 (43, 45), we investigated whether CD14 plays a role in TNF-α induction by MP2. To this end, we studied modulation of MP2-dependent TNF-α release by two anti-CD14 MAb, 60BCA and 3C10. TNF-α release induced by MP2 was significantly reduced by both MAb compared to the isotype controls ($P < 0.001$ [Fig. 4]). At present, it is not known whether this mechanism involves binding of LBP to MP. Moreover, other serum factors such as human mannose-binding protein may be interesting candidates in our MP-induced TNF-α. These are subjects of our current investigations. GalXM showed a close resemblance to MPs in inducing TNF-α, and in the future it might be useful to investigate the mechanism involved.

The clinical relevance of our findings remains speculative. We did not observe clinical deterioration in our patients upon starting treatment with amphotericin B, as can be seen, for instance, in pneumococcal meningitis and subsequent cell wall release upon treatment with penicillin. HIV-infected patients with cryptococcal meningitis need lifelong suppressive therapy because complete eradication of the fungus in the setting of profound immunosuppression is not possible. Therefore, there is a need to devise effective procedures for immunoreplacement or immunomodulatory therapy along with antifungal drugs to eradicate an ongoing infection. Appropriate immunotherapeutic protocols will not be developed without an understanding of the anticryptococcal host response mechanisms including the cells and cytokines involved.

In summary, our findings demonstrate that *C. neoformans* and the isolated envelope components GXM, MP1, MP2, and GalXM can stimulate TNF-α release from leukocytes in the presence of complement-sufficient serum. The TNF-α induction by MP1, MP2, and GalXM, however, is much less dependent on complement than is induction by whole *C. neoformans*, and for MP1 and MP2, a heat-stable factor is involved that is not Ig. In addition, we have found preliminary evidence that the CD14 receptor might play a role in MP-induced TNF-α secretion.

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