Capsular Polysaccharide of *Cryptococcus neoformans* Induces Proinflammatory Cytokine Release by Human Neutrophils

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Human polymorphonuclear leukocytes from normal subjects produced proinflammatory cytokines in response to stimulation with *Cryptococcus neoformans* yeast cells. The cytokines released after stimulation of neutrophils included interleukin-1 β (IL-1 β), IL-6, IL-8, and tumor necrosis factor alpha. The magnitude of the cytokine response was related to the yeast capsule size. Cells of a large-capsule isolate stimulated release of greater amounts of cytokine than did a thinly encapsulated isolate, which, in turn, stimulated release of greater amounts of cytokine than an acapsular isolate. Cytokine release was also stimulated by supernatant fluids from cryptococcal cells that were preincubated with 10% human serum, suggesting the generation of a soluble mediator. The major capsular polysaccharide, glucuronoxylomannan, stimulated release of tumor necrosis factor alpha, IL-1 β , and IL-8 in a dose-dependent fashion. These results differ from previous studies of cytokine secretion by human monocytes in several important respects, including the importance of encapsulation in stimulation of cytokine secretion and the ability of purified glucuronoxylomannan to induce cytokine secretion.

Cryptococcus neoformans is a ubiquitous yeast that has generated recent interest as a result of the increased incidence of cryptococcosis in the immunocompromised host (10, 20). Studies to date suggest that initial exposure occurs through the respiratory tract, and the fungus rapidly reaches the lung, where alveolar macrophages are an initial barrier that act in concert with locally recruited T lymphocytes. Studies of murine cryptococcosis indicate that CD4⁺ and CD8⁺ T cells are both critical in limiting infection and avoiding hematogenous dissemination (14).

Studies with experimental animal models or with human cells also suggest a critical role for natural effector cells such as natural killer cells (11, 12, 27), macrophages, and polymorphonuclear cells (25). An important role for phagocytic cells in host defense is suggested by the histopathological response to tissue invasion by *C. neoformans* that is characterized by the presence of polymorphonuclear leukocytes (PMN) and mononuclear cells (13).

The polysaccharide capsule is essential for virulence (17, 19, 24, 26), and the capability of phagocytic cells to clear *C. neoformans* in vitro is inversely related to the size of the capsule (25). Capsule-mediated inhibition of phagocytosis leads to impairment of antigen processing and presentation by macrophages, with a consequent reduction in the T-cell response (33). A potential role for the major capsular polysaccharide, glucuronoxylomannan (GXM), in immune regulation is through regulation of cytokine production. This is an important point because the majority of patients with cryptococcosis have soluble GXM in their body fluids. Our recent studies show that capsular polysaccharide downregulates interleukin-1 β (IL-1 β) and tumor necrosis factor alpha (TNF- α) production by human monocytes (34). The present study was un-

dertaken to examine the cytokine response of human PMN to *C. neoformans* or its purified capsular material. Our results show that cryptococcal cells and purified GXM induce proinflammatory cytokine production by PMN in the presence of human serum (HS).

MATERIALS AND METHODS

***C. neoformans* and cryptococcal polysaccharide.** The following strains of *C. neoformans* were obtained from J. Orendi: a *C. neoformans* var. *neoformans* serotype A thinly encapsulated strain (CBS 6995 = NIH 37; National Institutes of Health, Bethesda, Md.), a *C. neoformans* var. *neoformans* acapsular mutant (CBS 7698 = NIH B-4131), and a heavy encapsulated isolate of serotype A (NCPF 3168; National Collection of Pathogenic Fungi, London, England). The cultures were maintained by serial passage on Sabouraud agar (BioMérieux, Lyon, France) and harvested by suspending a single colony in RPMI 1640; the cells were washed twice, counted on a hemacytometer, and adjusted to the desired concentration. *C. neoformans* (CBS 6995, CBS 7698, and NCPF 3168) strains were inactivated by autoclaving. GXM was isolated from a serotype A isolate (ATCC 24064) by differential precipitation with ethanol and cetyltrimethylammonium bromide (6).

Reagents. RPMI 1640 and fetal bovine serum were obtained from Eurobio Laboratories (Paris, France). Triton X-100 was purchased from Sigma Chemical Co. (St. Louis, Mo.). HS was obtained from Biosource International (Camarillo, Calif.). Lipopolysaccharide (LPS) from *Escherichia coli* O55:135 was obtained from Difco Laboratories (Detroit, Mich.). The RPMI 1640, fetal bovine serum, HS, and *C. neoformans* cells (approximately 5×10^6) were tested for endotoxin contamination by *Limulus* amoebocyte lysate (LAL) assay (Sigma), which had a sensitivity of approximately 0.05 to 0.1 ng of *E. coli* LPS per ml. All reagents tested negative. The GXM was not examined by LAL because GXM preparations routinely test positive in LAL assays, even GXM prepared under stringent conditions designed to minimize or eliminate LPS contamination, suggesting that contamination of GXM with endotoxin cannot be accurately assessed by LAL (15a).

Preparation of polymorphonuclear cells and monocytes. Heparinized venous blood, obtained from healthy donors, was diluted with RPMI 1640. Then, mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation (32). The cells at the interface were washed twice in RPMI 1640, placed into cell culture petri dishes (Nunc Inter Med, Roskilde, Denmark) at a concentration of 2×10^6 to 3×10^6 /ml in RPMI 1640 supplemented with 5% fetal calf serum, and incubated at 37°C in 5% CO₂ for 1 h. The nonadherent cells were removed by washing the dishes three to five times with warm RPMI 1640. Adherent cells were carefully recovered with a rubber policeman. The harvested cells were 95 to 98% esterase positive and >98% viable as evaluated by trypan blue dye exclusion test. The pellet containing polymorphonuclear cells and erythrocytes was treated with hypotonic saline to lyse the erythrocytes (36). The granulocytes were col-

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FIG. 1. Time course of IL-1 β secretion by PMN that were nonstimulated (NS) or stimulated in vitro with LPS (10 μ g/ml) or with acapsular (CBS 7698), thinly encapsulated (CBS 6995), or heavily encapsulated (NCPF 3168) *C. neoformans* (E/T ratios = 1:1 [A] and 1:5 [B]). The results are expressed as the means $\pm$ standard deviations of four separate experiments with cells from four different donors. Differences in cytokine levels among various strains of *C. neoformans* (CBS 7698 versus CBS 6995 or NCPF 3168; CBS 6995 versus CBS 7698 or NCPF 3168; NCPF 3168 versus CBS 7698 or CBS 6995) after 18 h of incubation were significant ($P < 0.01$).

lected by centrifugation, washed twice in RPMI 1640, counted, and adjusted to the desired concentration. The purity of PMN isolated by this method was always greater than 98%, as determined by Giemsa staining. PMN preparations contained <1% monocytes. Cell viability in all determinations after 3, 6, and 18 h of culture was >98% for PMN as assessed by trypan blue exclusion.

Production of PMN culture supernatants. Isolated PMN were distributed in 1-ml volumes into 24-well flat-bottom tissue culture plates (Falcon; Becton Dickinson, Oxnard, Calif.) at 4×10^6 cells per ml and incubated for 3, 6, and 18 h at 37°C under a 5% CO₂ atmosphere with RPMI 1640 containing 10% HS and either (i) LPS (10 μ g/ml), (ii) heat-inactivated *C. neoformans* NCPF 3168, CBS 6995, or CBS 7698 at effector-to-target (E/T) ratios of 1:1 or 1:5, (iii) GXM (250, 25, or 0.25 μ g/ml), or (iv) buffer alone. Supernatant fluids were harvested at the indicated time intervals and stored at -20°C until assayed.

Cytokine determination. Cytokine levels in culture supernatant fluids were measured with enzyme-linked immunosorbent assay kits for human IL-1 β (Genzyme, Boston, Mass.), human IL-8, and TNF- α (Amersham). IL-6 was determined in a bioassay which assessed the ability to support the growth of a B-cell hybridoma as described by Van Snick et al. (31) with minor modifications. Briefly, B-cell hybridoma 7TD1 cells were incubated with serial dilutions of cell supernatant fluids in microtiter plates. After 4 days of incubation, the number of cells was evaluated by a colorimetric method. A 5-mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) was added to produce a final concentration of 10%. After 3 h of incubation, the supernatant fluids were discarded, and 0.2 ml of dimethyl sulfoxide (Sigma) was added to each well to solubilize the reduced MTT precipitate. After homogenization, the optical density was read in a multiwell spectrophotometer at 570 nm. All analyses were performed in duplicate, and concentrations were calculated by a probit analysis in comparison with a standard of recombinant human IL-6 (Sigma). The results were expressed in units per milliliter. The specificity of the test was controlled by inhibition of cell proliferation after the addition of a neutralizing anti-IL-6 rabbit antibody (Genzyme). In all cases, addition of specific antibody to PMN supernatants reduced cytokine-stimulated growth of the 7TD1 cell line by greater than 90%.

Statistical analysis. Statistical analysis was performed with Student's *t* test.

RESULTS

An initial experiment was done to evaluate secretion of IL-1 β after challenge of PMN with acapsular *C. neoformans* or *C. neoformans* with small or large capsules. The results (Fig. 1) show that *C. neoformans* induces IL-1 β secretion from PMN.

Appreciable levels were detected after 6 h of incubation, with greater levels noted after 18 h. The amount of IL-1 β detected depended on the size of the capsule. The greatest amount of secretion was observed after stimulation by the heavily encapsulated strain NCPF 3168, followed in order by the thinly encapsulated strain CBS 6995 and the acapsular strain CBS 7698.

We next determined whether PMN were able to produce IL-8, a major chemoattractant for neutrophils, in response to *Cryptococcus* challenge. IL-8 was detected in supernatant fluids of PMN cocultured for various incubation times with LPS or *C. neoformans*. The results (Fig. 2) show that large amounts of this cytokine were found after 18 h of incubation. Moreover, the amount of IL-8 production paralleled the quantity of capsular material present on the challenge yeast cells; maximum secretion was observed after challenge with the heavily encapsulated strain NCPF 3168.

The ability of PMN to produce IL-6 is controversial; several reports indicate that IL-6 is secreted by PMN (22, 23, 28), but other studies note an absence of IL-6 secretion by PMN (1, 15, 35). We examined IL-6 production by PMN in response to LPS or *C. neoformans*. The results (Fig. 3) show that IL-6 secretion was observed after 3 h of incubation, with increased synthesis noted after 6 and 18 h. Secretion was greatest in response to the encapsulated strains compared with that to the acapsular mutant CBS 7698.

TNF- α , with IL-8, IL-1 β , and IL-6, is an important proinflammatory cytokine that is secreted by PMN and has been associated with neutrophil recruitment, septic shock, and inflammatory disease. These cytokines also play a critical role in regulating many PMN functions, including antimicrobial activity (9). Consequently, it was of interest to ascertain whether TNF- α production by PMN is stimulated by *C. neoformans*. The results (Fig. 4) show that an appreciable level of TNF- α

FIG. 2. Time course of IL-8 secretion by PMN that were nonstimulated (NS) or stimulated in vitro with LPS (10 µg/ml) or with acapsular (CBS 7698), thinly encapsulated (CBS 6995), or heavily encapsulated (NCPF 3168) *C. neoformans* (E/T ratios = 1:1 [A] and 1:5 [B]). The data are expressed as the means $\pm$ standard deviations of three separate experiments with cells from three different donors. Differences in cytokine levels among various strains of *C. neoformans* (CBS 7698 versus CBS 6995 or NCPF 3168; CBS 6995 versus CBS 7698 or NCPF 3168; NCPF 3168 versus CBS 7698 or CBS 6995) after 18 h of incubation were significant ($P < 0.01$).

was detected 6 h after incubation with LPS and all cryptococcal cells and continued to rise up to 18 h. As with IL-1 β , IL-6, and IL-8, the heavily encapsulated strain induced more TNF- α secretion than weakly encapsulated or acapsular cryptococci. Microscopic examination of the cells after incubation for 1 h showed that acapsular yeast cells were observed primarily intracellularly, whereas encapsulated yeasts were rarely observed within PMN (data not shown). Thus, the ability to induce cytokine secretion was independent of ingestion of the yeast cells.

Enhanced stimulation of cytokine secretion by heavily encapsulated cryptococci suggested that the major capsular polysaccharide, GXM, might play a role in induction of cytokine secretion. As a consequence, an experiment was done to assess stimulation by purified GXM of PMN production of TNF- α , IL-1 β , and IL-8. The results (Table 1) showed that purified capsular material is able to induce proinflammatory cytokine

secretion in a dose-dependent manner. In an attempt to determine whether contamination of the PMN preparation with monocytes could influence the observed results, we performed experiments in which various amounts of monocytes (4×10^2 /ml to 4×10^4 /ml) were added to PMN culture. In our experimental conditions, even the higher dose of monocytes (as reported in Table 1) was not able to affect cytokine secretion by PMN in response to *C. neoformans*.

The ability of the GXM preparation to induce cytokine secretion might be due to the presence of contaminating LPS. This possibility was considered in an experiment in which stimulation of cytokine secretion by GXM and LPS was examined in the presence and absence of polymyxin B. The results (Table 2) showed that the addition of polymyxin B completely abrogated LPS-induced cytokine production. In contrast, polymyxin B did not affect cytokine production in response to GXM.

TABLE 1. Cytokine production by PMN in response to purified GXM

Cells	In vitro stimulus (concn) ^a	Cytokine production ^b		
		TNF- α (pg/ml)	IL-1 β (pg/ml)	IL-8 (ng/ml)
PMN		2.2 $\pm$ 0.1	21.2 $\pm$ 3.8	30.2 $\pm$ 5.4
	LPS (10 µg/ml)	191.7 $\pm$ 9.1	397.5 $\pm$ 35.2	245.3 $\pm$ 29.1
	GXM (250 µg/ml)	98.1 $\pm$ 5.9*	382.4 $\pm$ 30.1*	220.1 $\pm$ 33.5*
	GXM (25 µg/ml)	37.3 $\pm$ 2.8*	107.2 $\pm$ 12.5*	124.5 $\pm$ 10.1*
	GXM (0.25 µg/ml)	6.0 $\pm$ 1.2	18.3 $\pm$ 0.9	40.3 $\pm$ 8.9
PMN + monocytes (4×10^4 /ml)		2.0 $\pm$ 0.8	20.3 $\pm$ 2.9	31.9 $\pm$ 6.5
	GXM (250 µg/ml)	105.3 $\pm$ 6.9*	390.1 $\pm$ 35.3*	225.1 $\pm$ 29.1*

^a GXM was added to PMN at the indicated doses for 18 h.

^b Cytokines were tested in culture supernatant fluids. The data represent the means $\pm$ standard deviations of four separate experiments from four different donors. Monocytes (4×10^4 /ml) cultured alone in the absence or in the presence of GXM (250 µg/ml) did not produce appreciable levels of TNF- α , IL-1 β , or IL-8. *, $P < 0.01$ (GXM-treated versus untreated cells).

FIG. 3. Time course of IL-6 production by PMN stimulated in vitro with LPS (10 μ g/ml) or acapsular (CBS 7698), thinly encapsulated (CBS 6995), or heavily encapsulated (NCPF 3168) *C. neoformans* (E/T ratios = 1:1 [A] and 1:5 [B]). The results are expressed as the means $\pm$ standard deviations of four separate experiments with cells from four different donors. Differences in cytokine levels among various strains of *C. neoformans* (CBS 7698 versus CBS 6995 or NCPF 3168) after 6 or 18 h of incubation were significant ($P < 0.01$).

Previous studies found that purified GXM will bind to the surface of acapsular cryptococci to produce a GXM-coated yeast that resists phagocytosis in a manner similar to the limited phagocytosis noted with fully encapsulated cryptococci (3, 16, 18, 30). As a consequence, two experiments were done to determine the effect of purified GXM on the cytokine-inducing properties of the acapsular isolate CBS 7698. In the first experiment, isolated PMN were incubated for 18 h with cells of strain CBS 7698 in the absence of GXM or in the presence of GXM at 250, 25, or 0.25 μ g/ml. The results (Table 3) showed enhanced production of TNF- α , IL-1 β , and IL-8 relative to the stimulation observed with acapsular cryptococci alone. However, the level of stimulation achieved by treatment with GXM plus acapsular cryptococci (Table 3) was not greatly different than the level of stimulation observed with GXM alone (Table 1). This result suggested that adhesion of GXM to acapsular cryptococci per se was not a major factor in stimulation of cytokine production by GXM. This interpretation of the results was supported by an experiment in which acapsular cryptococci

were first incubated with various doses of GXM, washed to remove nonadherent GXM, and then added to PMN cultures. The results (Table 4) showed enhanced cytokine production by acapsular cryptococci pretreated with 25 μ g of GXM relative to that of untreated acapsular cryptococci or acapsular cryptococci pretreated with 0.25 μ g of GXM. However, there was only a minimal difference in cytokine production by acapsular cryptococci pretreated with either 25 or 250 μ g of GXM, suggesting that there was a threshold on the level of stimulation that could be achieved by pretreatment of acapsular cryptococci with GXM.

Encapsulated cryptococci are potent activators of the complement system. It is possible that cytokine secretion is stimulated by cleavage fragments of C3 or C5 which are produced as a consequence of incubation of the yeast cells in the presence of 10% HS. An initial experiment was done to consider this possibility. Encapsulated cryptococci (isolate CBS 6995 or NCPF 3168) were preincubated with RPMI 1640 containing 10% HS. The supernatant fluids were collected and filtered to

TABLE 2. Effect of polymyxin B addition on cytokine production by PMN treated with GXM

In vitro stimulus (concn) ^a	Polymyxin B addition	Cytokine production ^b		
		TNF- α (pg/ml)	IL-1 β (pg/ml)	IL-8 (ng/ml)
None	—	3.0 $\pm$ 0.8	18.3 $\pm$ 4.0	28.3 $\pm$ 4.0
None	+	2.9 $\pm$ 0.6	16.5 $\pm$ 3.5	22.5 $\pm$ 3.9
LPS (10 μ g/ml)	—	188.3 $\pm$ 10.2	369.3 $\pm$ 28.4	261.2 $\pm$ 20.9
LPS (10 μ g/ml)	+	2.3 $\pm$ 0.9*	29.1 $\pm$ 3.9*	35.3 $\pm$ 4.8*
GXM (250 μ g/ml)	—	90.3 $\pm$ 6.0	370.1 $\pm$ 35.3	235.3 $\pm$ 38.4
GXM (250 μ g/ml)	+	92.5 $\pm$ 4.9	358.2 $\pm$ 38.1	225.2 $\pm$ 29.2

^a GXM in the presence or absence of polymyxin B (50 μ g/ml) was added to PMN for 18 h.

^b Cytokines were tested in culture supernatant fluids. The data represent the means $\pm$ standard deviations of four separate experiments from four different donors.

*, $P < 0.01$ (polymyxin B-treated versus untreated cells).

FIG. 4. Time course of TNF- α production by PMN stimulated in vitro with LPS (10 μ g/ml) or acapsular (CBS 7698), thinly encapsulated (CBS 6995), or heavily encapsulated (NCPF 3168) *C. neoformans* (E/T ratios = 1:1 [A] and 1:5 [B]). TNF- α produced by unstimulated cells resulted in <5.5 pg/ml in all determinations performed. The results are expressed as the means $\pm$ standard deviations of four separate experiments with cells from four different donors. Differences in cytokine levels among various strains of *C. neoformans* (CBS 7698 versus CBS 6995 or NCPF 3168; CBS 6995 versus CBS 7698 or NCPF 3168; NCPF 3168 versus CBS 7698 or CBS 6995) after 18 h of incubation were significant ($P < 0.01$).

remove any remaining yeasts, and the yeast cells were collected and washed twice with RPMI 1640. The supernatant fluids or treated yeast cells obtained under the conditions described above were added to PMN for 18 h, and the culture supernatant fluids were recovered and tested for cytokine production. The results (Table 5) show that preincubation of encapsulated cryptococci with 10% HS releases products which induce secretion of TNF- α , IL-1 β , and IL-8 by PMN. In contrast, direct stimulation by yeast cells in the absence of human serum leads to only a modest induction of proinflammatory cytokines. In addition, when heat-inactivated (56°C for 30 min) HS was used, the production of proinflammatory cytokines by PMN in response to GXM was drastically reduced (data not shown).

TABLE 3. Cytokine production by PMN in response to acapsular strain CBS 7698 incubated with purified GXM

In vitro stimulus (concn) ^a	Cytokine production ^b		
	TNF- α (pg/ml)	IL-1 β (pg/ml)	IL-8 (ng/ml)
None	3.1 $\pm$ 0.8	20.2 $\pm$ 2.9	25.3 $\pm$ 4.1
LPS (10 μ g/ml)	189.3 $\pm$ 10.3	379.5 $\pm$ 40.1	198.5 $\pm$ 5.1
CBS 7698 alone	35.4 $\pm$ 5.2	70.1 $\pm$ 5.3	105.5 $\pm$ 10.1
CBS 7698 + GXM at:			
250 μ g/ml	171.6 $\pm$ 9.3*	220.1 $\pm$ 7.0*	290.3 $\pm$ 21.2*
25 μ g/ml	51.9 $\pm$ 4.6*	70.3 $\pm$ 7.0*	135.5 $\pm$ 10.1*
0.25 μ g/ml	35.4 $\pm$ 1.3	18.4 $\pm$ 2.5	98.5 $\pm$ 8.9

^a GXM was added to PMN at the indicated doses at the time of addition of acapsular strain CBS 7698. Cells of CBS 7698 were added at an E/T ratio of 1:1.

^b Cytokines were tested in culture supernatant fluids after 18 h of incubation. The data represent the means $\pm$ standard deviations of four separate experiments from four different donors. *, $P < 0.01$ (CBS 7698 + GXM-treated cells versus CBS 7698-treated cells).

DISCUSSION

The results reported here establish that PMN from normal subjects are able to produce proinflammatory cytokines in response to stimulation by *C. neoformans*. Regarding the ability of neutrophils to produce cytokines, our data are strongly supported by others (4, 5, 23, 28, 36). The cytokines released after stimulation of PMN included TNF- α , IL-1 β , IL-6, and IL-8. The magnitude of the cytokine response was related to the capsule size. Cells of a large-capsule isolate stimulated release of greater amounts of cytokine than did cells of a thinly encapsulated isolate, which, in turn, stimulated release of greater amounts of cytokine than an acapsular isolate. These results suggested that the capsular polysaccharide GXM was an important factor in stimulation of cytokine release. The importance of GXM as a stimulator of cytokine release was demonstrated when purified GXM was found to induce release of TNF- α , IL-1 β , and IL-8 in a dose-dependent fashion. The ability of purified GXM to stimulate cytokine release was not due to contamination of the GXM preparation with LPS, because treatment of the GXM with polymyxin B had no effect on cytokine release but the same treatment completely blocked LPS-induced cytokine release from PMN.

In a previous report, we demonstrated that human monocytes released TNF- α and/or IL-1 β after stimulation with *C. neoformans* yeast cells and that GXM is a potent inhibitor of cytokine secretion (34). The pattern of stimulation by cryptococcal cells of cytokine secretion from monocytes differed in several important respects from the pattern of cytokine secretion from PMN. First, acapsular cells were better stimulators of cytokine secretion from human monocytes than were encapsulated cells. In contrast, large-capsule isolates were the strongest stimulators of cytokine release by PMN. Second,

TABLE 4. Cytokine production by PMN in response to acapsular strain CBS 7698 treated with purified GXM and then washed to remove nonbound GXM

In vitro stimulus (concn) ^a	Cytokine production ^b		
	TNF- α (pg/ml)	IL-1 β (pg/ml)	IL-8 (ng/ml)
None	4.0 $\pm$ 0.9	22.5 $\pm$ 5.2	29.5 $\pm$ 3.0
LPS (10 μ g/ml)	178.1 $\pm$ 15.3	384.3 $\pm$ 20.4	210.1 $\pm$ 30.4
CBS 7698 alone	30.2 $\pm$ 2.8	78.3 $\pm$ 8.0	116.2 $\pm$ 4.4
CBS 7698 + GXM at:			
250 μ g/ml	50.5 $\pm$ 7.0	115.3 $\pm$ 4.0	172.3 $\pm$ 3.8
25 μ g/ml	47.5 $\pm$ 3.9	98.3 $\pm$ 3.8	150.5 $\pm$ 2.9
0.25 μ g/ml	29.1 $\pm$ 4.0	77.3 $\pm$ 5.0	110.1 $\pm$ 9.5

^a Cells of acapsular strain CBS 7698 were incubated with various doses of GXM for 2 h at room temperature, and then the cells were washed twice to remove nonadherent GXM and added to PMN cultures. Cells of strain CBS 7698 were added to PMN cultures at a ratio of 1:1. Control cultures of PMN incubated with CBS 7698 cells plus GXM under the same conditions reported above induced cytokine levels similar to those described in Table 3.

^b Cytokines were tested in culture supernatant fluids after 18 h of incubation. The data represent the means $\pm$ standard deviations of four separate experiments from four different donors.

there is a strong likelihood that phagocytosis plays a significant role in stimulation by cryptococcal cells of cytokine secretion from human monocytes but not from PMN. In the case with human monocytes, acapsular yeast cells, which are typically ingested by phagocytes, were stronger stimulators of cytokine secretion than encapsulated yeast cells, which are typically resistant to phagocytosis (2, 16). PMN, in contrast, were strongly stimulated by encapsulated yeast cells. Acapsular cells were less active in stimulation of PMN cytokine secretion, but the level of stimulation was increased by pretreatment of acapsular cells with GXM, a procedure which renders the yeast cells relatively resistant to phagocytosis (3, 16). The third difference is the effect of purified GXM on cytokine secretion. In the present study, purified GXM stimulated secretion of TNF- α , IL-1 β , and IL-8 by PMN. In contrast, purified GXM failed to induce secretion of detectable levels of TNF- α or IL-1 β by human monocytes. Indeed, the addition of purified GXM to cell cultures suppressed, in a dose-dependent manner, secretion of TNF- α and IL-1 β by human monocytes in response to stimulation with LPS.

We considered the possibility that the results from the study of PMN were masked by or due to cytokine secretion from contaminating monocytes. Two aspects of our study of PMN cytokine secretion suggest that this is not the case. First, the addition of limited numbers of monocytes to the PMN preparation did not produce quantitative or qualitative alterations in cytokine secretion after stimulation by GXM. Second, the distinctly different responses of monocytes and PMN to stimulation by encapsulated and acapsular cryptococci and by purified GXM argue that the PMN response is not due to contaminating monocytes.

The profile of PMN cytokine secretion in response to *C. neoformans* suggests an autocrine-paracrine network regulation of secretory activity. For example, TNF- α , which appears early in supernatant fluids (Fig. 4), could influence production of IL-1 β that is somewhat delayed in its release (Fig. 1). Our results are largely in agreement with a previous report by Cassatella et al. (5), which described the kinetics for release of IL-1 β and IL-8 after stimulation of PMN with LPS. IL-8 is an attractant or activating factor for PMN originally isolated from monocytes (29). Convincing evidence exists for IL-8 production by neutrophils (5, 36). Our results differ slightly from those of Cassatella et al. with regard to the kinetics of TNF- α secre-

TABLE 5. Cytokine production by PMN in response to cells of strains CBS 6995 or NCPF 3168 or to supernatant fluid from CBS 6995 or NCPF 3168 cells after incubation with RPMI 1640 plus 10% HS

In vitro stimulus ^a	Cytokine production ^b		
	TNF- α (pg/ml)	IL-1 β (pg/ml)	IL-8 (ng/ml)
None	3.5 $\pm$ 0.8	25.1 $\pm$ 4.0	30.2 $\pm$ 5.0
LPS	194.1 $\pm$ 16.8	371.3 $\pm$ 30.1	200.1 $\pm$ 10.9
CBS 6995	105.1 $\pm$ 14.3	135.3 $\pm$ 14.2	179.1 $\pm$ 4.3
NCPF 3168	148.3 $\pm$ 18.1	194.5 $\pm$ 20.0	190.5 $\pm$ 12.1
CBS 6995w	10.1 $\pm$ 5.4	35.1 $\pm$ 4.0	70.1 $\pm$ 5.3
NCPF 3168w	28.3 $\pm$ 4.5	41.2 $\pm$ 3.9	85.5 $\pm$ 4.2
sup. CBS 6995	93.1 $\pm$ 10.4*	100.1 $\pm$ 12.3*	168.3 $\pm$ 13.2*
sup. NCPF 3168	112.3 $\pm$ 11.2**	145.3 $\pm$ 14.1**	185.3 $\pm$ 14.0**

^a Cells of strain CBS 6995 or NCPF 3168 were added to PMN culture at an E/T ratio of 1:1 and incubated in RPMI 1640 plus 10% HS for 30 min at 37°C. The supernatant fluid was collected and filtered to remove any remaining yeast cells. The yeast cells (strain CBS 6995 or NCPF 3168) were collected and washed twice with RPMI 1640. Supernatant fluids (sup. CBS 6995 or sup. NCPF 3168) and washed yeast cells (CBS 6995w or NCPF 3168w) were added to PMN in RPMI 1640 without HS and incubated for 18 h at 37°C in 5% CO₂, and the supernatant fluids were recovered and tested for cytokine activity. LPS was added to PMN at a concentration of 10 μ g/ml.

^b The data represent the means $\pm$ standard deviations of four separate experiments from four different donors. *, $P < 0.01$ (PMN treated with supernatant fluid of CBS 6995 [sup. CBS 6995] versus CBS 6995-washed [CBS 6995w] treated cells). **, $P < 0.01$ (PMN treated with supernatant fluid of NCPF 3168 [sup. NCPF 3168] versus NCPF 3168-washed [NCPF 3168w] treated cells).

tion. We found an increase in the amount of TNF- α between the 6- and 18-h incubation periods. Cassatella et al. reported a decrease in the amount of LPS-induced TNF- α in supernatant fluids during the same interval (5). This difference may be due to the higher doses of LPS used in our study, which could prevent a drop in levels of secreted TNF- α . The copious amount of IL-8 released by *C. neoformans*-primed PMN could be the result of two distinct phases in a manner similar to that described by Cassatella et al. for stimulation of IL-8 by LPS (5). In this case, the first phase, which would be a consequence of early interaction between *C. neoformans* and PMN, would account for the early small amount of IL-8 production. The later, more abundant production would be the result of paracrine regulation by endogenous TNF- α and/or IL-1 β .

The molecular and/or cellular basis for induction of PMN cytokine secretion by cryptococcal cells and GXM is not known at this time. Our initial experiments in this regard raise the possibility that cytokine secretion is a consequence of activation of the complement system. Secretion of TNF- α , IL-1 β , and IL-8 was observed after treatment of PMN with supernatant fluids from cryptococcal cells that were preincubated with RPMI 1640 containing 10% HS. Encapsulated and nonencapsulated cryptococci are activators of the complement system which generate biologically active cleavage products such as C5a following incubation in normal HS (7, 21). Of particular interest is the observation that encapsulated cryptococci are more powerful activators than acapsular cryptococci (37). The relationship between capsule production and enhanced complement activation parallels our observation that large-capsule isolates are more powerful stimulators of PMN cytokine release than small-capsule or acapsular isolates. It is more difficult to evoke complement activation as an explanation for the ability of purified GXM to stimulate PMN cytokine release. GXM is a relatively weak activator of the complement system, requiring large amounts of GXM to produce evidence of complement activation. Typically, GXM concentrations of 1 mg/ml or greater are required to produce evidence of complement

activation, such as production of sufficient numbers of cleavage fragments to induce chemotaxis of neutrophils and monocytes (7), cleavage of factor B (21), or depletion of hemolytic complement activity (8). However, such assays of complement activation are relatively insensitive. It is quite possible that assays of cytokine secretion which measure a positive experimental outcome are more sensitive measures of complement activation than depletion assays that require a substantial level of activation before an effect can be accurately measured. It is clear, however, that the issue of complement activation and its relationship to induction of cytokine secretion by PMN is in need of further study. Rigorous experiments to clarify the possible contribution of the complement system to the observed results are currently under way.

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