

Downregulation by Cryptococcal Polysaccharide of Tumor Necrosis Factor Alpha and Interleukin-1 β Secretion from Human Monocytes

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The regulation by *Cryptococcus neoformans* encapsulation of interleukin 1 β (IL-1 β) and tumor necrosis factor alpha (TNF- α) production by human monocytes was investigated. By using encapsulated and acapsular *C. neoformans*, we demonstrated that both strains induce cytokine production, although the acapsular strain was a better stimulator than the thinly encapsulated strain. The cytokine levels produced by cells stimulated by the two strains were lower and followed a different kinetic than those stimulated by lipopolysaccharide (LPS). Purified capsular polysaccharide inhibits TNF- α secretion induced by LPS or acapsular *C. neoformans*. In contrast, no regulatory effect on IL-1 β was observed when LPS was used. The secretory response of these cytokines follows different pathways of macrophage activation; in fact, complete inhibition of TNF- α does not affect IL-1 β production and vice versa. These data indicate that purified capsular polysaccharide of *C. neoformans* could contribute to the in vivo progress of cryptococcosis by suppressing cytokine production of macrophages and suggest that a therapeutic approach to address the suppressive effect of cryptococcal polysaccharide could be devised.

Cryptococcus neoformans is a ubiquitous fungus that is responsible for severe infection in the immunocompromised host (18). The growing interest in *C. neoformans* infection is attributable to a life-threatening mycosis that often occurs in patients with AIDS (7, 33). This encapsulated fungus enters the body via inhalation and reaches the lung, which is the first target organ. Although an important role for T lymphocytes (21) and natural killer cells has been described (8, 9, 28), alveolar macrophages (AM) are the first line of defense against *C. neoformans* infection (36), and their functional status contributes to control of infection. In a previous report, we demonstrated that human AM from normal subjects play a significant role as antigen-presenting cells to T lymphocytes, while their effector function seems to be less relevant, at least in the afferent arm of the immune response to this fungus (36).

The capsule is a prominent virulence factor with antiphagocytic and toleragenic properties, as described by many authors (14, 16, 24, 27). The size of the capsule affects phagocytosis by professional phagocytic cells, which can easily ingest the encapsulated fungus after opsonization with normal human serum (HS) (25). The fungicidal activity of human macrophages is influenced by the presence of capsular material (37). Moreover, the presence of a capsule greatly influenced the requirements for opsonization and macrophage activation in the fungistatic activity of murine macrophages (20). Such opsonization by HS is due to activation and binding of opsonic fragments of C3 to the capsular surface. The rate of accumulation of C3 on encapsulated cryptococci is influenced by the capsular serotype. C3 accumulates at a higher rate on cells of

serotypes A and D than on cells of serotypes B and C (39). The nonencapsulated yeast cell initiates complement activation by utilizing classical and alternative pathways; in contrast, encapsulated cryptococci initiate complement activation only via the alternative complement pathway (17).

Other immunomodulatory effects ascribed to encapsulation of *C. neoformans* have been reported by Levitz et al., who found that cryptococci with small capsules were potent stimulators of tumor necrosis factor (TNF) by human leukocytes (22). In contrast, large-capsule yeast cells were poor stimulators of TNF production. A regulatory effect of capsular polysaccharide on human lymphocyte proliferation in response to *C. neoformans* has been demonstrated by Mody et al. (26).

Taken together, numerous lines of study indicate that the capsule of *C. neoformans* plays an important role in regulating humoral and cell-mediated immunity. However, very little is known about cytokine secretion by macrophages in response to *C. neoformans* and whether differences exist in the presence or absence of capsular polysaccharide. This study investigates the role of the purified capsular polysaccharide on TNF- α and interleukin-1 β (IL-1 β) secretion by human monocytes.

MATERIALS AND METHODS

Reagents and media. RPMI 1640 medium and fetal calf serum (FCS) were obtained from Eurobio Laboratories, Paris, France. Glucuronoxylomannan (GXM) was prepared from serotype A (strain ATCC 24064) as described before (12). Lipopolysaccharide (LPS) from *Escherichia coli* O55:135 was obtained from Difco Laboratories (Detroit, Mich.). Rabbit anti-human IL-1 was purchased from Janssen Biochimica, Beerse, Belgium, and mouse monoclonal anti-human TNF- α was from Biosource International, Camarillo, Calif.

Preparation of human AM. AM were collected from healthy nonsmoking informed volunteers of both sexes (35 to 65 years of age) as described elsewhere (36).

Preparation of PBM. To prepare peripheral blood monocytes (PBM), heparinized venous blood obtained from normal volunteers was diluted with RPMI 1640. Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation (35). The cells were washed twice in RPMI, placed into cell culture

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TABLE 1. Cytokine production by human AM and PBM in response to encapsulated and acapsular *C. neoformans*^a

Effector cells	In vitro stimulus	TNF- α (U/ml)	IL-1 β (pg/ml)	% Phagocytic cells	Phagocytic index	% Killing
PBM	NS ^b	11.2 \pm 3.4	9.3 \pm 2.8			
	LPS	252.3 \pm 40.2	425.3 \pm 69.2			
	6995	70.3 \pm 10.4	80.3 \pm 16.1	53.2 \pm 4.2	1.10 \pm 0.02	36.1 \pm 4.9
	7698	165.2 \pm 32.1	178.5 \pm 30.2	69.4 \pm 3.8	1.50 \pm 0.1	57.9 \pm 3.8
AM	NS	10.3 \pm 2.8	8.9 \pm 6.2			
	LPS	184.2 \pm 28.4	251.2 \pm 35.1			
	6995	48.3 \pm 15.9	60.0 \pm 10.2	45.3 \pm 4.9	1.11 \pm 0.01	19.0 \pm 3.7
	7698	90.4 \pm 16.2	100.3 \pm 28.8	59.3 \pm 5.4	1.43 \pm 0.09	45.3 \pm 4.0

^a AM or PBM (2×10^6 /ml) were cultured for 18 h with or without encapsulated (6995) or acapsular (7698) *C. neoformans*, and the supernatants were collected and tested for cytokine activity. Phagocytosis and phagocytic index were determined as described in Materials and Methods. Killing activity was determined by mixing monocytes and live encapsulated (6995) or acapsular (7698) *C. neoformans*, followed by 4 h of incubation at 37°C in 5% CO₂. Percent killing was evaluated at an E:T ratio of 10:1 in a CFU inhibition assay, as previously described (36, 37). The results are the means \pm standard deviations of four separate experiments with cells from four different donors.

^b NS, not stimulated.

petri dishes (Nunc Inter Med, Roskilde, Denmark) at a concentration of 2×10^6 to 3×10^6 /ml in RPMI 1640 medium supplemented with 5% FCS (cRPMI) 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 medium. Adherent cells were carefully recovered with a rubber policeman.

Microorganisms. Two strains of *C. neoformans* were obtained from J. Orendi (Central Bureau Schimmel Cultures [CBS], Delft, The Netherlands): the *C. neoformans* variant *neoformans* serotype A, thinly encapsulated (CBS 6995; NIH 37; National Institutes of Health, Bethesda, Md.), and *C. neoformans* variant *neoformans*, acapsular mutant (CBS 7698; NIH B-4131). The morphological characteristics and growth conditions of the two strains of cryptococci have been described previously (29, 36, 37). The cultures were maintained by serial passage on Sabouraud agar (Bio Merieux, 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* 6995 and 7698 were inactivated by autoclaving.

Phagocytosis. Phagocytosis assays were performed as previously described (36, 37). Briefly, live *C. neoformans* 6995 or 7698 cells were added to AM or PBM monolayers cultured in RPMI plus 10% HS on the coverslip at an effector-to-target cell (E:T) ratio of 1:1. After 1 h of incubation at 37°C in 5% CO₂, excess microorganisms were removed by extensive washing. The percent phagocytosis was calculated as the proportion of PBM or AM cells containing one or more yeast cells after 100 AM or PBM had been counted. The phagocytic index was calculated as the average number of yeast cells per phagocytosing AM or PBM.

Production of PBM culture supernatants. Harvested PBM were placed in 24-well flat-bottomed tissue culture plates (Falcon; Becton Dickinson, Oxnard, Calif.) at 2×10^6 cells per ml and incubated for 3, 18, or 48 h with (i) lipopolysaccharide (LPS; 10 μ g/ml); (ii) *C. neoformans* 6995 at an E:T ratio of 1:1 or *C. neoformans* 7698 at an E:T ratio of 1:1; (iii) polysaccharide (500, 250, and 0.1 μ g/ml); or (iv) buffer alone. After incubation in RPMI plus 10% HS at 37°C under a 5% CO₂ atmosphere, supernatants were harvested and stored at -20°C until assayed.

Quantification of cytokines. Cytokine levels in the culture supernatants were measured with an enzyme-linked immunosorbent assay (ELISA) kit for human IL-1 β (Seromed, Biochrom KG, Berlin, Germany) and a bioassay for TNF- α . TNF was determined by using actinomycin D-treated L929 cells as targets. Serial dilutions (0.1 ml) of assay supernatants were added to 4×10^4 L929 cells seeded in 0.1 ml in flat-bottomed microtiter plates. The determination of TNF activity was made in comparison to commercially available preparations with known titers, and the results were expressed as units per milliliter. Direct addition of GXM to the TNF bioassay mix does not affect the response of L929 cells to TNF- α standards of known titers. To verify the specificity of TNF activity, selected samples were incubated with monoclonal anti-TNF- α antibody before the cytokine assay was performed.

RESULTS

In our previous study, we demonstrated that encapsulation of *C. neoformans* regulates the antigen presentation process and fungicidal activity of human AM (37). To verify whether the presence of capsular material is involved in the regulation of cytokine production by human macrophages, we performed experiments to evaluate TNF- α and IL-1 β secretion by monocytes and AM from normal subjects.

Table 1 shows the levels of TNF- α and IL-1 β produced by PBM and AM in response to LPS or *C. neoformans* encapsulated (6995) and acapsular (7698) strains. Cytokine levels were higher in the supernatants of monocyte cultures than in those

of AM, indicating that PBM are more responsive to these stimuli. Moreover, the amount of TNF- α and IL-1 β produced in response to the acapsular strain was greater than that obtained with the encapsulated yeast strain. To evaluate the kinetics of cytokine secretion by PBM in response to *C. neoformans*, we performed experiments in which supernatant fluids from monocyte cultures challenged with LPS or *C. neoformans* were harvested at various times (3, 18, and 48 h). The results reported in Fig. 1 show that appreciable cytokine levels were detected within 3 h of LPS stimulation and reached a maximum by 18 h. In contrast, TNF- α and IL-1 β secretion in response to *C. neoformans* reached appreciable levels after 18 h of culture and then declined. The time course of TNF- α and IL-1 β secretion by PBM in response to encapsulated (6995) and acapsular (7698) *C. neoformans* strains showed a similar pattern even if TNF- α seems to decrease more rapidly. Comparing the two stimuli, LPS seems to be a more efficient stimulus for inducing an earlier and longer-lasting response than *C. neoformans*.

To investigate the effect of capsular polysaccharide on TNF- α and IL-1 β secretion, experiments were performed by adding GXM to LPS- or *C. neoformans* 7698-stimulated cells. The results (Fig. 2) show that GXM is able to modulate TNF- α secretion induced by LPS or *C. neoformans* 7698. Downregulation is evident 3 h after LPS addition (Fig. 2A) and within 18 h after *C. neoformans* challenge (Fig. 2C). In contrast, capsular polysaccharide is unable to regulate IL-1 β following LPS stimulation, while it inhibits *C. neoformans* 7698-induced IL-1 β (Fig. 2B, D, and F). The kinetics for early induction of TNF- α and IL-1 β secretion have a similar pattern, but it seems that TNF- α production by *C. neoformans* 6995- or 7698-stimulated cells declines more rapidly than IL-1 β production (Fig. 2E and F). It is unlikely that any of the observed effects of GXM on cytokine secretion are due to contamination of the GXM preparation with LPS, because addition of GXM alone to PBM cultures was unable to induce detectable levels of TNF- α or IL-1 β secretion.

In many experimental systems, TNF- α is able to regulate IL-1 β secretion and vice versa (19). Hence, experiments were performed in which monoclonal anti-TNF- α or anti-IL-1 β antibodies were added to monocytes challenged with LPS or *C. neoformans* 6995 or 7698. Table 2 shows that treatment of cell cultures with anti-TNF- α or anti-IL-1 β was unable to affect IL-1 β or TNF- α activities, respectively.

DISCUSSION

The results reported here establish that *C. neoformans* is able to induce cytokine secretion by monocytes and AM. The amount of IL-1 β and TNF- α production is greatly influenced

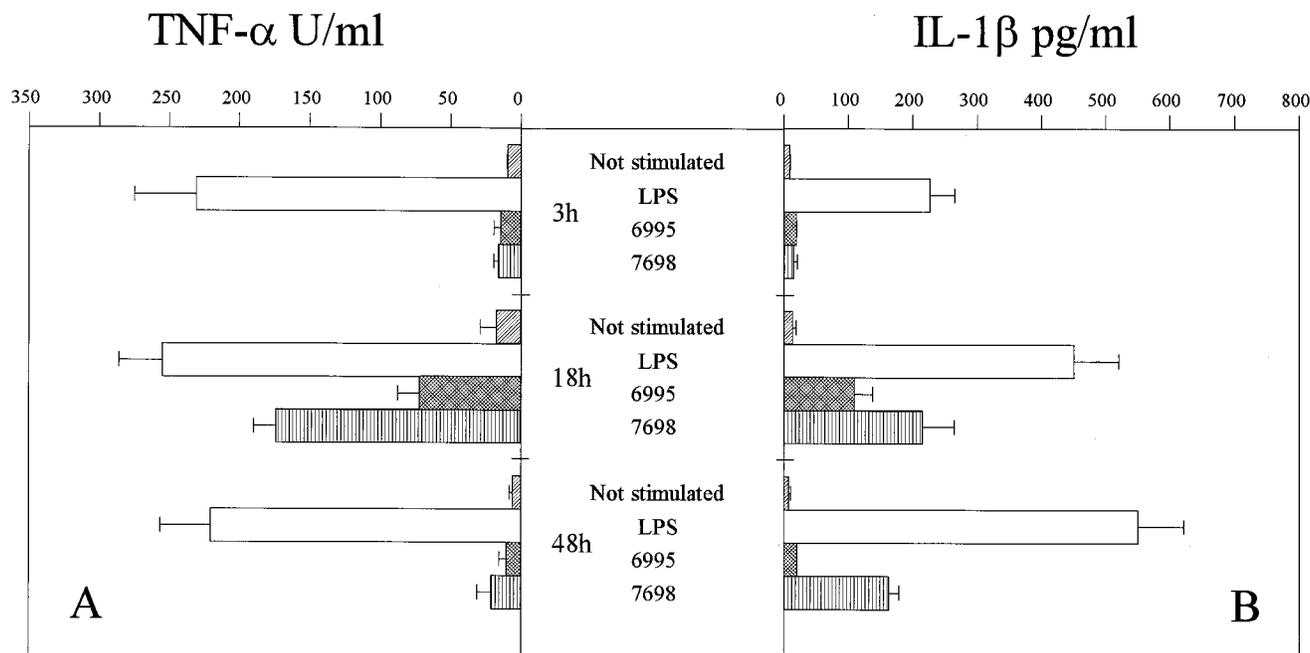


FIG. 1. Time course of TNF- α and IL-1 β secretion by monocytes in vitro stimulated with LPS (10 μ g/ml) or encapsulated (6995) or acapsular (7698) *C. neoformans* (E:T ratio, 1:1). The results are the means \pm standard deviations of five separate experiments with cells from five different donors.

by encapsulation of *C. neoformans*. Nonencapsulated cryptococci induce much higher levels of cytokine secretion than encapsulated cryptococci. Cytokine secretion by monocytes in response to the fungus was delayed and decreased more rapidly than cytokine secretion in response to LPS, a classical stimulus of macrophages. In addition, the purified capsular polysaccharide is able to downregulate the amount of TNF- α and IL-1 β induced by the acapsular strain and to suppress LPS-induced TNF- α in a dose-dependent manner. In our experimental system, the presence of TNF- α could not induce IL-1 β secretion and vice versa.

Recently, Levitz et al. reported that TNF- α is produced by human leukocytes stimulated with *C. neoformans* and that the size of the cryptococcal capsule affects the amount of TNF secreted (22). Our results confirm and extend these observations to IL-1 β . However, under our experimental conditions, we found that TNF- α production by monocytes in response to *C. neoformans* challenge decreased after 18 h instead of 48 h of culture. This may be due to different cellular populations involved in the observed response.

Purified GXM suppressed the induction of TNF- α by LPS but had no effect on LPS induction of IL-1 β secretion. In contrast, GXM suppressed the induction of both TNF- α and IL-1 β secretion by acapsular *C. neoformans*. These results suggest that the effects of GXM on TNF- α and IL-1 β secretion are fundamentally different. One explanation is the possibility that, in contrast to the effects of GXM on stimulation of TNF- α by LPS, GXM has no direct effect on IL-1 β secretion. If it is assumed that IL-1 β secretion is a response to phagocytosis of the acapsular yeast cells, there is a strong possibility that GXM acts by inhibiting phagocytosis of the yeast. Addition of purified GXM to acapsular cryptococci leads to binding of polysaccharide to the cells (16, 34), with a consequent inhibition of phagocytosis (3, 12).

C. neoformans is a ubiquitous encapsulated yeast that most likely enters the body as a thinly encapsulated fungus by inha-

lation (2). Disseminated disease in the immunocompetent host is rare, although under immunodepressed conditions, cryptococcosis represents an increasingly serious problem (30, 40) and is a common lethal mycosis among AIDS patients (7). The polysaccharide capsule is essential for virulence (3, 13) and determines the serotype specificities of the yeast. It is generally accepted that CD4⁺ (10) and CD8⁺ (11) cells are crucial in clearing the yeast from the lung; moreover, an effector function has been attributed to IL-2-activated T cells (21) and natural killer cells (28). The presence of capsular material influences phagocytic activity and the antigen presentation process by macrophages (5, 37). In addition, encapsulation of *C. neoformans* downregulates stimulation of cytokine production by human monocytes. TNF- α and IL-1 β production by monocytes stimulated with either encapsulated or acapsular cryptococci is delayed with respect to production after LPS stimulation, suggesting that different mechanisms of activation are involved in the observed phenomenon. Endogenous TNF- α and IL-1 β could exert autocrine stimulation in macrophages inducing up-regulation of binding and phagocytosis and killing of the fungus (1, 6, 38). Our results show that secretory activity is markedly influenced by encapsulation of *C. neoformans* as well as by the presence of polysaccharide. During cryptococcosis, synthesis of a large capsule and capsular polysaccharide released from encapsulated cryptococci may contribute to progression of disease by inhibiting effector function and secretory activities by macrophages. TNF- α stimulates human immunodeficiency virus type 1 (HIV-1) replication (23), and hence its production by macrophages in response to *C. neoformans* could be deleterious in AIDS patients. However, Orendi et al. (29) recently reported that enhanced HIV-1 replication is not dependent on TNF release by PBM in response to the fungus and is dependent on cryptococcal components other than capsular polysaccharide (31). IL-1 β is able to induce HIV expression in a human promonocytic cell line (32), and hence in AIDS

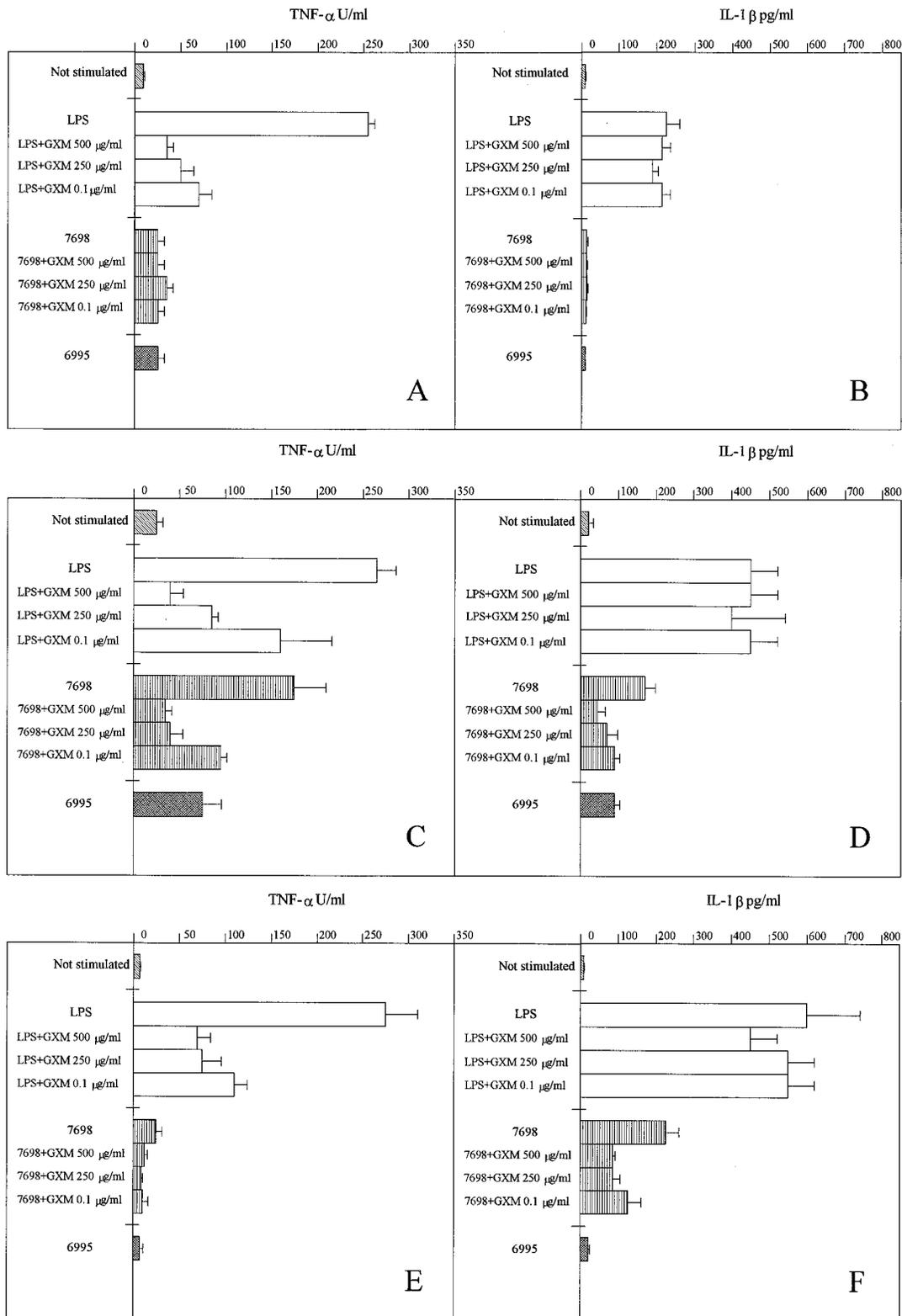


FIG. 2. Time course of the effect of GXM addition on TNF-α and IL-1β production from monocytes treated with LPS (10 μg/ml) or acapsular *C. neoformans* 7698. Encapsulated *C. neoformans* 6995 was tested as a control. The fungi were added to PBM at an E:T ratio of 1:1. Results were determined after 3 h (A and B), 18 h (C and D), and 48 h (E and F). The results are the means ± standard deviations of five experiments with cells from five different donors.

TABLE 2. Effects of monoclonal anti-human TNF- α or IL-1 β antibodies on cytokine secretion by monocytes^a

Stimulus	Monoclonal antibody	TNF- α (U/ml)	IL-1 β (pg/ml)
None	None	13.2 \pm 4.0	9.0 \pm 3.2
LPS	None	234.3 \pm 38.2	395.3 \pm 48.4
	Anti-TNF- α	4.2 \pm 2.0	404.3 \pm 50.2
7698	Anti-IL-1 β	190.2 \pm 29.4	3.8 \pm 1.5
	None	115.3 \pm 28.2	183.1 \pm 25.9
	Anti-TNF- α	2.9 \pm 1.0	190.2 \pm 30.4
	Anti-IL-1 β	98.4 \pm 8.2	2.8 \pm 1.2

^a Monoclonal antibodies were added to monocytes at the time of culture preparation. Supernatants for cytokine determination were harvested after 18 h of culture. The results are the means \pm standard deviations of five separate experiments with cells from five different donors.

patients, the induction of IL-1 β release after *Cryptococcus* challenge could contribute to the progression of HIV infection.

It seems that TNF- α does not induce IL-1 β and vice versa. In our experimental system, it seems that TNF- α does not affect IL-1 β and vice versa, but it is possible that similar kinetics of production makes reciprocal regulation difficult. Nevertheless, both cytokines may synergize in inducing fever among AIDS patients with cryptococcosis (4). The significance of these results in AIDS patients remains to be determined, in particular, the possible involvement of *Cryptococcus*-induced IL-1 β in HIV replication. Taken together, these results indicate new levels of complexity in the pathogenesis of *C. neoformans* infection and provide indications that could lead to new therapeutic approaches aimed at neutralizing the suppressive effect exhibited by capsular polysaccharide.

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