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Interleukin-6 Production by Human Monocytes Stimulated with *Cryptococcus neoformans* Components

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In order to ascertain if *Cryptococcus neoformans* components can induce interleukin-6 (IL-6) production, we stimulated human whole blood with purified capsular products. Their potencies in stimulating IL-6 release were mannoproteins > galactoxylomannan = glucuronoxylomannan > α(1-3)glucan. IL-6 production was tumor necrosis factor alpha independent and required the presence of monocytes and plasma. Since IL-6 can stimulate replication of the human immunodeficiency virus in monocytic cells, these findings may be clinically relevant.

Cryptococcus neoformans is an opportunistic yeast that can cause life-threatening infections in patients with AIDS. The capsule, which is composed of glucuronoxylomannan (GXM) and two minor constituents, galactoxylomannan (GalXM) and mannoprotein (MP), is an important virulence factor (8, 11). The role of cytokines in cryptococcosis is complex. On the one hand cytokines, such as tumor necrosis factor alpha (TNF-α) and granulocyte-macrophage colony-stimulating factor, may amplify antifungal responses (1, 2). On the other hand, some of these mediators may increase virus replication in AIDS patients infected with *C. neoformans* (16).

Whole cryptococci, as well as some purified components, can induce TNF-α in human monocytes (2, 9). This cytokine may increase human immunodeficiency virus (HIV) replication through nuclear factor activation (13). Less is known about the role of interleukin-6 (IL-6) in cryptococcal infections. IL-6 can be induced by TNF-α (19) and participates in a negative-feedback loop regulating TNF-α production (17). However, IL-6 can also be induced directly by microbial products via TNF-α-independent pathways (6). Interestingly, IL-6 can directly upregulate replication of HIV in monocytic cell lines and synergizes with TNF-α as well as granulocyte-macrophage colony-stimulating factor in the induction of latent HIV expression (14). Therefore, better understanding of the mechanisms whereby *C. neoformans* induces IL-6 may be of help in devising therapeutic strategies for AIDS patients. This study was undertaken to investigate IL-6 production by *C. neoformans* and its major surface components.

GXM, GalXM, MP, and cell wall α(1-3)glucan, obtained as previously described (3), were added in increasing concentrations to cultures of whole blood obtained from healthy volunteers and diluted with equal volumes of RPMI 1640 medium (Life Technologies, Milan, Italy) containing heparin at 20 U/ml. Diluted blood was incubated for 24 h at 37°C in 5% CO_2 in the presence of soluble cryptococcal products or whole heat-inactivated (80°C for 30 min) yeasts. Culture supernatants were assayed for IL-6 activity by using the IL-6-dependent 7TD1 cell line, exactly as described elsewhere (20). Bioactivity in selected samples was totally abrogated by a neutralizing mouse anti-human IL-6 monoclonal antibody (Genzyme, Milan, Italy), indicating that only IL-6 bioactivity was being measured in our assay.

Data were expressed as means ± standard deviations of separate experiments, each performed in duplicate with cells from different donors. Statistical significance was assessed by one-way analysis of variance and the Student-Newmann Keuls test. Figure 1A shows that MP, GXM, and GalXM were capable of inducing dose-dependent IL-6 release. Maximal IL-6 release was similar to that induced by the positive control, *Salmonella enteritidis* lipopolysaccharide (LPS) (Sigma, Milan, Italy) (Fig. 1B). In contrast, glucan induced only minimal IL-6 secretion. MP appeared more potent than GXM or GalXM, since significant stimulation was attained with lower stimulating doses ($P < 0.05$) (Fig. 1A).

It is unlikely that the observed IL-6 responses were due to endotoxin contamination of the preparations used. In fact, endotoxin contamination was below the limits of detection of a sensitive *Limulus* assay (E Toxate; Sigma). Endotoxin contamination was further ruled out as a cause of IL-6 induction by observing that the endotoxin-inactivating agent polymyxin B (10 or 25 μg/ml) did not affect IL-6 induction by any of the cryptococcal constituents (not shown). Polymyxin B alone did not induce IL-6 production (not shown).

In further experiments we sought to assess the influence of capsule, a known virulence factor, on IL-6 production. To this end, the stimulating activity of A 9759, a type A strain (3), was compared with that of CAP 67, an acapsular mutant which is unable to produce GXM (7). Heat-inactivated cells from both the acapsular and the encapsulated strains were capable of stimulating IL-6 (Fig. 1B). However, the unencapsulated strain produced significantly higher cytokine levels relative to the encapsulated one ($P < 0.05$). These data are compatible with the hypothesis that GXM, the main capsular component (Fig. 1A), may act by masking surface components, with higher cytokine-inducing activities, possibly MP. Alternatively, increased IL-6 production may be related to increased phagocytosis of unencapsulated yeasts relative to encapsulated ones (9). Indeed, phagocytosis may be an important cofactor in promoting *C. neoformans*-induced TNF-α release. Further studies will be needed to better clarify these issues.

Since IL-6 can be induced by TNF-α (19) and the latter is

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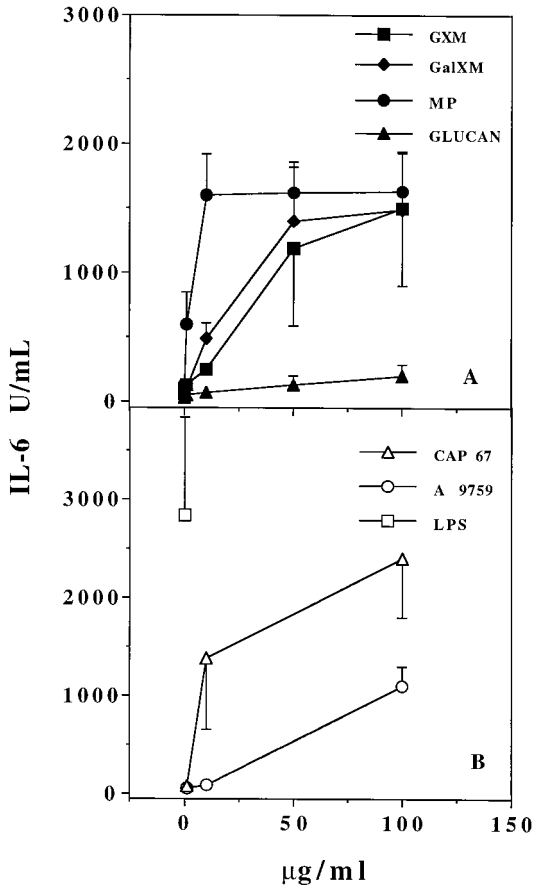

FIG. 1. Production of IL-6 in supernatants of whole-blood cultures stimulated with *C. neoformans* components (A) or heat-killed yeast cells (B). *S. enteritidis* LPS (0.1 μg/ml) was used as a positive control. Results represent the means and standard deviations of five independent observations conducted with blood from different donors.

produced in response to *C. neoformans* (9), we assessed the effects of TNF-α blockade on IL-6 production. The addition of neutralizing anti-TNF-α rabbit serum (Genzyme) at a final dilution of 1:100 did not affect IL-6 levels in supernatants of whole-blood cultures, while totally abrogating TNF-α bioactivity (not shown). These data indicated that the release of IL-6 may be largely TNF-α independent. Next, cellular separation experiments were performed to identify the main cytokine-producing population in whole blood. Blood was separated by centrifugation over Ficoll-Hypaque gradients (Pharmacia), as described elsewhere (10), and the interface cells (mononuclear leukocytes [PBMN]) were compared with cells in the pellet (polymorphonuclear leukocytes [PMN]) for their ability to produce IL-6. Purity of all of these cell populations was >90% by morphology. Figure 2A shows that PMN released considerably lower amounts of the cytokine, relative to unseparated leukocytes or PBMN, in response to any of the tested agents. PBMN were further fractionated on the basis of their ability to adhere to plastic surfaces. As shown in Fig. 2B, IL-6-producing activity was almost exclusively found in the adherent cell fraction, which consisted of >90% monocytes by morphology. These findings indicated that monocytes are predominantly responsible for IL-6 release in response to *C. neoformans* components. These data, however, do not exclude the possibility that PMN can produce moderate amounts of IL-6 upon exposure to GXM, as suggested by a recent study (15). Indeed, slight but significant elevations were detected in supernatants of PMN cultures in the presence of GXM, relative to unstimulated controls (32.8 ± 5.4 versus 11 ± 5.4; $P < 0.05$).

Since it was previously shown that TNF-α production in response to cryptococcal products was markedly influenced by plasma factors (3), it was of interest to determine if the same applied to IL-6 production. Washed, unseparated leukocytes were cultured without plasma and in the presence of either untreated or heat-inactivated (56°C for 30 min) autologous plasma. Heat-labile factors, presumably complement, were not an absolute requirement for IL-6 responses induced by MP, since comparable IL-6 levels were observed in the presence of complement-sufficient or heat-inactivated plasma (Fig. 3A). In the absence of plasma, however, there was a drastic decrease in IL-6 levels. With GXM, significant IL-6 production was observed only in the presence of complement-sufficient plasma (Fig. 3B), and the same was true for GalXM. In conclusion, our studies indicate that both whole yeasts and soluble cryptococcal constituents are potent IL-6 inducers. These data may be clinically relevant since high numbers of organisms, as well as high concentrations of soluble extracellular products, are frequently present in AIDS patients affected by cryptococcosis (5).

Little is known about the role of IL-6 in these infections. Elevated levels of IL-6 as well as of TNF-α and IL-1 are found

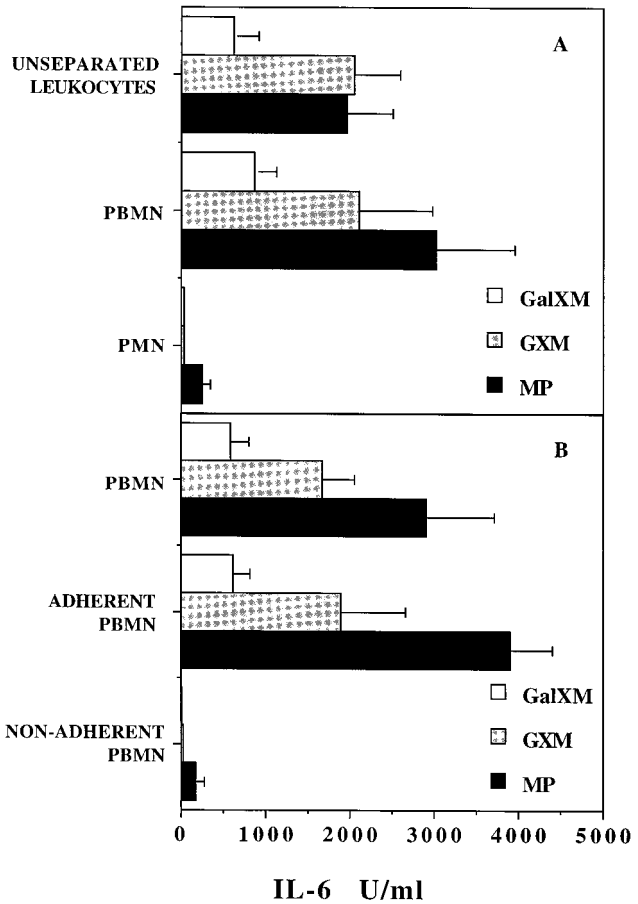

FIG. 2. Release of IL-6 from unseparated leukocytes, PBMN, and PMN (A) stimulated with *C. neoformans* components. PBMN further separated in adherent and nonadherent cells were stimulated with the same components (B). Results represent means and standard deviations of five separate experiments conducted with blood from different donors.

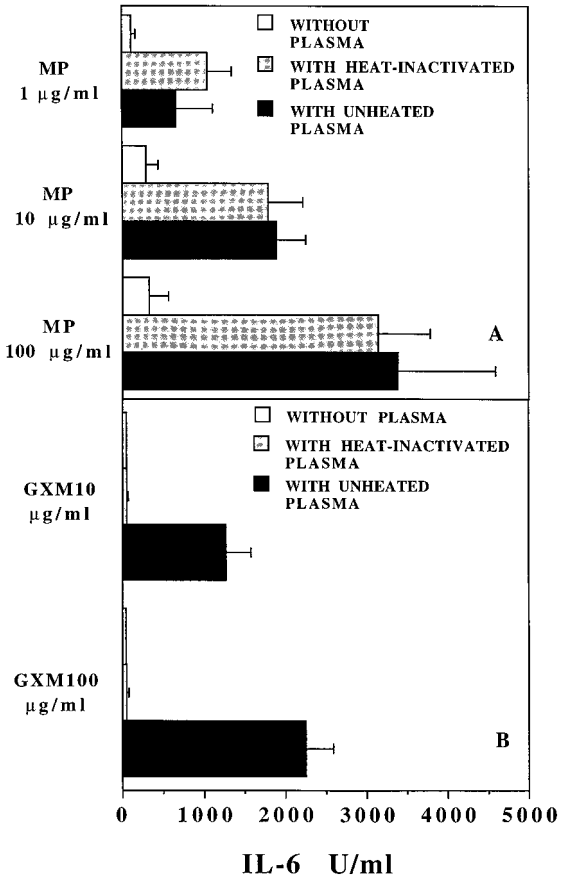

FIG. 3. IL-6 production from monocytes cultured without plasma or in the presence of heat-inactivated or unheated autologous plasma. Cells were incubated with the indicated concentrations of MP (A) or GXM (B). Results represent means and standard deviations of five different experiments conducted with blood from different donors.

in the cerebrospinal fluid of AIDS patients (18), raising the possibility of a pathogenic role for these cytokines. In *Mycobacterium avium* infections, another common cause of death in AIDS patients, IL-6 can promote bacterial growth and therefore be detrimental (4). It was shown that IL-6 can directly upregulate HIV replication in both acutely and chronically infected cells of human monocytic lineage (14). Moreover, IL-6 mRNA-expressing cells are found in patients with clinical AIDS (12). Therefore, *C. neoformans*-induced IL-6 production may be clinically relevant in AIDS patients. Further studies, however, will be needed to better clarify the mechanisms and the significance of IL-6 production in cryptococcal infections.

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