CHAPTER 8

Potent Inhibition of Neutrophil Migration by Cryptococcal Mannoprotein-4 -induced Desensitization


Chapter 8

ABSTRACT
Cryptococcal capsular antigens induce the production of pro-inflammatory cytokines in patients with cryptococcal meningitis. Despite this, their cerebrospinal fluid typically contains few neutrophils. Capsular glucuronoxylomannan (GXM) is generally considered to mediate the inhibition of neutrophil extravasation. In the current study culture supernatant harvested from the non GXM-producing strain CAP67 was found to be as potent as supernatant from wild-type strains in preventing migration. We identified capsular mannoprotein-4 (MP-4) as the causative agent. Purified MP-4 inhibited migration of neutrophils toward platelet-activating factor (PAF), IL-8 and fMLP, probably via a mechanism involving chemoattractant receptor cross-desensitization as suggested by its direct chemotactic activity. Supporting this hypothesis, MP-4 elicited Ca$^{2+}$ transients that were inhibited by pre-incubation with fMLP, IL-8 or C5a, but not PAF - and vice versa. Moreover, MP-4 strongly decreased the neutrophil surface expression of L-selectin and induced shedding of TNF receptors p55/p75, while CD11b/18 increased. Finally, MP-4 was clearly detectable in both serum and cerebrospinal fluid of patients suffering from cryptococcal meningitis. These findings identify MP-4 as a novel capsular antigen prematurely activating neutrophils and desensitizing them toward a chemoattractant challenge.

INTRODUCTION
Neutrophil infiltration into the central nervous system is a dual-edged sword in that it protects the tissue from infection and injury but is also detrimental to the host. In diseases such as bacterial meningitis (BM) or cerebrovascular ischemia there is evidence that a high influx of polymorphonuclear neutrophils (PMN) plays an adverse role in the pathogenesis of neurological damage (1;2). In BM, the phagocytic capacity of PMN in the cerebrospinal fluid (CSpF) is often insufficient and the harmful effects of their cytotoxic products may outweigh their beneficial effects (3). Accordingly, it is now assumed that adjunctive therapeutic strategies with anti-inflammatory agents may favor neurological recovery (4-6). Interestingly, the opportunistic fungus Cryptococcus neoformans has developed a means of infection evoking only minimal PMN infiltration.

Disseminated cryptococcosis is characterized by the presence of high levels of capsular glucuronoxylomannan (GXM), galactoxylomannan (GalXM), mannoprotein-1 (MP-1), and MP-2, in the CSpF and serum of affected patients (7;8). Titers in both serum and CSpF from AIDS patients reach levels equivalent to several hundred micrograms per milliliter, in exceptional cases rising to 20 mg/mL (9). GXM, GalXM and MP have been shown to induce the production of the early pro-inflammatory cytokines TNF-α and IL-1β by peripheral blood monocytes (10-12) and PMN (13) in the presence of pooled human serum (PHS). TNF-α then plays a pivotal role (14) in
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initiating a protective cell-mediated immune response. In healthy subjects, TNF-α enables the host to overcome the anti-phagocytic properties of the fungus, which usually suffices to clear systemic infection (15). In patients in whom this accurate response is hampered, the disease disseminates and induces inflammation. This reaction theoretically could be a very purulent one since the induced TNF-α has several properties that strongly promote PMN extravasation. First, TNF-α can cause profound systemic vasodilatation and subsequent hypotension via the induction of prostaglandins (16). Hypotension is a prerequisite to allow the transient L-selectin (CD62L)-mediated adherence of leukocytes to endothelial cells (17-19). In addition, TNF-α and IL-1β can induce the expression of adhesion molecules on endothelial and glial cells (4). Furthermore, TNF-α and IL-1β activate mononuclear cells, endothelial cells, and astrocytes to produce IL-8 (20) in the brain of patients with meningitis.

Despite the elevated IL-8 CSpF/serum ratio (13;21) and the elevated serum levels of TNF-α and IL-1β, the CSpF of patients with cryptococcal meningitis typically contains few mononuclear cells and virtually no PMN. To date GXM is considered to account for this inhibition of leukocytosis. In a mouse model study, Dong and Murphy showed that the inhibition of PMN infiltration elicited by intravenous administration of the cryptococcal culture filtrate (CneF) is due mainly to GXM (22). Using a modified Boyden chamber, we previously demonstrated that GXM significantly inhibited PMN migration toward IL-8 (23). In addition, we confirmed Dong and Murphy’s earlier observation that GXM mediates the chemotactic activity for PMN of whole encapsulated yeast cells and unfractionated CneF derived from these cells (24). Moreover, we found a significant inverse correlation between the GXM ratio (serum/CSpF) and the CSpF leukocyte count in patients with cryptococcosis (25). More recently, we showed that GXM delays translocation of PMN across the blood-brain barrier in a rabbit BM model (26). Therefore, the initial aim of this study was to investigate the molecular mechanism by which GXM prevents PMN migration toward chemoattractants.

Several studies have investigated the mechanisms regulating the passage of leukocytes through endothelial cells and their infiltration at inflammatory foci. The exact nature of the signaling mechanisms in brain inflammation still remains to be elucidated but undoubtedly involves TNF-α and IL-1β, chemoattractants (IL-8, PAF), as well as the expression of adhesion molecules and proteases that together promote cell recruitment and vascular permeability (6). Initially, localized inflammation results in hypotension allowing selectin-mediated tethering and rolling of PMN along the vessel wall (17;18). Rolling precedes a further functional up-regulation of PMN following exposure to pro-inflammatory cytokines and chemoattractants (27), resulting in firmer integrin-mediated adherence and shedding of CD62L (17;27). Further stimulation of PMN phenotypically high in CD11b/CD18 (CR3, Mac-1)
initiates migration through the interendothelial junctions (28). Finally, chemotactic gradients guide PMN to the site of infection.

Currently, the discrepancy between raised cytokine levels and hampered PMN influx into the brains of patients is explained by the observation that GXM is able to induce shedding of CD62L and TNF-α receptor (TNF-R) molecules from PMN (29). The key observation underlying this paper, i.e. a quantitatively similar prevention of PMN migration by CneF harvested from GXM-producing and non-producing (ΔCneF) strains, seriously questions the opinion that GXM is the sole cryptococcal antigen preventing extravasation. Therefore, we set out to characterize the component of ΔCneF responsible for the inhibition. To assess this, we first optimized the current protocol for isolating capsular antigens and purified the most important constituents of ΔCneF (GalXM, MP-1, MP-2 and MP-4) to near homogeneity (30). We found that MP-4 was primarily responsible for the inhibition of PMN migration. We then investigated the intrinsic chemotactic capacity of MP-4 and analyzed its ability to influence the expression of PMN surface-receptors involved in PMN migration. As a result of the action of MP-4 the surface expression of CD62L was down-regulated and both TNF-R p55 and p75 were shed into the surrounding medium. Furthermore, MP-4-induced signaling caused Ca²⁺ transients that could regulate inflammatory reactions by desensitizing chemoattractant receptors.

MATERIALS AND METHODS

Cryptococcal strains and antigen preparations

The cryptococcal culture filtrate antigens ΔCneF and CneF were prepared from cultures of the non-GXM-producing C. neoformans mutant strain, CAP67 (E.S. Jacobson, Medical College of Virginia), or the isogenic capsular strain NIH B3501, respectively. Inocula (10^8 colony forming units) of these strains were added to 500 ml RPMI-1640 supplemented with 10 μg/ml gentamycin and allowed to propagate for 5 days at 37°C. Next, supernatant fluids were isolated by centrifugation and concentrated by ultra-filtration (Stirred Cell Concentrator Millipore, 3.5 kDa cut-off). The concentrated (Δ)CneF was filtered sterilized and adjusted to 0.1 mg of protein per ml as determined by the BCA (Pierce; Rockford, Illinois, USA) protein concentration assay. (Δ)CneF used in the assays was diluted threefold. Mannoproteins were purified from ΔCneF as described recently (30). Briefly, ΔCneF was dialyzed against excess mQ water, freeze-dried and applied to a 100 ml Con A Sepharose-4B column. The column was washed and stepwise eluted with α-methyl-D-mannose pyranoside (αmDm). GalXM flows through this column, whereas MP-1 and -2 are eluted at 0.2 M and MP-4 at 0.4 M αmDm. These fractions were concentrated (MP-4) or further separated (MP-1 and -2) by DE52 or source Q anion exchange columns. All purified components were finally dialyzed against excess PBS and kept frozen at -20°C.
Maintenance of endotoxin-free conditions

Preparations of the various cryptococcal components were negative for endotoxin contamination using a Limulus assay (Kabi Diagnostica, Mölndal, Sweden) with a sensitivity of 100 pg/ml *E. coli* LPS. Nevertheless, all experiments were carried out at least once in the presence of 10 µg/ml polymyxin B sulfate in order to neutralize any undetected LPS contamination.

Isolation of human PMN

PMN were isolated as previously described (31). Briefly, blood from healthy volunteers was collected into Vacuette tubes containing sodium heparin, diluted with an equal volume of pyrogen-free PBS and centrifuged through a gradient of Ficoll and Histopaque. PMN were collected from the Histopaque phase, briefly shocked with mQ water, washed and suspended at 5 x 10⁶ cells/ml in RPMI supplemented with 0.05% human serum albumin (RPMI-HSA).

Transwell assay for measurement of chemotaxis

PMN were labeled by incubating them for 30 min. at 20°C with 3.3 µM 2',7'-bis-(2-carboxyethyl)-5-(6)-carboxyfluorescein acetoxymethyl ester (BC-ECF AM; Molecular Probes Europe, Leiden, Netherlands) in RPMI-HSA. Cells were washed and suspended in HBSS-HSA; 200 µl aliquots were incubated with an equal volume of each of the cryptococcal antigens being assessed for chemoattractive activity or buffer control for 30 min. at 37°C under constant agitation. Just prior to the end of this incubation transwell filters (pore size 3.0 µm; Costar type 3415, Cambridge, MA) were pre-wetted in HBSS/HSA. To each bottom compartment we added 600 µl of the indicated chemoattractants: human recombinant IL-8 (10 nM; Pepro Tech Inc., Rocky Hill, NJ-USA); fMLP (10 nM; Sigma Chem. Co., St. Louis, MO-USA) or PAF (platelet activating factor, 100 nM; Calbiochem, Cambridge, MA-USA). Filter compartments containing 100 µl of PMN suspension were placed on top of the bottom compartments. Migration was allowed to proceed for 1h at 37°C (5% CO₂) and analyzed by reading the fluorescence of the wells with a Cytofluor II multi-well Plate-reader (PerSeptive Biosystems; Farmingham, MA-USA) equipped with a 485 nm excitation and a 530 nm emission filter. Control wells containing BCECF-labeled cells were included to obtain the maximal fluorescence value. The fluorescence values of the samples are expressed relative to this maximum. Fluorescence was linear with cell number with a detection limit of 2,500 cells (1% of input), as determined by serial dilution.

Immunostaining and FACS® analysis of PMN antigen expression

To measure the expression of selected surface antigens, 5 x 10⁵ PMN were suspended in 40 µl RPMI-HSA containing purified cryptococcal antigens at the designated concentrations. Mixtures were incubated for 30 min. at 37°C on a rotation wheel after
they had been heated for 3 min. in a water-bath at 37°C. Then 10 µl of RPMI-HSA containing 0.25 - 0.5 µg of FITC-conjugated mAbs directed against CD11b (IgG1; ATCC 44a), CD18 (IgG2a; BD, Mountain View, CA-USA), or CD62L (IgG2a; Leu-8, BD) was added and the incubation was continued for 30 min. on ice. Heparinized or EDTA-treated whole blood samples were analyzed using an identical staining protocol with a final incubation step in Lysing solution (BD) to eliminate red cells. Neutrophils were distinguished from other leukocytes by differences in forward (FSC) and side scatters (SSC) in FACS® analyses.

Expression levels of chemoattractant and TNF receptors on PMN were measured using the same methodology. We determined the expression of IL8-R type A and PAF-R using mAbs to CDw128A of the IgG2b subclass and to PAF-R (IgG), both from Alexis Corp. (San Diego, USA). Surface expression levels of C5a-R and fMLP-R were determined using an anti CD88 mAb (IgG2a, Serotec, Oxford, UK) and anti-fMLP-R mAb (IgG1, PharMingen/BD), respectively. Since these different mAbs were not FITC-labeled, binding was visualized by incubation with FITC-conjugated goat F(ab') anti-mouse IgG (DAKO, Carpinteria, CA USA) as a secondary reagent to recognize the mAbs. To quantify the surface expression of the two receptors for TNF (p55 and p75) we used FITC-labeled antibodies of IgG1 and 2a subclasses, respectively, which were purchased from R&D Systems (Minneapolis, MN USA). Isotype-matched conjugated mAbs (mouse IgG1-FITC and mouse IgG2a/b-RPE) not specifically reacting with PMN were purchased from DAKO and used as controls to exclude non-specific or Fc-related binding. The labeled cells were washed twice and analyzed with a FACS® or incubated with the secondary antibodies prior to analysis.

**Recording of cytosolic Ca²⁺ concentration**

To measure chemoattractant-induced Ca²⁺ fluxes, PMN were loaded with 2 µM Fluo-3-AM in RPMI/HSA for 15 min. at 37°C under agitation, washed with buffer and suspended at 10⁶/ml in RPMI/HSA. The fluorescence (530 nm) of each 0.5-ml sample was measured to determine the basal Ca²⁺ level. Since Ca²⁺ fluxes are very rapid and transient, 5 µl reagent was added under vortexing and the sample was analyzed immediately by FACS®. For each sample nine measurements (0 through 8) of 2,000 cells were performed, each requiring an average time of ten seconds for sampling and data saving before the next acquisition was started. Samples were analyzed after gating the PMN population, thereby excluding cell debris and nonspecific staining.

**TNF-R ELISA**

Soluble TNF-R p55 and p75 shed from the surface of PMN were measured using a commercially available human ELISA kit (R & D Systems Europe, Abingdon, UK) according to the manufacturer's protocol. The minimum detectable doses of sTNF-R for these assays were typically less than 3 pg/ml for p55 or 1 pg/ml for p75. All data are expressed as means ± SEM.
Generation of antibodies directed against MP-4 and immunodetection

A rabbit antiserum directed against MP-4 was prepared by primary subcutaneous injection of two New Zealand White rabbits with 100 µg of MP-4 in complete Freund's adjuvant followed by a booster of 50 µg MP-4 in incomplete FA. The serum specifically recognized MP-4 migrating between 25 and 28 kDa, as shown (Figure 5 panel A) by standard Western blotting and ECL detection (Amersham Pharmacia Biotech, Uppsala, Sweden). When indicated, samples were pre-adsorbed to rabbit IgG purified from pre-immune serum using a protein G Sepharose 4 Fast Flow column (Amersham Pharmacia Biotech). Shortly, isolated pre-immune IgG was coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) beads. Immobilized IgG was added to the patient serum and CSpF samples and non-specific IgG-antigen interactions were allowed to occur for 30 min. at room temperature (batch binding).

RESULTS

Effects of cryptococcal capsular antigens on PMN migration toward IL-8, PAF and fMLP

IL-8, fMLP and PAF are examples of inflammatory mediators causing neutropenia because of rapid sequestration of PMN within the post-capillary venules of target organs. We evaluated the relative potency of capsular antigens to inhibit the migration of PMN toward these chemoattractants. Therefore, we first purified GalXM and three different MP from ∆CneF. Next, we confirmed that PMN were able to migrate toward IL-8, PAF, or fMLP in a dose-dependent manner (not shown). In our initial experiments we obtained almost identical results with CneF and ∆CneF, which was unexpected since GXM is considered to be the antigen exclusively responsible for the inhibition of PMN migration into the CSpF. We were able to detect a statistically significant inhibition of migration by adding ∆CneF to PMN migrating toward fMLP (18% inhibition, P=0.002) or IL-8 (23% inhibition, P=0.025; Figure 1A). We then added PMN incubated with MP-4 or other capsular antigens at the indicated optimal concentrations to the upper chamber and compared the inhibitory effect on PMN migration for each of them. GalXM (89%), GXM (46%) and MP-4 (98%) all caused significant (P<0.01) inhibition of migration toward IL-8, with MP-4 being the most potent (Figure 1A). GXM, GalXM and MP-4 all impaired migration toward fMLP, albeit to a somewhat lesser extent, with migration being inhibited by 66% (P=0.05), 60% (P=0.006) and 56% (P=0.0004), respectively. Migration toward PAF was completely blocked by MP-4 (P=0.015) and significantly inhibited by MP-2 (51%, P=0.007), GalXM (28%, P=0.0003) and GXM 70% (P=0.004). Because this inhibition could be caused by down-regulation of the receptors for these chemoattractants by MP-4, we analyzed the expression of the receptors for IL-8, fMLP, and PAF on PMN. There were no significant changes in the expression of
these receptors, although the number of fMLP and PAF receptors was slightly increased and the number of IL-8 receptors slightly decreased (not shown). Addition of polymyxin B to exclude any possible influence of trace amounts of LPS yielded identical results.

**FIGURE 1. Cryptococcal capsular antigens affect PMN migration.**

A. Inhibition of PMN migration toward chemoattractants. BCECF-AM-labeled PMN in the presence or absence of fungal antigens were added to the upper chamber of a transwell device. The migration of cells toward chemoattractants added to the lower chamber was quantified after 1 hour. MP were added to a concentration of 100 µg/ml, at which inhibition reached a plateau. GXM and GalXM were present at a final concentration of 1 mg/ml. Y-axis values represent migration toward chemoattractants in the presence of the indicated antigens relative to migration in the presence of buffer only (value = 1). Data are the means ± SEM derived from 3 (PAF; 100 nM final concentration), 5 (fMLP; 10 nM final concentration) to 7 (IL-8; 10 nM final concentration) independent experiments, except for GXM and GalXM (n = 2). Statistically significant data (2-tailed Student's t-Test) are marked by asterisks. B. MP-4 has intrinsic chemotactic activity. A concentration series of MP-4 was added to the lower compartment. Values (± SEM; n = 6) represent migration as percentage of the total amount of PMN (value = 1) added to the upper chamber.

**MP-4 has intrinsic chemotactic properties**

To determine the nature of the potent blocking effect of MP-4 on PMN migration we assayed the intrinsic chemoattractive activity of MP-4. MP-4 attracted PMN in a concentration-dependent manner (Figure 1B). Maximum migration (61% of input; P=0.007) was reached at an MP-4 concentration of 30 µg/ml whereafter migration started decreasing, probably due to saturating diffusion of MP-4 into the upper chamber. This observation was confirmed by the conventional under-agarose assay (32) for detection of chemotaxis (not shown). In this assay, the potency of MP-4 at 200 µg/ml equaled that of the positive control (PHS). Chemotaxis toward GXM (1
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mg/ml) could not be detected with this less-sensitive assay. Importantly, the underagarose assay permits measurement of both chemotaxis and spontaneous migration. The directed migration toward MP-4 observed in this assay, demonstrates that MP-4 is truly a chemoattractant and not simply acting as a chemokinetic agent. Furthermore, when 0.1 ng to 10 µg LPS/ml was added to the medium, in both assays no migration of PMN was observed, thus ruling out the possibility that the effects of MP-4 were caused by LPS contamination (not shown).

Ca²⁺ mobilization data indicate cross-desensitization of chemoattractant receptors by MP-4

The above results indicated that MP-4 might exert its effect by acting as a chemoattractant or by mimicking the action of chemoattractants. We therefore determined whether MP-4 could trigger an intracellular Ca²⁺ signal as occurs when chemoattractants bind to their G-protein coupled receptors. Figure 2A shows a steep increase in [Ca²⁺], in Fluo-3-AM-loaded PMN following stimulation by MP-4.

FIGURE 2. Interference of MP-4 with chemoattractant-induced intracellular Ca²⁺ mobilization.
A. MP-4 transiently increases [Ca²⁺], in human PMN. Fluo-3-AM-loaded PMN were stimulated with MP-4 (10 µg/ml) and immediately analyzed for the induction of Ca²⁺-fluxes. Y-axis represents fluorescence values (E 530 nm). Nine subsequent measurements (0 through 8) were performed (X-axis); time point zero refers to the non-stimulated control. Each time point required an average time of ten seconds for sampling and data saving before the next acquisition was started.

B. Desensitization by MP-4 of Ca²⁺ mobilization induced by fMLP, C5a and IL-8, but not PAF. Duplicate samples of PMN were incubated with MP-4, after which one tube was immediately used for Ca²⁺-flux measurements. Once fluorescence decreased to baseline levels fMLP (10 nM), C5a (200 nM), IL-8 (10 nM), or PAF (100 nM) was added to the parallel tube and responses were recorded. Depicted is the Ca²⁺ mobilization elicited by the indicated chemoattractants after pre-incubation with MP-4 relative to the Ca²⁺ pattern obtained after chemoattractant stimulation alone, calculated by the following equitation: Relative Ca²⁺ mobilization = (response value after preincubation–direct response value)/ response value after preincubation. Depicted data are from one representative experiment of at least 3 independent experiments with qualitatively similar results.
Activation of chemoattractant receptors can result in homologous and heterologous desensitization, i.e. down-regulation of cellular responses after a second challenge with a G-protein coupled receptor ligand (33). In desensitization experiments, pretreatment of PMN with MP-4 almost completely prevented the IL-8-, fMLP- or C5a-, but not PAF-induced Ca^{2+} flux (Figure 2B). Inversely, pretreatment of PMN with the mentioned chemoattractants abrogated the Ca^{2+} flux induced by MP-4. These results suggest that MP-4 exerts its anti-inflammatory activity by interfering with chemoattractant-receptor signal transduction pathways.

**Effects of cryptococcal antigens on the expression of CD11b/18 and L-selectin in the presence or absence of serum and bivalent cations**

Stimulation of leukocytes with different chemoattractants such as fMLP, C5a and LTB4 causes a rapid decrease in the number of surface CD62L molecules and a concomitant inverse regulation of Mac-1/CR3 (27). Given the observed functional resemblance of MP-4 and other chemoattractants, we investigated whether MP-4 resembles these true-type chemoattractants in this respect. Culture of isolated PMN with purified MP-4 at 2 µg/10^6 cells resulted (Figure 3C-D) in a significant decrease in the amount of cell-surface CD62L (68%; P=6E-5) and in up-regulation of surface Mac-1 (2.2-fold; P=7E-4). MP-4 was more potent than GXM or CneF in evoking this response (Figure 3A-B). The MP-4 response almost equaled that induced by fMLP, which caused a decrease in surface CD62L of 74% and an almost 3-fold increase in Mac-1 expression. ∆CneF caused a significant down-regulation of CD62L (47%; P=4E-9) and up-regulation of CD11b (64%, P=9E-8). Neither GXM, GalXM (not shown), MP-1 (not shown), MP-2 (not shown), nor CneF induced significant CD62L loss or CD11b gain in the absence of serum. These data provide evidence that MP-4 is the capsular component that has the greatest effect on the expression of CD62L and Mac-1.

In order to study the effect of MP-4 on PMN under physiological conditions, we incubated heparinized blood with purified MP-4 and compared the observed effects to those obtained with CneF, ∆CneF, and GXM. Under these circumstances, ∆CneF (75%; P=0.02), GXM (149%; P=0.02), and MP-4 (195%; P=0.008) significantly increased the expression of CD11b (Figure 3A and C). The relative values for CD18 (not shown) were similar to those observed for CD11b. In addition, GXM (37%; P=0.02) and MP-4 (43%; P=2E-4) induced a significant loss of CD62L (Figure 3B and D). However, both have been reported to activate the complement cascade in serum to generate C5a, which then can induce the observed effects on the expression of surface molecules (24;29). To investigate the contribution of complement activation to MP-4 activity, we studied the regulation of CD62L and Mac-1 expression in the absence of bivalent cations by using EDTA-treated blood. Apart from fMLP, which was used as positive control, MP-4 was the only component able
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FIGURE 3. Cryptococcal capsular antigens induce CD62L shedding and up-regulation of CR3 expression on human PMN by a mechanism not requiring serum or bivalent cations.

The surface expression of CD62L and CR3 of PMN either isolated as described, or present in heparinized or EDTA-treated blood was compared. A, B. Bars indicate the expression (± SEM) of L-selectin and CD11b after incubation of PMN with fMLP (10 nM), GXM (1 mg/ml), CneF, or ∆CneF relative to the expression after incubation with RPMI/HSA (value = 1). C, D. Lines represent the level of expression of the indicated surface molecules after incubation with MP-4 at 0, 1, 3, 10, 30, or 100 µg/ml. Changes in the expression of CD18 were similar to those measured for CD11b. For clarity reasons, expression values in heparinized blood are depicted - SEM, values in EDTA blood are depicted + SEM and values of isolated PMN ± SEM. Statistically significant data (2-tailed Student's t-Test) are marked by asterisks.

to alter the expression of CD11b or CD62L significantly under these circumstances (Figure 3). MP-4 dose-dependently increased the expression of CD11b to a maximum of 3.8-fold (P=0.015; Figure 3C) and decreased surface CD62L expression by 48% (P=0.005; Figure 3D). The same data for GXM were 7% and 2%, respectively. To exclude any donor-specific influence, isolated PMN, heparinized blood and EDTA-treated blood were collected from the same donors. For heparinized blood and PMN absolute fluorescence values were in the same range. EDTA-treated blood showed an almost 2-fold reduction in basal CD11b/CD18 expression, whereas CD62L levels were 2-fold higher. These observations demonstrate that MP-4 affects PMN function by a mechanism not requiring serum proteins nor extracellular Ca²⁺ or Mg²⁺.
MP-4 induces shedding of TNF-R from the surface of PMN

Results showed that MP-4 desensitized PMN, which explains the inhibited PMN influx into the brains of patients with meningitis. In conflict with this clinical observation is the induction of TNF-α expression observed in vitro (10-13) because TNF-α is generally assumed to promote extravasation. Since at least two reports (34;35) demonstrated a substantial reduction in PMN influx at the site of infection after neutralization of TNF, we investigated the possible involvement of TNF scavenging in reducing CSF leukocyte counts. To examine the possible role of TNF-R in cryptococcal meningitis, we measured the influence of capsular antigens on the generation of soluble TNF-R for the two types of TNF-R expressed on the surface of PMN: p55 and p75 (36). With a soluble TNF-R ELISA kit, the medium from freshly isolated PMN cultured for 1 h at 37°C contained clearly detectable levels of TNF-R (108 pg/ml and 186 pg/ml p55 and p75, respectively), suggesting that cell surface proteolysis is part of the normal turnover of TNF-R. Stimulation of PMN with MP-4 resulted in a significant increase of both sTNF-R p55 (4-fold; P = 0.0002) and p75 (1.8-fold; P = 0.002) in the supernatants (Figure 4A). None of the other tested capsular components had an effect on TNF-R shedding. Since soluble TNF-R
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compete with TNF-α at the cellular receptor, we also measured the density of p55 and p75 on PMN after treatment with MP-4. We observed a significant (P = 0.01) down-regulation of surface p55 on PMN after addition of MP-4. p75 was also down-regulated however due to the large variability between individual donors this was not significant (Figure 4B).

MP-4 levels in Cryptococcosis patients

Using a rabbit polyclonal antiserum directed against MP-4, we measured the levels of MP-4 present in the serum and CSpF of patients suffering from cryptococcal meningitis by standard Western blot analysis.

In panel A, we analyzed and compared the amount of MP-4 detectable in serum collected from a cryptococcosis patient and pooled human sera. Although the non-adsorbed antiserum directed against MP-4 is cross-reactive to several serum proteins we clearly detected a specific band at the putative molecular weight of MP-4 in patient- but not pooled serum. The identity of this putative MP-4 band was confirmed in panel B lanes 1 to 4 where we applied purified MP-4 in a concentration of 30, 10, 3 and 1 μg/ml, respectively, as a reference. In this panel, all samples were pre-adsorbed to pre-immune IgG, which removed all cross-reactive proteins except for one doublet at a high molecular weight position. In lanes 5-14 we analyzed and compared the

FIGURE 5. Detection of MP-4 in the serum and CSpF of patients suffering from cryptococcal meningitis. Immunoblotting. (A). Comparison of the amount of MP-4 detectable in serum collected from a cryptococcosis patient and pooled human sera; separation by SDS-PAGE on a 15% PA gel. (B). Quantification of MP-4 in the serum and CSpF of patients suffering from cryptococcal meningitis. Lanes 1-4 contain 7.5 μl of purified MP-4 in a concentration of 30, 10, 3 and 1 μg/ml, respectively, resolved by SDS-PAGE on a 20% gel. In lanes 5 to 14 - which were all pre-adsorbed to pre-immune IgG - we added matched CSpF (7.5 μl initial volume; lanes 5-9) and serum (1.5 μl; lanes 10-14) samples. Lane 15 contains PHS (1.5 μl initial volume). Patient samples are from HIV-infected cryptococcosis patients (5-8; 10-13) and from a non-HIV-infected patient (9; 14). Proteins were electroblotted to nitrocellulose membranes and incubated with the described primary antiserum in a 1:100 dilution. Filters were subsequently incubated with goat ant-rabbit IgG linked to HRP. Bound HRP was visualized by ECL. Arrowheads indicate the position of MP-4.
amounts of MP-4 detectable in paired samples of CSpF and serum collected from
HIV-infected cryptococcosis patients (5-8, 10-13) or a non-HIV-infected patient (9
and 14). MP-4 is clearly detected in the serum of these patients at a level strongly
exceeding that present in CSpF, providing evidence for the formation of a gradient of
MP-4 over the blood-brain barrier. The amount of MP-4 present in the serum of these
patients was calculated relative to the standards after densitometric scanning of the
blots and ranged between 10 and 55 µg/ml, respectively, by densitometric scanning of
the autoradiograph. The concentration present in CSpF varied between < 1 µg/ml and
7 µg/ml.

DISCUSSION

The potency of C. neoformans culture supernatants to inhibit leukocyte migration was
recognized almost half a century ago (37). Using purified cryptococcal antigens we
now show that MP-4 is the most active capsular antigen in inhibiting migration
toward chemoattractants. Moreover, we provide direct evidence for the presence of
MP-4 in the serum of patients with disseminated cryptococcosis. Below we discuss
the role of these and other recent findings in the pathogenesis of the disease and focus
on the mechanism of action of MP-4.

MP-4 strongly inhibited PMN migration toward fMLP, PAF and IL-8 (Figure 1A).
Conversely, MP-4 was found to be a potent chemoattractant in vitro (Figure 1B). The
paradox emerging here can be explained by a phenomenon called cross-
desensitization of chemoattractant receptors, as initially described by Sabroe et al
(33). This hypothesis was confirmed by our observation that pre-incubation of PMN
with MP-4 impaired Ca\(^{2+}\) mobilization by fMLP, IL-8 and C5a - but not PAF (Figure
2B). Although speculative at present, this difference in the regulation of
chemoattractant-mediated responses is likely to be related to G-protein usage, as
suggested by the distinct pertussis toxin sensitivity of the receptors for IL-8, fMLP,
and C5a versus PAF, with the former being sensitive and the latter at least partially
resistant (38;39).

The pathophysiological role of MP-4 is thus to functionally mimic the action of
chemoattractants, resulting in a premature activation of PMN. Such a role for MP-4 is
consistent with the observed loss of CD62L and concomitant up-regulation of
CD11b/18 (Figure 3), because chemoattractants affect the expression of these
molecules (27;40;41). Kishimoto et al. showed that PMN shed CD62L from the cell
surface within minutes after activation with chemotactic factors. Both the expression
and activity of CD11b/18 were greatly increased (27). This work founded the current
view that by the time chemoattractants arrive in the circulation, CD62L-mediated
neutrophil rolling has already taken place and the integrin-mediated attachment takes
over. In addition, Luscinskas et al. showed that IL-8, fMLP and C5a, besides
inhibiting the attachment of PMN to cytokine-activated endothelial monolayers, promoted the rapid detachment of tightly adherent PMN from activated endothelial cells, and abolished transendothelial migration (40). Finally, in IL-8 transgenic mice, but not in non-transgenic littermates, PMN migration into the inflamed peritoneal cavity was severely inhibited (41). Thus, although fMLP, IL-8, and C5a have been characterized primarily as chemoattractants, they can exert a wide range of modulatory effects on PMN-endothelial adhesive interactions.

The association between MP-4 and down-regulation by shedding of surface TNF-R (Figure 4) is also in agreement with the proposed role of MP-4 as chemoattractant. Several in vitro studies (42;43) demonstrated that within minutes of chemoattractant incubation both TNF-R p55 and p75 were down-regulated from the surface of PMN. At the same time, soluble TNF-R appeared in supernatants, in amounts proportional to the extent of down-regulation. This suggests that shedding is the major mechanism leading to the loss of p55 and p75 upon chemoattractant activation. Our demonstration of increased amounts of shed receptors in conditioned media of neutrophils exposed to MP-4 supports this hypothesis. In vivo, scavenging of TNF-α by soluble TNF-R is likely to contribute to the observed lack of leukocytes in the CSF of patients for at least two reasons. First, others and we have shown that cryptococcal antigens stimulate the production of TNF-α by leukocytes in the presence of serum (12;13). Second, TNF-α is known to play a crucial role in the control of organ infiltration. TNF-α activates the endothelium to cause leukocyte adherence (4;44) and triggers leukocyte infiltration of lung, liver, and kidney in control mice but not p55-deficient mice (44). Finally, TNF-α can cause profound systemic vasodilatation and subsequent hypotension (16), allowing CD62L-mediated adherence of leukocytes to the vessel wall (17-19). Together, these in vivo data indicate that the presence of TNF-α positively correlates with leukocyte organ infiltration.

In a series of studies by Dong and Murphy and our group, GXM has been shown to possess anti-inflammatory properties that prevent PMN from accumulating in C. neoformans infected tissues (22-25;45). The present work shows that MP-4 shares most of these properties with GXM, but is more potent. Although analyzed before (22;24;45) no direct effects of MP on PMN migration have been reported. These conflicting results can be explained by differences in the purification strategy used here. Previously, MP were eluted from the Con A column using a 0.2 M α-methyl-D-mannopyranoside (α-mDm) step elution protocol. Recently, however, we reported that MP-4 elutes from this column at 0.4 M α-mDm (30), thus indicating that MP-4 was never present in the previously used MP preparations. Since analysis of MP-4 reveals a signal eluting between polysaccharide calibration standards of 18 and 26 kDa in gel filtration chromatography (not shown), this component also was not
present in ΔCneF used in the studies mentioned above, because the culture filtrates were concentrated using a 30 kDa cut-off cassette.

Our analysis of the concentrations of MP-4 present in the serum of patients with cryptococcal meningitis has revealed a concentration range matching that required to obtain the described in vitro effects - thereby confirming the biological significance of our data. The concentrations required are high when compared to serum concentrations for mannoproteins from other fungi but are in line with experiments - both in vitro and in vivo - performed with other mannoproteins from C. neoformans (46;47). The relatively high concentrations of MP-4 detected in 4 out 5 patients can be partly explained by the fact that these patients were all newly admitted patients for whom treatment had not been started at the moment of sampling. In order to further explain the relatively high concentrations required for cryptococcal mannoproteins, experiments are in progress to determine the biological activity and half-life of MP-4 in vivo as well as in vitro.

In summary, we have shown that MP-4 has potent anti-inflammatory activity that may be responsible for the poor cellular infiltration of PMN into the CSpF of cryptococcosis patients. Given our hypothesis that MP-4 exerts its action largely by its intrinsic chemoattractive properties, the balance between pro-inflammatory and anti-inflammatory effects will depend critically on the temporal and spatial distribution of MP-4. Interestingly, even in patients with cryptococcal meningitis MP-4 concentrations in serum strongly exceed the levels detected in CSpF. We hypothesize that this distribution might be due to active drainage of MP-4 from CSpF to blood, resulting in increasing blood concentrations of MP-4 in the course of the disease. Active drainage will prevent both the migration of PMN toward MP-4 present in the CSpF, as well as the proper response to extra-vascular chemoattractants because of the premature activation of PMN. Alternatively, MP-4 expression might be strongly up-regulated during cryptococcal trafficking in the vascular compartment, before the brain section is reached. Environmentally induced expression of capsule genes is not unprecedented. Several nutrients, e.g. iron and CO₂, have been demonstrated to affect the composition of the cryptococcal capsule (48). We are currently testing the ability of MP-4 to limit neutrophil infiltration in vivo using a rabbit model for BM.
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