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Cryptococcal Polysaccharides Bind to CD18 on Human Neutrophils

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CD18, the β chain of the β2 integrin family of adhesion molecules, is associated with three different α chains (CD11a, -b, and -c) and is expressed on the surface of all types of leukocytes. CD18-containing molecules are up-regulated on the surface of neutrophils (polymorphonuclear cells [PMN]) in response to chemotactic agents and are implicated in mediating adhesion to an inflamed endothelium, which is a prerequisite to migration of PMN into infected tissues. In a previous study, we found that a cryptococcal culture filtrate (CneF), when injected into the bloodstream of mice to simulate the antigenemia in cryptococcosis, inhibits PMN accumulation at the site of an inflammatory stimulus. In the present study, we assessed the ability of CneF and its individual components, i.e., glucuronoxylomannan (GXM), galactoxylomannan (GalXM), and mannoprotein (MP), to interact with CD18 on human PMN. CneF labeled with ^{14}C was shown to bind to human PMN in a dose-dependent manner. Pretreatment of PMN with anti-CD18, but not an isotype-matched control monoclonal antibody (MAb) or anti-CD11a MAb, blocked the binding of ^{14}C-labeled CneF to PMN. In addition, CneF, GXM, and GalXM but not MP significantly blocked the binding of the anti-CD18 MAb to CD18 on the surface of unactivated and formyl methionyl leucyl phenylalanine-activated PMN as determined by indirect immunofluorescence staining and flow cytometric analysis. In the same experiments, the cryptococcal polysaccharides did not affect the binding of an anti-CD11a or anti-L-selectin MAb to the surface of PMN at 4°C. The results suggest that CneF and its components GXM and GalXM bind to CD18 on human PMN. Based on our findings, we propose that CD18 is a possible molecular target of cryptococcal polysaccharides and that binding of the polysaccharides to CD18 has the potential to inhibit leukocyte infiltration into inflammatory sites.

Cryptococcus neoformans is the only encapsulated yeast-like organism which is pathogenic to humans. Approximately 5 to 10% of adults (25) and 1% of children (30) with AIDS become infected with *C. neoformans*. Hallmarks of disseminated cryptococcosis are the presence of high concentrations of circulating capsular polysaccharides, such as glucuronoxylomannan (GXM), and less cellular infiltration into the infected tissues than is typically seen in other infectious diseases (11, 12, 17, 28, 29). With the mouse model, we have found that intravascular cryptococcal culture filtrate (CneF), which contains 88% GXM and 12% galactoxylomannan (GalXM) and mannoprotein (MP), is a potent inhibitor of leukocyte accumulation at the site of the *C. neoformans*-induced inflammatory response as well as into sites of inflammation induced by components of other organisms, such as *Mycobacterium tuberculosis*, or by the proinflammatory cytokine tumor necrosis factor (13). Our findings indicate that the minimal cellular infiltrates observed in infected tissues of cryptococcosis patients may be due, in part, to the circulating cryptococcal polysaccharides.

The mechanisms responsible for the inhibitory effects of circulating cryptococcal polysaccharides on leukocyte influx are not clear. According to a recent model, leukocyte attachment to endothelial cells is an important step for leukocyte extravasation, and leukocyte-endothelial cell interactions are viewed as active processes requiring at least three sequential steps (7, 33). First, leukocyte-endothelial cell interactions are initiated by binding of constitutively functional leukocyte adhesion molecules, such as L-selectin, to their ligands on endothelial cells (7). This initial L-selectin-mediated adhesion to its ligands is transient and reversible and causes the leukocytes to roll along the endothelial surface. Second, the rolling leukocytes are activated by specific chemoattractants or leukocyte-endothelial cell contact-mediated signals. This activation results in conformational changes in secondary adhesion molecules (33). Third, interactions of the activation-dependent adhesion molecules with their ligands on endothelial cells cause a strong, sustained attachment of the leukocytes to the endothelial cells. The best-characterized activation-dependent adhesion molecules are heterodimeric integrins of the β2 (CD18) class. The CD18 complex is composed of three members, CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1 or CR3), and CD11c/CD18 (p150,95) (18). A current model of leukocyte migration into tissues implies that leukocyte-endothelial cell interactions and extravasation of leukocytes can be controlled at any one of the three steps involved. Therefore, if cryptococcal polysaccharides down-regulate surface expression of functionally important adhesion molecules such as L-selectin and/or block receptor-ligand interactions by binding to the receptor or the ligand, the polysaccharides will diminish leukocyte attachment to the endothelial cells, resulting in inhibition of leukocyte migration into inflammatory sites.

In earlier studies, we found that GXM, but not GalXM and MP, triggers human neutrophils to shed surface L-selectin within 1 h at 37°C (14). Loss of surface L-selectin from neutrophils before the neutrophils interact with inflamed endothelial cells prevents the migration of neutrophils into sites of inflammation (2, 21).

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The main objective of the current study was to determine whether CneF or individual components of CneF (GXM, GalXM, or MP) could bind to the β2 integrins. We found that ^{14}C-labeled CneF was able to bind to neutrophils and that the binding was inhibited by an anti-CD18 monoclonal antibody (MAb), indicating that CneF binds to CD18, the β chain of the β2 integrins. We also observed that CneF, GXM, and GalXM but not MP blocked binding of anti-CD18 MAb but not anti-CD11a MAb to their targets on the neutrophil surface. The binding of CneF, GXM, and GalXM to CD18 has the potential to block the binding of CD18-containing molecules such as LFA-1 to their natural ligand ICAM-1 on the endothelial cell surfaces, thereby preventing or reducing migration of the neutrophils into tissue (7, 33).

MATERIALS AND METHODS

Maintenance of endotoxin-free conditions. To prevent endotoxin from influencing the experimental results, all experiments were performed under conditions which minimize endotoxin contamination. This included the use of plastic ware that was endotoxin free, glassware that had been heated for 3 h at 180°C, and reagents that had been found to contain less than 8 pg of endotoxin per ml (minimal detectable level) with the *Limulus* assay (Whittaker Bioproducts, Inc., Walkersville, Md.).

MAbs. The following MAbs were purchased from Becton Dickinson Immunocytometry Systems (Mountain View, Calif.): anti-L-selectin MAb (anti-Leu-8; mouse immunoglobulin G2a [IgG2a]), phycoerythrin (PE)-conjugated anti-CD11b (mouse IgG2a), PE-conjugated anti-L-selectin MAb (anti-Leu-8), and a PE-conjugated mouse irrelevant IgG2a for an isotype control. Anti-human CD11a (mouse IgG1) and anti-human CD18 (mouse IgG1) were purified on a G-bind column from supernatants from American Type Culture Collection hybridoma cell lines HB202 and HB203, respectively. Anti-GXM MAb 439 (mouse IgG1) was a generous gift of T. R. Kozel (University of Nevada, Reno). The mouse myeloma proteins MOPC21 (IgG1) and UPC10 (IgG2a) were obtained from Organon Teknika-Cappel (Malvern, Pa.) and were used as isotype control antibodies. Fluorescein isothiocyanate (FITC)-conjugated goat F(ab′)$_2$ anti-mouse IgG, used as a secondary reagent to recognize mouse IgG, was purchased from Caltag Laboratories (South San Francisco, Calif.).

Cryptococcal culture filtrate (CneF). CneF was prepared as previously reported by culturing *C. neoformans* isolate 184A (serotype A) in a defined medium (5). Briefly, *C. neoformans* was cultured in a liquid defined medium for 5 days, after which time the *C. neoformans* cells were removed with a Millipore OM-141 Pellicon Tangential Flow System and a 0.45-μm-pore-size filter. The culture filtrates were washed with 10 volumes of sterile physiologic saline solution with a 30,000-molecular-weight-cutoff cassette in the Pellicon System, and then the material retained was concentrated to 1/10 of the original volume. The protein concentration of the CneF used in this study was 153 μg per ml on the basis of a bicinchoninic acid assay, and the carbohydrate concentration was 4 mg per ml as determined by the phenol-sulfuric acid assay. A 1:16 dilution (carbohydrate concentration of 250 μg per ml) of this lot of CneF had a titer of approximately 1:16,384 with the standard latex particle agglutination assay (Latex-Crypto Antigen Detection System; Immuno-Mycologics, Inc., Norman, Okla.). CneF was analyzed by anodic 10% polyacrylamide slab gel electrophoresis and displayed three bands previously characterized as GXM, GalXM, and MP after the gels were stained with periodic acid-Schiff stain (27).

Preparation of GXM, GalXM, and MP. GXM, GalXM, and MP fractions of CneF were prepared as previously described (15). Briefly, GXM was isolated from CneF by gel filtration on a Sephacyl S-300 high-resolution column. The GalXM fraction was separated from the MP fraction by concanavalin A chromatography. All fractions were subjected to electrophoresis and concanavalin A blotting to determine the efficiency of the fractionation, and then the fractions were lyophilized and reconstituted in endotoxin-free sterile physiologic saline solution to 2 mg (dry weight) per ml.

Preparation of ^{14}C-labeled CneF. *C. neoformans* isolate 184A was grown in a defined medium (5). When growth reached the early log phase, the medium was removed and replaced with 5 ml of defined medium containing 13.6 mg of ^{14}C-labeled glucose (ICN catalog no. 11048.5). After 3 days in culture with the ^{14}C-labeled glucose, the supernatant from the *C. neoformans* culture was collected and filtered through a Centricon-10 with a 10,000-molecular-weight-cutoff filter. The material retained was concentrated to one-half of the original volume and was the undiluted ^{14}C-labeled CneF. The undiluted ^{14}C-labeled CneF had a specific activity of 8.9×10^6 cpm/mg of carbohydrate. When the ^{14}C-labeled CneF was subjected to electrophoresis on 10% native polyacrylamide gels and stained with periodic acid-Schiff stain (27), three bands which had positions in the gel similar to those of the GXM, MP, and GalXM bands of unlabeled CneF were observed. The gel containing the ^{14}C-labeled CneF was exposed to photographic film. The autoradiogram showed a prominent GXM band at the top of the gel and faint bands where the MP and GalXM ran in the gel. With the latex agglutination assay for cryptococcal antigen, the undiluted ^{14}C-labeled CneF had a titer of 1:16,384, which is equivalent to approximately 250 μg of carbohydrate per ml. Consequently, the carbohydrate concentration of the undiluted ^{14}C-labeled CneF was equivalent to the carbohydrate concentration of a 1:16 dilution of the lot of unlabeled CneF used in this study.

Human neutrophils. Human polymorphonuclear cells (PMN) were obtained by a method described previously (15). Briefly, buffy coats of human peripheral blood were diluted with phosphate-buffered saline (PBS), layered on Ficoll-Hypaque, and centrifuged. The cell pellet was collected from the Ficoll-Hypaque and sedimented with 6% dextran solution for 30 min. The PMN-rich population was collected from the upper layer of the dextran solution. Residual erythrocytes were lysed with a 0.85% NH_4Cl solution. PMN were washed and resuspended in Hanks balanced salt solution (HBSS) before being diluted to a concentration of 10^7 cells per ml. The cell populations were comprised of >95% neutrophils as determined by differential counts on Diff-Quick (Baxter Healthcare Co., Miami, Fla.)-stained smears. The viability of cells in the PMN-enriched fraction was >95% as determined by trypan blue dye exclusion.

Cell culture. PMN (10^6) were incubated in 0.5-ml polypropylene microcentrifuge tubes for 1 h at 37°C either in 100 μl of HBSS alone or in 100 μl of HBSS containing formyl methionyl leucyl phenylalanine (FMLP) at 10^{-6} M, 100 μg of GXM, or a 1:4 dilution (100 μg of carbohydrate) of CneF. After culture, PMN were immunostained for surface expression of L-selectin, CD11a, CD11b, and CD18 and subjected to flow cytometric analysis. The viability of each PMN population was determined by trypan blue dye exclusion after incubation with FMLP, GXM, or CneF for 1 h at 37°C and found to be the same as when the PMN were incubated similarly in HBSS (>95% viable).

Detection of L-selectin, CD11a, CD11b, and CD18 expression on the surface of human neutrophils after culturing with FMLP, CneF, or GXM. The medium used for the immunofluorescence staining and binding assays was PBS supplemented with 0.1% bovine serum albumin and 0.1% sodium azide. For direct immunofluorescence analysis of L-selectin and CD11b on the surface of human PMN, 10^6 PMN were suspended in 50 μl of medium containing 0.2 μg of PE-conjugated mouse anti-Leu-8 MAb or 0.5 μg of PE-conjugated mouse anti-CD11b MAb and incubated for 30 min at 4°C. Isotype-matched PE-conjugated MAbs that did not specifically react with human PMN were used as controls to exclude nonspecific antibody binding. For indirect immunofluorescence analysis of CD11a and CD18 on the surface of human PMN, 10^6 PMN were suspended in 50 μl of medium with 1 μg of goat IgG (Sigma Chemical Co.) as a preblocking antibody and incubated for 30 min at 4°C. After being washed twice in medium, the cells were suspended in 50 μl of medium containing 0.5 μg of mouse anti-human CD11a, anti-CD18 (IgG1), or the isotype-matched control antibody and incubated for 30 min at 4°C. After three washes with medium, the cells were suspended in medium containing 1 μg of FITC-conjugated goat F(ab′)$_2$ anti-mouse IgG and incubated for 30 min at 4°C. Following staining by either the direct or indirect method, the cells were washed three times in medium, fixed in 1% paraformaldehyde (wt/vol) in PBS, and stored at 4°C in the dark until analyzed. All samples were analyzed within 24 h after immunolabeling. The immunofluorescently stained cells were analyzed with a FACStar PLUS (Becton Dickinson & Co.) flow cytometer as previously described (14).

FMLP-activated neutrophils. PMN (10^7/ml) were activated by incubating the PMN with FMLP at 10^{-6} M for 30 min at 37°C. For the nonactivated control PMN, the PMN (10^7/ml) were incubated in HBSS for 30 min at 37°C. After incubation, the PMN were washed three times and adjusted to a concentration of 10^7 cells/ml in HBSS.

Anti-CD18, anti-CD11a, or anti-L-selectin MAb binding assay. Freshly isolated PMN or FMLP-activated PMN (10^6) were suspended in 50 μl of PBS with 0.1% bovine serum albumin, 0.1% sodium azide, and 1 μg of goat IgG (Sigma Chemical Co.) as a preblocking antibody and incubated for 30 min at 4°C. After being washed in medium twice, the cells were suspended in 50 μl of medium containing 0.5 or 2 μg of mouse anti-human CD18 (IgG1), anti-CD11a (IgG1), anti-Leu-8 (IgG2a), MOPC (IgG1) protein, or UPC10 (IgG2a) protein alone or with different concentrations of CneF, GXM, GalXM, or MP and incubated for 30 min at 4°C. After three washes in medium, the cells were suspended in medium containing 1 μg of FITC-conjugated goat F(ab′)$_2$ anti-mouse IgG and incubated for 30 min at 4°C. The cells were washed and fixed as described above for flow cytometry. The concentration of each MAb used in this study was a saturating concentration as determined by preliminary flow cytometric experiments.

^{14}C-labeled CneF binding assay. Freshly isolated PMN (10^6) were incubated for 30 min at 4°C in 50 μl of either medium alone or medium containing 0.5 μg of anti-CD11a, anti-CD18, or the isotype-matched mouse myeloma protein MOPC21. After being washed twice with medium, the cells were suspended in 50 μl of medium alone or medium containing different dilutions of ^{14}C-labeled CneF and were incubated for 30 min at 4°C. The PMN were washed two additional times with medium, and then the sedimented PMN were lysed by being incubated with 50 μl of 0.25% Triton X-100 for 15 min at 37°C. The supernatants were collected, and radioactivity was counted in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). In the blocking assays with anti-GXM MAb, different dilutions of ^{14}C-labeled CneF were preincubated with anti-GXM (IgG1) (40 μg/ml) or the isotype-matched MOPC21 protein (40 μg/ml) for 30 min at 4°C before being added to the PMN.

TABLE 1. Effects of FMLP, CneF, and GXM on surface molecule expression on human neutrophils[a]

Treatment of PMN	Mean channel fluorescence intensity after staining with:						
	Anti-L-selectin[b]	Anti-CD11b		Anti-CD11a		Anti-CD18	
		Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
Medium	38 ± 4	656	750	12	14	21	17
FMLP	9 ± 2[c]	949	1,120	12	14	27	24
CneF or GXM	12 ± 1[c]	1,203	1,405	13	14	18	15

[a] PMN (10^6) were incubated with 100 μg of GXM or a 1:4 dilution of CneF (100 μg of carbohydrate) in 0.1 ml of HBSS for 1 h at 37°C, and then levels of L-selectin, CD11a, CD11b, and CD18 on the PMN were determined.
[b] Means ± standard errors of the means for results from four normal donors.
[c] $P < 0.002$ compared to medium controls.

Statistical analysis. Means, standard errors of the means, and Student's *t* test results were used to analyze the data. When two groups were being compared, a *P* of ≤0.01 was considered to indicate a significant difference between the groups.

RESULTS

Effects of CneF and GXM on surface L-selectin, CD11a, CD11b, and CD18 expression. L-selectin and CD18 are constitutively expressed on the surfaces of the majority of blood PMN from normal individuals (8). CD18 on PMN is associated with CD11a, CD11b, or CD11c to form three different β2 integrins (8). Most chemoattractants induce a concomitant down-regulation of L-selectin expression and up-regulation of CD11b/CD18 expression on PMN (23). As expected, after 1 h of stimulation at 37°C with FMLP, a well-characterized chemoattractant, the PMN lost a significant amount of their surface L-selectin (Table 1, compare medium to FMLP [$P = 0.003$]) and expressed higher-than-control levels of surface CD11b ($P < 0.01$) and CD18 ($P < 0.01$) but not CD11a (Table 1). In the same experiment, both CneF (containing 88% GXM) and purified GXM, which are chemotactic to human PMN (15), also induced a significant loss of L-selectin (Table 1, compare medium with CneF or GXM [$P < 0.001$]) and an increase in CD11b ($P < 0.01$) on the surfaces of human PMN as compared to controls (Table 1). However, the CD18 density on the PMN treated with CneF or GXM at 37°C for 1 h was not increased but was similar to control levels (Table 1). These results could be due to lack of modulation of CD18 expression on the PMN treated with CneF or GXM. However, this explanation seems unlikely, considering that CD11b was increased and CD11b is generally associated with CD18 on the surfaces of activated PMN (8, 22). Furthermore, the lack of up-regulation of CD18 on the PMN surface after treatment with CneF or GXM seems to be in conflict with the behavior of PMN in response to other stimuli (22). Alternatively, it is possible that CneF and GXM may bind to CD18 and block the binding site for anti-CD18 MAb. If this is true, an increase in CD18 would not be detected with an anti-CD18 MAb whose binding site was blocked by CneF or GXM even if an increase in CD18 occurred after stimulation with CneF and GXM. The purpose of the subsequent experiments was to determine if CneF and GXM block binding of anti-CD18 to CD18 on PMN.

Effects of CneF on the binding of anti-CD18, anti-CD11a, or anti-L-selectin to the surface of human PMN. To prevent CneF or its constituents from activating PMN and altering the PMN surface molecules, the binding assays were done at 4°C. By an indirect immunofluorescence staining procedure and flow cytometric analysis, we found that CneF blocked the binding of the anti-CD18 MAb to CD18 on the surfaces of PMN (Fig. 1). In the presence of CneF at a concentration of 0.5 or 1 mg of carbohydrate per ml (1:8 or 1:4 dilution, respectively), the amount of anti-CD18 MAb bound to PMN was reduced by 20% ($P < 0.05$) compared to that for PMN incubated in the absence of CneF, whereas with CneF at a concentration of 0.25 mg of carbohydrate per ml (1:16 dilution), anti-CD18 binding was reduced by 40% compared to binding of anti-CD18 in the absence of CneF ($P < 0.01$). In the same experiments, none of the concentrations of CneF tested affected the binding of the anti-CD11a or anti-L-selectin MAb to PMN compared to controls ($P > 0.05$).

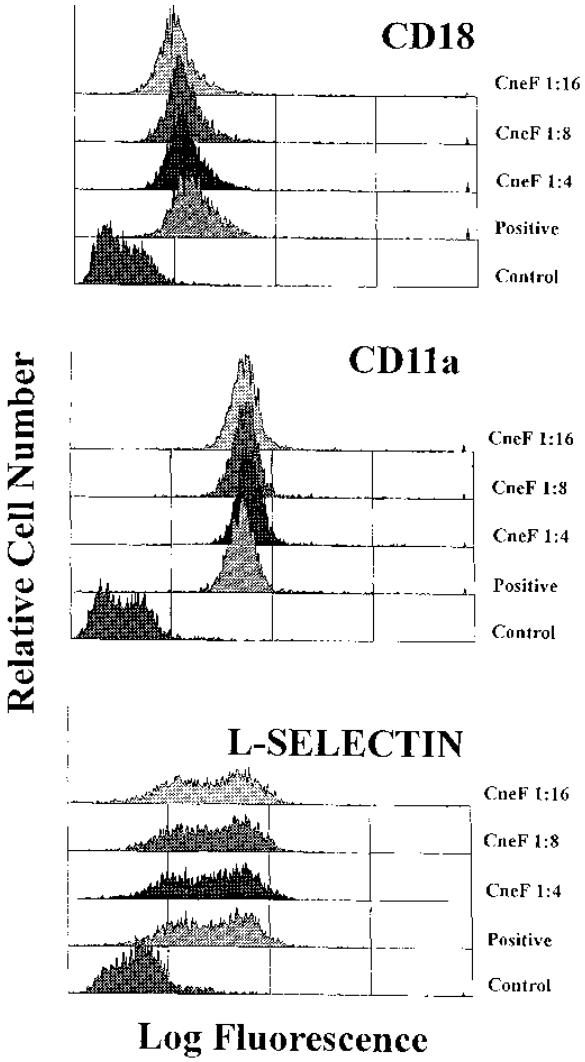

FIG. 1. Effects of CneF on the binding of anti-CD18, anti-CD11a, and anti-L-selectin MAbs to their antigens on human PMN. Each MAb (10 μg/ml) was incubated with PMN at 4°C for 30 min in the absence of CneF (positive) or in the presence of different dilutions of CneF. A 1:4 dilution of CneF was equivalent to 1 mg of carbohydrate per ml, a 1:8 dilution was equivalent to 0.5 mg of carbohydrate per ml, and a 1:16 dilution was equivalent to 0.25 mg of carbohydrate per ml. The MAb bound to PMN was detected by an indirect immunofluorescence staining procedure and flow cytometric analysis. Controls consisted of PMN treated with an isotype-matched irrelevant MAb. Data are from a representative experiment. The experiment was repeated two times with similar results.

Identification of CneF components which blocked the binding of anti-CD18 MAb to CD18 on the surfaces of human PMN. It is well documented that CneF is composed of at least three components (27). The major component is GXM. Two other minor components are GalXM and MP. As shown in Fig.

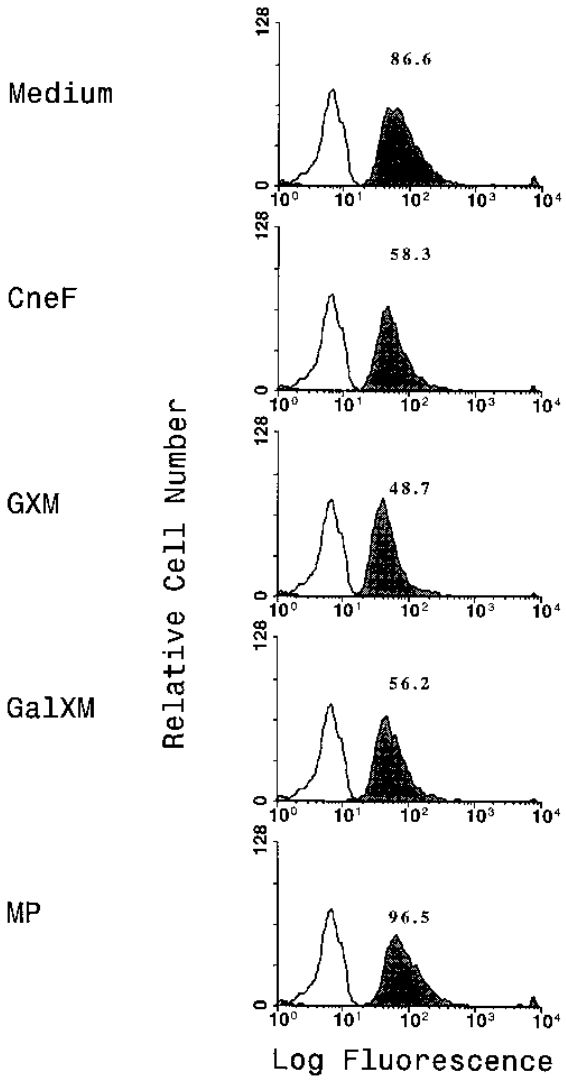

FIG. 2. Effects of CneF, GXM, GalXM, and MP on the binding of anti-CD18 MAb to CD18 on human PMN. The anti-CD18 MAb (40 μg/ml) was incubated with PMN at 4°C for 30 min in the presence of medium, CneF, GXM, GalXM, or MP. The MAb bound to PMN was detected by indirect immunofluorescence staining and flow cytometric analysis. Shaded areas indicate cells labeled with anti-CD18; unshaded areas represent cells binding the IgG1 isotype control. The number above each shaded area is the mean fluorescence intensity for that peak. CneF (1:32 dilution) had a carbohydrate concentration of 0.125 mg per ml. The concentration of GXM, GalXM, and MP was 0.125 mg (dry weight) per ml. Data are from a representative experiment. The experiment was repeated two times with similar results.

2, both GXM and GalXM at a concentration of 0.125 mg (dry weight) per ml displayed similar levels of activity in blocking the binding of the anti-CD18 MAb to PMN ($P < 0.01$ compared to the medium control), and the blocking was similar to that observed when CneF at a concentration of 0.125 mg of carbohydrate per ml was used. The reduction in the binding of the anti-CD18 MAb to PMN in the presence of CneF, GXM, or GalXM was 30 to 40% as compared to binding in medium alone. In contrast, MP at 0.125 mg (dry weight) per ml did not affect the binding of the anti-CD18 MAb to CD18 on the surface of PMN ($P > 0.05$).

Effects of CneF and individual components of CneF on the binding of anti-CD18 MAb to CD18 on the surfaces of FMLP-activated PMN. It is known that CD18 complexes are activation-dependent adhesion molecules, and according to a recent

TABLE 2. Effects of CneF, GXM, GalXM, and MP on binding of anti-CD18 and anti-CD11a MAbs to FMLP-activated PMN[a]

PMN	Blocking agent	Mean channel fluorescence intensity[b] with:			
		Anti-CD18		Anti-CD11a	
		Expt 1	Expt 2	Expt 1	Expt 2
Nonactivated	Medium	17	16	12	14
Activated[c]	Medium	40	32	13	13
	CneF	29 (28)	22 (31)	14	14
	GXM	24 (40)	21 (34)	14	16
	GalXM	25 (38)	22 (31)	14	14
	MP	38	36	14	16

[a] An anti-CD18, anti-CD11a, or irrelevant isotype (IgG1) control MAb was incubated with PMN in the presence of medium, CneF, GXM, GalXM, or MP (125 μg/ml) at 4°C for 30 min, and then each MAb bound to PMN was detected by indirect immunofluorescence staining and flow cytometric analysis.

[b] Values in parentheses indicate the percent reduction in binding of MAb to PMN in the presence of CneF, GXM, or GalXM as compared to binding in medium.

[c] PMN were activated by incubation of PMN with FMLP at 37°C for 30 min.

PMN-endothelial cell interaction model (7, 33), the CD18 complexes on PMN are activated by specific chemoattractants. The activation of the CD18 complexes on PMN results in strong binding to the appropriate ligands on the endothelium. To mimic the activation conditions, we treated PMN with FMLP for 30 min at 37°C and then assessed the surface expression of CD18 and CD11a. Results similar to those shown in Table 1 were observed. FMLP up-regulated CD18 but not CD11a expression on human PMN (Table 2). In the absence of CneF, GXM, or GalXM, more anti-CD18 MAb bound to FMLP-activated PMN than bound to unactivated PMN ($P < 0.01$). However, in the presence of CneF, GXM, or GalXM but not MP, the amount of anti-CD18 MAb bound to FMLP-activated PMN was reduced by approximately 30 to 40% compared to that of controls. These results were similar to those that we had obtained when unactivated PMN were treated with CneF, GXM, GalXM, or MP and the anti-CD18 MAb for 30 min at 4°C. In the same experiments, none of the polysaccharide preparations affected the binding of the anti-CD11a MAb to FMLP-activated PMN.

Effects of anti-CD18 MAb on the binding of ^{14}C-labeled CneF to human PMN. To confirm that CneF was binding to CD18, a ^{14}C-labeled CneF preparation was made and used in the binding assay. As shown in Fig. 3, ^{14}C-labeled CneF could bind to PMN at 4°C. Within the concentration range studied, the amount of ^{14}C-labeled CneF bound to the PMN was directly related to the concentration of ^{14}C-labeled CneF in the assay. When an anti-GXM MAb was added, the binding of ^{14}C-labeled CneF to the PMN was partially blocked (at a carbohydrate concentration of 0.031 mg per ml, $P > 0.05$; at 0.063 mg per ml, $P < 0.05$; and at 0.125 mg per ml, $P < 0.01$), indicating that GXM, the major component of CneF, was participating in the binding. Pretreatment of PMN with the anti-CD18 MAb, but not with the isotype-matched control or anti-CD11a MAb, significantly reduced ($P < 0.01$) the amount of ^{14}C-labeled CneF bound to the PMN as compared to the amount of ^{14}C-labeled CneF binding in the absence or presence of the relevant isotype control antibody (Fig. 4). The reduction in the binding of ^{14}C-labeled CneF to PMN in the presence of anti-CD18 MAb was 30%. These results indicate that CD18 is one receptor on PMN through which CneF or its components can bind.

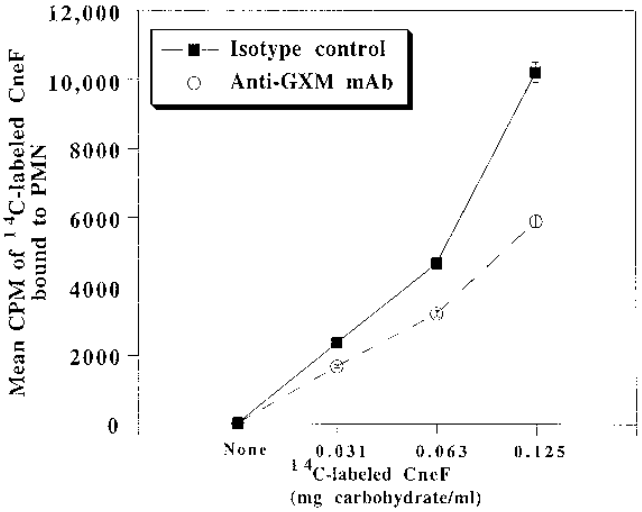

FIG. 3. Effects of anti-GXM MAb on the binding of ^{14}C-labeled CneF to human PMN. ^{14}C-labeled CneF was incubated with the IgG1 isotype control (40 μg/ml) or anti-GXM MAb (40 μg/ml) as described in Materials and Methods. After incubation and centrifugation, the supernatants were added to the PMN, and cell suspensions were incubated at 4°C for 30 min. The amount of ^{14}C-labeled CneF that bound to human PMN was detected by measuring the radioactivity on the PMN with a liquid scintillation counter. Data are from a representative experiment and are reported as the mean counts per minute for duplicate samples. The experiment was repeated two times with similar results.

DISCUSSION

PMN possess a variety of cell surface receptors which are involved in the different responses and have different functions (4). Among these receptors, adhesion molecules have been characterized as a group of molecules which play an important role in mediating PMN-endothelial cell interactions and extravasation of PMN. The best-characterized adhesion molecules on PMN are L-selectin and the heterodimeric integrins of the β2 (CD18) family (7, 33). L-selectin mediates the initial contact of PMN with an inflamed endothelium by binding to its unidentified ligands on the surface of the endothelial cells (21), whereas the CD18 complexes, such as LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18), mediate a stronger adhesion of PMN to the endothelium by binding to their ligand ICAM-1 (CD54) on the surface of the endothelial cells (7, 9). After finding that PMN did not migrate into foci of known chemoattractants when cryptococcal polysaccharides were in the bloodstream (13), we began studies to define the mechanisms associated with this phenomenon. We performed a series of experiments to examine the effects of cryptococcal polysaccharides on surface expression of L-selectin and CD18 on PMN and to determine the ability of cryptococcal polysaccharides to block binding of antibodies to L-selectin or CD18 to human PMN.

In this study and a previous study (14), we found that CneF and its major component GXM induce a loss in L-selectin from the surface of human PMN when stimulated with the cryptococcal antigens for 1 h at 37°C. The reduction in PMN surface L-selectin induced by GXM was due to shedding of L-selectin from the membrane as evidenced by the fact that surface L-selectin reduction was accompanied by an increase in L-selectin in the supernatant from PMN treated with GXM (14). It is well documented that stimulation of PMN with most chemoattractants induces parallel down-regulation of L-selectin and up-regulation of the surface CD11b/CD18 complex (22, 23). Consistent with this concept, in this study we showed that FMLP, a well-characterized chemoattractant, down-regulated L-selectin and up-regulated CD11b/CD18 expression on human PMN. CneF and GXM have been found to directly induce migration of PMN in vitro (15) and to induce migration of PMN in vivo when the CneF or GXM is injected into a gelatin sponge implanted in mice (13). Consequently, CneF and GXM act as chemoattractants. When CneF or GXM is injected into the bloodstream, these cryptococcal antigens inhibit PMN influx into inflammatory sites (13), which is another characteristic typical of chemoattractants. In in vitro experiments, CneF and GXM activate PMN to shed surface L-selectin in a manner similar to that of other well-known chemoattractants, such as FMLP, interleukin-8, and C5a (19, 24, 31). Considering the similarities of CneF and GXM to known chemoattractants, it seems reasonable to predict that CneF or GXM would up-regulate CD11b/CD18 expression on human PMN. Indeed, this is the case, because after stimulation with CneF or GXM for 1 h at 37°C, PMN expressed higher levels of surface CD11b than did PMN incubated similarly in the absence of CneF or GXM. However, to our surprise, considering that chemoattractants typically up-regulate both CD11b and CD18 simultaneously (22, 23), we found that under the same conditions in which CD11b was up-regulated, neither CneF nor GXM up-regulated CD18 expression on PMN.

As mentioned earlier, CD18 on the leukocyte cell surface is noncovalently associated with three CD11 molecules. These complexes are the three forms of β2 integrins, i.e., LFA-1 (CD11a/CD18), Mac-1 or CR3 (CD11b/CD18), and p150,95 (CD11c/CD18) (18). Expression of the β2 integrins is restricted to leukocytes, but the distribution of CD11/CD18 differs among the leukocyte types. For instance, peripheral blood lymphocytes express primarily CD11a/CD18, whereas PMN, monocytes, and NK cells express all three of the β2 integrins (8). Intracellular storage pools of CD11b/CD18 and CD11c/CD18 are present in PMN and monocytes, but there are no storage pools of CD11a/CD18 (8, 9). Surface expression of

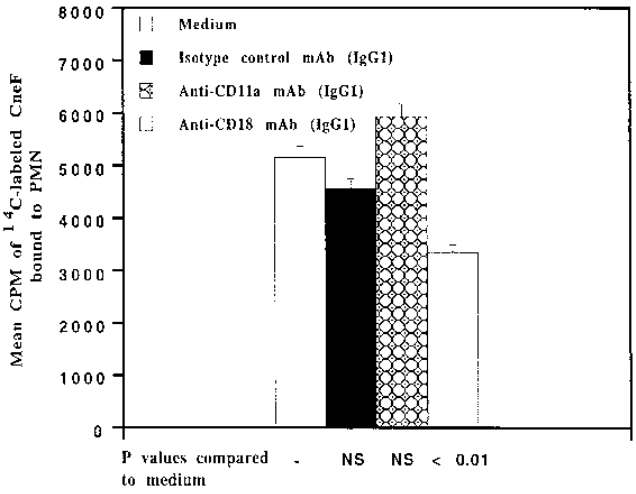

FIG. 4. Effects of irrelevant IgG1 isotype control, anti/CD11a, or anti/CD18 MAb on the binding of ^{14}C-labeled CneF to human PMN. PMN were preincubated with the isotype control protein, anti/CD11a MAb, or anti/CD18 MAb (10 μg/ml) at 4°C for 30 min. After being washed, the PMN were treated with ^{14}C-labeled CneF at a 1:4 dilution (0.063 mg of carbohydrate per ml) for 30 min at 4°C. The ^{14}C-labeled CneF that bound to human PMN was detected by measuring the radioactivity on the PMN in a liquid scintillation counter. Data are from a representative experiment and are reported as the mean counts per minute (with standard errors of the means) for quadruplicate samples. The experiment was repeated four times with similar results. NS, not significant.

CD11b/CD18 and CD11c/CD18 but not CD11a/CD18 on PMN is increased by a variety of agonists (9). Up-regulation of CD11b/CD18 expression is associated with degranulation of PMN (9). In the present study, our findings that FMLP stimulated PMN to express more CD11b/CD18 but not CD11a are consistent with those of others (9). The increase in CD11b on CneF- or GXM-activated PMN would be expected to be associated with an increase in expression of CD18; however, this was not the case. The level of CD18 on the surface of PMN activated by treatment with CneF or GXM at 37°C for 1 h was similar to control levels.

Based on the above-described analysis, it seems possible that CneF and GXM bind to CD18 and block the binding site for the anti/CD18 MAb. This would give the appearance that CD18 expression was not up-regulated even if CneF or GXM stimulated an increase in CD18 on the PMN surface. Consequently, one objective of this study was to determine if CneF or components of CneF were capable of blocking anti/CD18 MAb binding to CD18 on the PMN surface. When PMN were treated at 4°C with CneF, GXM, or GalXM for 30 min, the amounts of anti/CD18 MAb that bound to unactivated and FMLP-activated PMN were significantly decreased. MP did not alter the binding of the anti/CD18 MAb to PMN. In the same experiments, CneF did not affect binding of other MAbs, such as anti/CD11a or anti-L-selectin, to their antigens on PMN. Since anti/CD11a binding was not blocked by CneF, it seems unlikely that CneF nonspecifically interferes with β2 integrin molecules on PMN. The anti/CD18 MAb used in this study does not bind to encapsulated cryptococci (data not shown), so the cryptococcal capsular polysaccharides are probably not associating with the anti/CD18 MAb, thereby preventing anti/CD18 from binding to CD18. Our findings suggest that CneF and GXM bind to CD18 on the PMN.

Higher concentrations of CneF (0.5 or 1 mg of carbohydrate per ml) were not as effective in blocking the binding of the anti/CD18 MAb to PMN as were the lower concentrations of CneF (0.125 or 0.25 mg of carbohydrate per ml). Considering that cryptococcal polysaccharides are high-molecular-weight molecules with many side chains (11) and have the potential to polymerize, we suggest that at the higher concentrations the polysaccharide molecules may be aggregating with one another, making it difficult for the large polysaccharides to associate with CD18.

Evidence to support the conclusion that CneF can bind to CD18 was also obtained from binding assays with radioactive CneF. Because ^{14}C-labeled CneF is a mixture with at least three heterogeneous molecules, it is impossible to determine its avidity for binding to PMN. We did observe increased binding of ^{14}C-labeled CneF to PMN as we increased the concentration of labeled CneF in the binding assay, indicating that CneF does indeed bind to human PMN. GXM, which is the major constituent of CneF, appears to be one of the components of CneF binding to the PMN, because an anti-GXM MAb partially neutralized the binding of ^{14}C-labeled CneF to PMN. Anti/CD18 but not anti/CD11a MAb was able to block the binding of ^{14}C-labeled CneF to PMN at concentrations of 0.125 mg (1:2 dilution) (data not shown) or 0.063 mg (1:4 dilution) of carbohydrate per ml, supporting the idea that cryptococcal antigen binds to CD18.

β2 leukocyte integrins have been demonstrated to be involved in different responses and have different functions. Mac-1 is characterized as the complement component C3bi receptor (CR3) and mediates CR3-dependent phagocytosis (26). Both LFA-1 and Mac-1 are involved in PMN binding to the endothelium (32). The significance of the β2 integrins in mediating PMN extravasion is emphasized by the fact that patients with a genetic defect in CD18 (leukocyte adhesion deficiency) fail to mobilize leukocytes to sites of inflammation (1). Furthermore, infusion of an anti/CD18 MAb into animals blocks migration of PMN into brains and peripheral sites in response to both noninfectious and infectious stimuli (3, 35). Therefore, it is reasonable to predict that cryptococcal polysaccharides, especially GXM and GalXM, through binding to the common β chain (CD18) of the three β2 integrins, may interfere with the functions of the β2 integrins, resulting in blockage of CR3-dependent phagocytosis and inhibition of PMN influx into inflammatory sites.

To our knowledge, these data are the first indication of cryptococcal polysaccharides binding to the CD18 molecule on human PMN. However, the finding that polysaccharides bind to CD18 is not unique. Other investigators have found that polysaccharide moieties from *Leishmania mexicana*, *Histoplasma capsulatum*, and *Escherichia coli* bind to the molecules containing CD18 (6, 20, 34).

GXM, GalXM, and MP are well-characterized molecules. GXM is composed of a linear α-1-3-linked mannose backbone with xylose residues linked β-1-2, glucuronic acid residues linked β-1-2, and *O*-acetyl groups possibly attached to C-6 (11). In GalXM, galactose instead of glucuronic acid is linked to a mannan backbone (10). MP is composed of a mannose-rich part (80%) and a peptide part (20%) (10). Since both GXM and GalXM, but not MP, can affect the binding of an anti-CD18 MAb to CD18 on the surface of PMN, it is reasonable to suggest that sugar residues with side chains of xylose and glucuronic acid or galactose in the configuration found in GXM or GalXM may interact with CD18.

It is well known that cryptococcal capsular polysaccharides, especially GXM, are readily detected in serum and spinal fluid specimens of patients with disseminated cryptococcosis (12). Indirect evidence suggests that both GalXM and MP are in patients' body fluids during infection (28). The concentration of CneF or GXM (0.125 mg/ml) which we found to block the binding of the anti/CD18 MAb to CD18 of PMN is equivalent to a cryptococcal antigen titer of 1:8,192 as determined by the standard latex particle agglutination assay. This concentration is within the range of cryptococcal polysaccharide concentrations reported to be in sera and spinal fluids from most AIDS patients with cryptococcosis and similar to the concentrations of cryptococcal polysaccharides found in some non-AIDS cryptococcosis patients (12, 16). For example, cryptococcal polysaccharide titers in sera from non-AIDS patients with cryptococcosis typically range from 1:64 to 1:32,768 (12). In patients with titers of 1:8,192 or greater, one would expect, based on our data, that PMN migration into infected tissues would be prevented due to blockage of CD18 by cryptococcal polysaccharides (12, 13). When titers of cryptococcal polysaccharides in serum reach exceptionally elevated levels, as seen in some AIDS patients with cryptococcosis (titers sometimes exceeding 1:100,000) (16), then one might expect PMN migration into infected tissues to be prevented by the polysaccharides causing L-selectin to shed from the surface of the leukocytes (14). In short, intravascular cryptococcal polysaccharides can block extravasation of neutrophils by different means depending on the concentration of the polysaccharide. The concentrations of CneF and GXM that block migration of neutrophils into tissues by blocking CD18 or by stimulating L-selectin loss are clinically relevant concentrations of cryptococcal polysaccharides.

In summary, the results obtained from this study, combined with the findings obtained from our previous work (14), indicate that the mechanisms responsible for inhibitory effects of cryptococcal polysaccharides on PMN influx into tissues are

complex. There are at least two mechanisms involved in cryptococcal polysaccharide-mediated inhibition of PMN migration. The first is down-regulation of surface L-selectin expression on PMN by cryptococcal polysaccharides (14). The second is binding of cryptococcal polysaccharides to CD18 on PMN, thereby blocking the interaction of CD18 with its ligands on the endothelium. Through induction of L-selectin loss from the surface of PMN, cryptococcal polysaccharides can prevent initial adhesion of PMN to the endothelium. Through binding to CD18 molecules, cryptococcal polysaccharides can block CD18 complex-mediated strong adhesion of PMN to the endothelium. Both L-selectin and CD18 interactions with their ligands on the endothelium are prerequisites for PMN migration through the endothelial cells. Inhibition of PMN attachment to endothelial cells by cryptococcal polysaccharides through these combined mechanisms would be expected to result in little to no PMN infiltration into infected tissues, as has been reported for disseminated cryptococcosis (17).

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