Cryptococcus neoformans and neutrophil migration

Cryptococcus neoformans en de migratie van neutrofiele granulocyten

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(met een samenvatting in het Nederlands)

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And since you know you cannot see yourself,
so well as by reflection, I, your glass,
will modestly discover to yourself,
that of yourself which you yet know not of.

W. Shakespeare

Aan Prof. W. Erkelens
Aan mijn ouders

Contents

CHAPTER 1

GENERAL INTRODUCTION

The capsular polysaccharides of *Cryptococcus neoformans* and their effects on phagocyte migration and inflammatory mediators

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Effects of the capsular polysaccharides of *Cryptococcus neoformans* on phagocyte migration and inflammatory mediators.

Contents

I. The fungus and capsular virulence

Cryptococcus neoformans is an encapsulated fungus that mainly causes infections in the immunocompromised host with T-cell defects (e.g., AIDS, lymphoproliferative disorders, immunosuppressive therapy), but which can also infect immunocompetent individuals[65,174]. The incidence of disseminated cryptococcosis has increased considerably in the last decades because of the AIDS pandemic, and still rises in developing areas such as Sub-Saharan Africa and Asia were highly active anti-retroviral therapy (HAART) and anti-fungal therapy are not readily available[14,96,204]. Inhalation of desiccated cryptococci from the surroundings is the generally accepted route of infection. From the lungs, the cryptococci might enter the bloodstream and spread hematogenously to the brain, which is the preferred site of infection, leading to an often life-threatening meningoencephalitis with a high relapse-rate[175.]

Two varieties are recognized, *C. neoformans* var. *grubii* and var. *neoformans*[75], which are predominantly found in temperate regions. Based on genetic characterization it has recently been recognized that the former third variety (*C. neoformans* var. *gattii*), which prevails in (sub)tropical regions, is a different species and is currently known as *Cryptococcus gattii*[243]. The varieties differ somewhat in virulence and in the clinical course of infection. The most frequent occurring serotype in AIDS-associated cryptococcosis is the *C. neoformans* var. *grubii*, whereas var. *neoformans* causes a minor percentage of infections[65,229]. *C. gattii* has a relative predilection for the immunocompetent host[236].

Numerous virulence factors of *C. neoformans* have been identified that contribute to the infectivity of the fungus and favor its survival in the host (reviewed in[18,21]). One unique virulence factor is the thick antigenic polysaccharide capsule. Several experimental animal studies have demonstrated that capsular strains induce more severe infection when compared to acapsular mutants[80,140]. The identification of several genes that are involved in capsule synthesis and the consecutive targeted disruption as well as complementation experiments have further improved the insight into the relevance of the capsule for virulence[28-31,258]. More recently, Fries and Goldman were able to demonstrate the ability of cryptococci to change its capsule size and composition *in vitro* as well as *in vivo* during infection, leading to changes in polysaccharide composition and resulting in enhanced virulence and lethal outcome in mice[78,79,85].

Capsular virulence is acquired by a number of mechanisms. First, the capsule is able to mask the fungus from host phagocytes[133,136,166,221]. Second, during infection its polysaccharide components are abundantly shed into the blood and other body fluids of the patient[51,71,72], from where they modulate several aspects of the host-response, which are described in section IV and V. High serum titers of polysaccharides have been associated with progressive disease in humans[51,89]. Murphy *et al* demonstrated that this is not merely due to a higher burden of organisms by showing that the injection of additional polysaccharides in an animal model of cryptococcosis worsened the outcome of the infection[193]. In addition, pulmonary inoculation with a cryptococcal isolate that produces high serum polysaccharide levels caused more severe infection in mice when compared to a low-producing strain[10].

Several lines of research have associated the presence of limited inflammatory responses during cryptococcosis with heavy encapsulation or highly virulent strains[5,73,77,95,123,126,224]. The research outlined in this thesis has focused on the contribution of the capsular polysaccharides to this reduced leukocyte recruitment. Earlier research on this topic is summarized in this review in section **V**.

II. Structure of the capsule and its polysaccharide components

The cell envelope of *C. neoformans* consists of a rigid cell wall composed of glucans and a polysaccharide capsule. The capsule is mainly composed of polysaccharides, although enzymes and proteins are also present. Three-dimensional studies of the capsule show a dense web of intertwining polysaccharide strands attached to the cell wall[226]. Capsule growth depends on both genetic and environmental factors and its thickness may vary between 1 μm and 50 μm[69,91]. Whereas low iron concentration, high temperature, high CO2 content and neutral pH are the optimal conditions for capsule growth, an acidic pH or high osmolarity will lead to a decreased capsule size[69,91,247,262]. In the environment, the yeast cells possess minimal capsules. It has been generally accepted that the inhaled yeast starts to generate a thick capsule during the course of infection cryptococci[262]; while new material is added from the inside, the older material is relocated outwards[213].

Although the assimilation process of the capsule remains insufficiently understood (reviewed in[55,165]), several genes that are important for capsule synthesis and virulence have been identified (CAP59, CAP64, CAP60, CAP10, and the STE12-alpha gene[28-32]). Additionally, the recognition of genes encoding the major cyclic AMP (cAMP)-dependent protein kinase catalytic subunit (PKA1 gene) and the protein kinase A (PKA) regulatory subunit (PKR1 gene) has revealed the importance of the G-alpha protein-cAMP-PKA signaling pathway in the regulation of capsule synthesis[44]. Disruption of any of these genes leads to decreased virulence.

The major polysaccharide component of the capsule is glucuronoxylomannan (GXM; 88%); the two minor carbohydrate antigens are galactoxylomannan (GalXM; 10%) and mannoprotein (MP; 2%)[35].
The reported molecular weight of GXM varies considerably between 10 and 1400 kDa, depending on the methods used for purification and sonication. Biochemically, GXM consists of a polymannose backbone (linear (1→3)-α-D mannopyran; Man*p*) with β-linked monomeric side chains of glucuronic acid (β-D-glucopyranosyluronic acid; Glc*p*A) and variable amounts of xylose (β-D-xylopyranosyl; Xyl*p*) (Figure 1[9,21,244]). Several important enzymes and genes involved in the synthesis and assimilation of mannose and xylose have been recognized[6,54,183,259].
The mannan backbone of GXM is modified by 6-*O*-acetylation (Figure 1B), and the arrangement of 6-*O*-acetyl substituents determines the antigenic activity[7]. GXM elicits an antibody response in the host and *O*-acetyl is the major epitope for antibody binding[37,70]. Janbon and coworkers recently identified a gene involved in GXM *O*-acetylation that encodes for a putative glucosyl transferase (*CAS1*)[121].

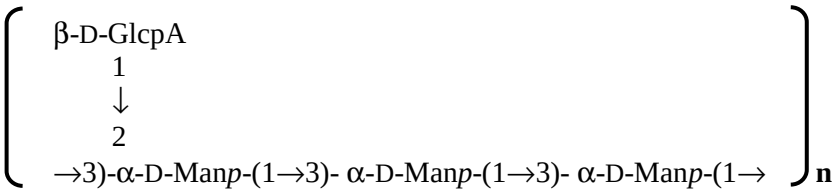
Historically, *C. neoformans* strains were classified into four serotypes (Serotypes A to D) according to the degree of *O*-acetylation and xylose substitution of GXM, which determine the antigenic activity. However, currently this classification is heavily doubted, since even within these serotypes variations in structure (ratio of xylose: mannose: glucuronic acid) and function were recognized. Cherniak[39] recognized six structural motifs in GXM (structure reporter groups; Figure 1C) on the basis of which eight chemotypes could be discerned, which has blurred the boundaries between the serotypes (Figure 1D). Based on genetic characterization it has recently been recognized that the former third variety (*C. neoformans* var. *gattii*; former serotypes B and C), which prevails in (sub) tropical regions, is a different species (now *Cryptococcus gattii*)[243].

GalXM consists of a heterogeneous group of polysaccharides composed of Xyl, Man, and Gal and has a more complex structure than GXM. GalXM is a branched polysaccharide with a α(1,6)-galactan backbone. Oligosaccharide side chains originate at the *O*-3 position of alternate Gal units and consist of β(1,3)-linked galactose, α(1,4)-linked mannose and α(1,3)-linked mannose, substituted with zero to three terminal β–Xyl residues[245]. *O*-acetyl groups are also present on GalXM, but the residue that is modified is unknown[245].

Mannoproteins (MPs) encompass the other minor component of the capsule. Like GalXM, most of the MP is present in the inner layer of the cell wall from where it diffuses towards the outer part of the capsule[248]. The main component of MP is mannose, although Gal and Xyl are also present[27,38]. The exact structure of MP is not yet known, but different MPs have been discerned based on their elution patterns in affinity, anion exchange and size chromatography; they differ in molecular mass, protein, and sugar ratios.
The first MPs described were named according to the order in which they eluted from an affinity chromatography column (MP-1, MP-2, MP-4). The molecular weights of these MPs are 35.6 kDa (MP-1), 8.2 kDa (MP-2) and 18-26 kDa (MP-4), respectively[255]. More recently, three more mannoproteins and their encoding genes have been have been identified and named after their molecular weight (MP-88, MP-98, MP-105[108,147,217]).

GXM, GalXM and MPs are all excreted by cryptococci into culture medium[36,41,245] as well as into serum and other body fluids of infected patients[8,33,41,51]. The presence of GXM in serum or cerebrospinal fluid of patients is a diagnostic marker of the disease[8,13,235]. Serum levels of GXM tend to be higher in AIDS patients than in non-HIV-infected individuals and titers corresponding with levels up to several milligrams per ml (1 mg/ml=titer 1:125.000) have been measured[51,72,169,227,257]. Usually, however, these levels are a hundred fold lower[51,72,169]. The half-life time of GXM in serum ranges between 14 and 48 hours[71,87,94,144,185]. In contrast, GXM is retained in the organs for weeks[144,185], especially in the liver and spleen where it is stored in the macrophages[94]. Studies have also revealed that macrophages[94] and anti-capsular antibodies[87,144] participate in GXM clearance. GalXM, MP-1, MP-2 and MP-4 have been detected in the serum and CSF of patients as well[41,219], however, little is yet known about their tissue localization and clearance.

Figure 1A. The core repeating structure of GXM



1B. Site of backbone *O*-acetylation

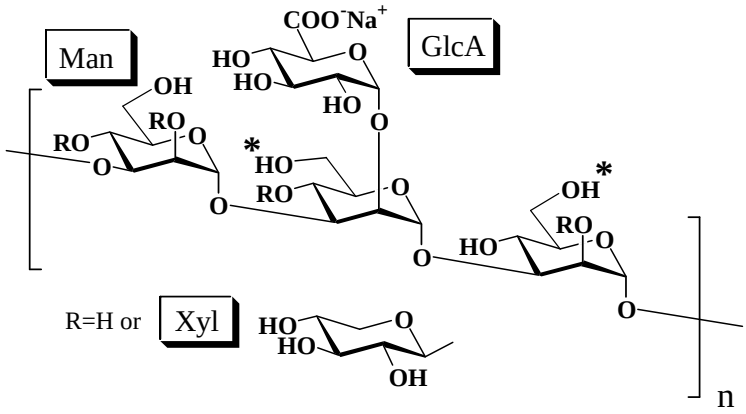
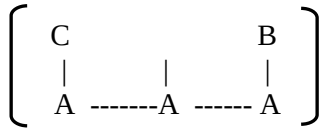


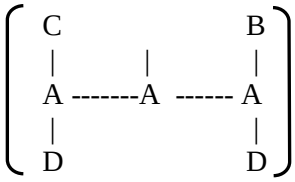
Figure 1. Structure of GXM[39].
1A. Schematic representation of the repeating core-unit of the linear mannose backbone.
1B. GXM is variably acetylated at the 6-*O* position of the mannose residues, where the hydrogen (H) is replaced by an acetyl group (*).
1C. The six "structure reporter groups (SRG)". These are structural motifs found within GXM molecules that are distinguished by the pattern of side-chain substitution.
1D. The eight GXM chemotypes are differentiated by the most frequent repetitive SRG or SRG combination. An approximate correlation is provided between the chemotypes and the older system of serotyping which depends on the degree of *O*-acetylation and xylose substitution**.**

1C. Structure reporter groups

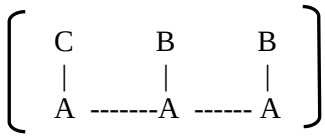
M1



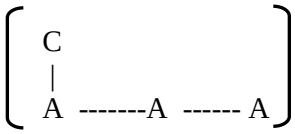
M5



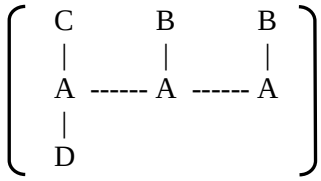
M2



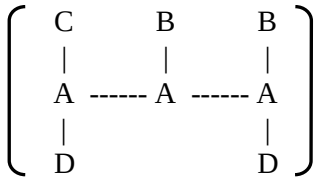
M6



M3



M4



A = α-D-Man (1→3)
B = β-D-Xylp (1→2)
C = β-D-GlcpA (1→2)
D = β-D-Xylp (1→4)

1D. The 8 chemotypes of GXM

Chemotypes:	**Structure Reporter groups:**		**Corresponding to serotypes:**
1	M1	M5/M6	D
2	M1>M2	M5/M6	D, A
3	M1>M2>M3	M5/M6	A
4	M2	M6	A
5	M2>M3	(M6)	A
6	M3>M2>M4		C
7	M3		B, (C)
8	M3>M4		B, C

III. The host response during cryptococcosis

Encounters between the human host and cryptococci will not be rare due to the common presence of these fungi in our environment (reviewed in[145]). The inhalation of aerolized basidiospores or poorly encapsulated cryptococci is the generally accepted route of infection. If not cleared by the local cellular defenses in the lung, some cryptococci might eventually migrate into the bloodstream and preferentially disseminate to the brain by means still largely unknown, but ultimately resulting in meningoencephalitis. For years, it was assumed that the physical and innate defense mechanisms in the airways of an immunocompetent host would immediately clear the fungus after entrance. However, recent evidence points toward the ability of cryptococci to reside within alveolar macrophages for years (dormant infection) before causing infection[67,82,86,237].

Cell-mediated immunity is the cornerstone of the host's defense mechanisms against cryptococci. Intact phagocytosis (innate immunity)[83,182], T-cell function (adaptive immunity; reviewed in[195]) and the subsequent production of cytokines[2,43,46,106,116,207] and chemokines[112,113,115] have proven to be essential for protection against cryptococci and the prevention of dissemination.
The innate host-defense is the initial line of defense in the lower respiratory tract. Here, cryptococci are attacked and phagocytosed by alveolar macrophages[83,182], later assisted by neutrophils[131,162,170], monocytes[146], natural killer cells[194], and lymphocytes (T-cells, B-cells)[149,198,203], which are capable of killing or inhibiting cryptococci. Macrophages are the predominant cells in the process of phagocytosis and killing of cryptococci, which occurs with or without opsonisation[43,92,129,138,150]. Furthermore, macrophages produce proinflammatory cytokines and promote T-lymphocyte proliferation by presenting antigens of phagocytosed cryptococci to T-cells. In the brain, microglia, which are macrophage-like cells, and astrocytes additionally participate in local cellular anti-cryptococcal defense[3,12,143].

The most important element of nonspecific humoral immunity involved in anti-cryptococcal defense is the complement system, which provides opsonins (mainly C3) for phagocytosis and chemotactic factors (mainly C5a) for the recruitment of inflammatory cells. Nonencapsulated cryptococci activate complement through both the antibody-dependent classical pathway and the alternative pathway[137,139]. Encapsulated yeasts exclusively activate complement through the alternative pathway since their thick capsule is able to prevent the binding of antibodies to epitopes in the cell wall[137,139,260].

The adaptive (specific) cellular defense against *C. neoformans*, consisting of the development of T-cell immunity[195], has proven to be a prerequisite for initiating and activating a pulmonary inflammatory response, which ultimately clears the infection and controls dissemination [23,118,119,157]. Both CD4+ and CD8+ T cells participate in various aspects of the host's response to cryptococci[16,111,118,176-178]. They display direct fungistatic effects *in vitro*[149,198], recruit and subsequently activate phagocytic cells[111], and play a role in the development of an antibody response[186].

Capsular polysaccharides and proteins can elicit a specific humoral immune response[98,99,102]. These antibodies have been in infected individuals [63,102], but also in normal healthy subjects without a history of cryptococcosis[1,34,49,86]. Despite extensive research, the significance of these naturally occurring anti-cryptococcal antibodies is not clear. It is apparent, however, that anti-cryptococcal antibodies indeed function as opsonins and assist in the clearance of high levels of polysaccharide antigens[87,144].

Cytokine and chemokine responses link the innate and adaptive host responses and promote phagocytosis[161]. *In vivo* patterns of cytokine and chemokine responses in the defense against cryptococci depend highly on the experimental model used (reviewed in [110,112,117,195]). Successful clearance of cryptococci from the lungs has been associated with the production of macrophage and neutrophil-derived cytokines (TNFα, IL-1β, IL-12, and GM-CSF) that are able to stimulate macrophages, to induce chemokine production[125,126] and to induce protective Th1-type (IFNγ, IL-2) cell-mediated immunity[2,46,104,106,116,128]. Both Th1-type cytokine responses and early TNFα production appear to prevent dissemination to the brain[112]. However, Th2-related cytokines (IL-4, IL-5, IL-10) are also expressed in the early stages of infection [109]. Infections by highly virulent cryptococcal strains have been associated with a dominant non-protective Th2 response, low titers of the Th1 cytokines, IL-12, and TNFα as well as lethal infection in animal models[127,207].
The formation of chemoattracting gradients mediates the mobilization of leukocytes to the site of inflammation. Studies using knockout animals have shed some light on the complex interplay between chemokines and cells during experimental cryptococcosis (reviewed in[112,117]). During primary infection in the lungs, the C-C chemokines MCP-1, its receptor CCR2 and MIP-1α appear to be important players in the polarization towards Th-1 type cytokine responses, the recruitment of a cellular infiltrate and the control of dissemination to the brain[114,115,117,207,242]. During disseminated cryptococcosis MIP-1α and chemokine receptor CCR5 (which binds MIP-1α, MIP-1β and RANTES) mediate leukocyte recruitment to the brain[112,113] and are associated with the development of protective Th1 type mediated immunity. Other possibly important chemokines during disseminated cryptococcosis are RANTES, MIP-1β and IL-8 which are upregulated during cryptococcosis[24,154,158]. In the brain, these are produced by microglia upon stimulation with cryptococci[84,154]. The T-cell-derived C-C chemokine TCA3 primarily attracts neutrophils and plays a role in the anti-cryptococcal cell-mediated response[62]. Finally, complement factor C5a has been shown to be essential for the recruitment of neutrophils and for the clearance of cryptococci in murine models of cryptococcosis[66,160,223].

IV. Effects of the capsular polysaccharides on the host-response

Several mechanisms have been described by which the capsular polysaccharides are able to down-regulate the host response (reviewed in [18,21,196,249].
The capsule as well as isolated GXM have been shown to prevent the phagocytosis of cryptococcal cells by acting as a mechanical shield that is able to mask cell wall antigens or bound opsonins[133,136,166,221] and interfere with the presentation of antigens[42,221].

Thus, opsonisation with complement or specific antibodies appears to be important for the phagocytosis of encapsulated cryptococci[132,250]. Although encapsulated cryptococci are able to activate the complement pathways, purified GXM appears to be a weak stimulator *in vitro*[142].

Both mixtures of cryptococcal polysaccharides and isolated GXM have been demonstrated to suppress T-cell mediated immunity. First, interference with antigen presentation by phagocytes leads to the inhibition of lymphocyte proliferation[179,239]. Second, cryptococcal culture filtrate (CneF; containing cryptococcal polysaccharides) and GXM induce T-suppressor cell responses, which are associated with repressed delayed-type hypersensitivity reactions to cryptococcal antigens[11,130,199,200,202], decreased clearance of cryptococci during infection[193], inhibition of phagocytosis and differentiation towards non-protective Th2-type cytokine responses by lymphocytes[4,17].
In contrast, however, mannoproteins enhance several aspects of cell-mediated immunity. Mannoproteins are strongly immunogenic and are probably the main inducers of the delayed-type hypersensitivity phenomenon observed in human and experimental cryptococcosis[201]. Further, MP-1 and MP-2 have been shown to promote *in vitro* proliferation of lymphocytes and mononuclear cells in response to *Cryptococcus neoformans*[217] and are strong inducers of IL-12 production resulting in protective Th1 type cytokine responses by lymphocytes[214-216]. Both MP-88 and MP-98 activate T-cells[108,147].

GXM affects antibody responses in different ways. On one hand, purified GXM has been shown to be poorly immunogenic in animals and to induce antigen-specific immunogenic tolerance, which has been mainly related to the administration of high doses of GXM (> 400 μg)[15,197] and might be caused by the induction of T-suppressor cells[15]. This antibody unresponsiveness to cryptococcal polysaccharide has been demonstrated in cured cryptococcosis patients as well as in GXM-primed mice[15,102,103,134,197,238]. On the other hand, vaccination with GXM-protein conjugates has shown to elicit an antibody response[20,50] and passive immunization with anti-GXM antibodies in experimental cryptococcosis has resulted in improved survival, enhanced clearance of cryptococci [64,186,189-191,228] and enforcement of anti-fungal therapy[88,187]. These antibodies have been shown to increase phagocytosis and killing[93,192], to activate complement[120], to assist in GXM clearance from tissues[87,144], and to modulate cellular responses[93, 252]. The protective nature of these anti-GXM antibodies has raised the interest in the production and the application of a vaccine in human cryptococcosis[19,22,74]. Even though GalXM (~100 kDa) elicits an antibody response[219,246], it appears to play a minor role in virulence.

Furthermore, cryptococcal polysaccharides enhance *in vitro* HIV replication[101,208,209,211,212] by inducing TNFα secretion[101] as well as by TNFα independent mechanisms[208]. Finally, cryptococcal polysaccharides affect cytokine production and interfere with leukocyte migration by several mechanisms, which are discussed in the next chapters.

V. The effects of *C. neoformans* and its capsular components on the events leading to leukocyte recruitment

The inflammatory tissue responses in cryptococcal disease may vary considerably from scant infiltrates to fulminant inflammatory reactions. Several lines of research have associated the presence of limited inflammatory responses during cryptococcosis with heavy encapsulation or highly virulent strains[5,73,77,95,123,126,224]. First, although acapsular strains are rarely isolated in clinical infections, a few case reports have described fulminant inflammatory responses in patients infected by poorly encapsulated C. neoformans strains, as opposed to much milder reactions in infections caused by encapsulated strains[5,73,95]. Second, the induction of infection in mice with various cryptococcal strains differing in capsule size revealed that the magnitude of the inflammatory response is inversely correlated to capsule size[123]. Finally, the administration of hyaluronidase - a compound capable of disrupting the capsule - to rats infected with a capsular strain led to stronger inflammatory reactions in these animals[218].

The observation of limited inflammatory responses in the infected tissues during cryptococcosis[5,73,77,95,123,224] as well as the large quantities of polysaccharides present in the circulation of infected patients[51,71,72], have brought about the theory that these polysaccharides interfere with the recruitment of leukocytes into inflammatory sites. Leukocyte migration requires intact chemotaxis by chemotactic substances produced at the site of inflammation and unhindered transmigration of the endothelium (Figure 2). In the last decade, a substantial number of reports have focused on how cryptococci and their capsular polysaccharides affect the events involved in leukocyte migration, such as cytokine and chemokine production, chemokinesis, and leukocyte adhesion to endothelium (Figure 2, Table 2).

(a) Interference with leukocyte migration *in vivo* ('Proof of the principle')

Dong *et al*[59] implanted gelatin sponges into mice, subsequently injected the sponges with CneF, fMLP, or TNFα, and analyzed the leukocyte infiltration after retrieval. In these experiments, the intravenous administration of either CneF or GXM to the mice considerably inhibited leukocyte infiltration into the sponges. Further, Lipovsky demonstrated a correlation between a high ratio of GXM in serum/CSF and a low CSF leukocyte count[156]. A single dose of GXM administered intravenously to a rabbit with bacterial meningitis led to a significant delay in the influx of leukocytes into the CSF as well as a reduction in the TNFα levels in the CSF and a reduction in inflammatory damage[155]. Finally, the anti-inflammatory properties of CneF were confirmed in models of experimental septic arthritis, immune complex glomerulonephritis and Adriamycin-induced nephropathy in rats, in which the intraperitoneal or intravenous administration of CneF resulted in a significant decrease in both leukocyte infiltration and disease-related tissue destruction[171-173].

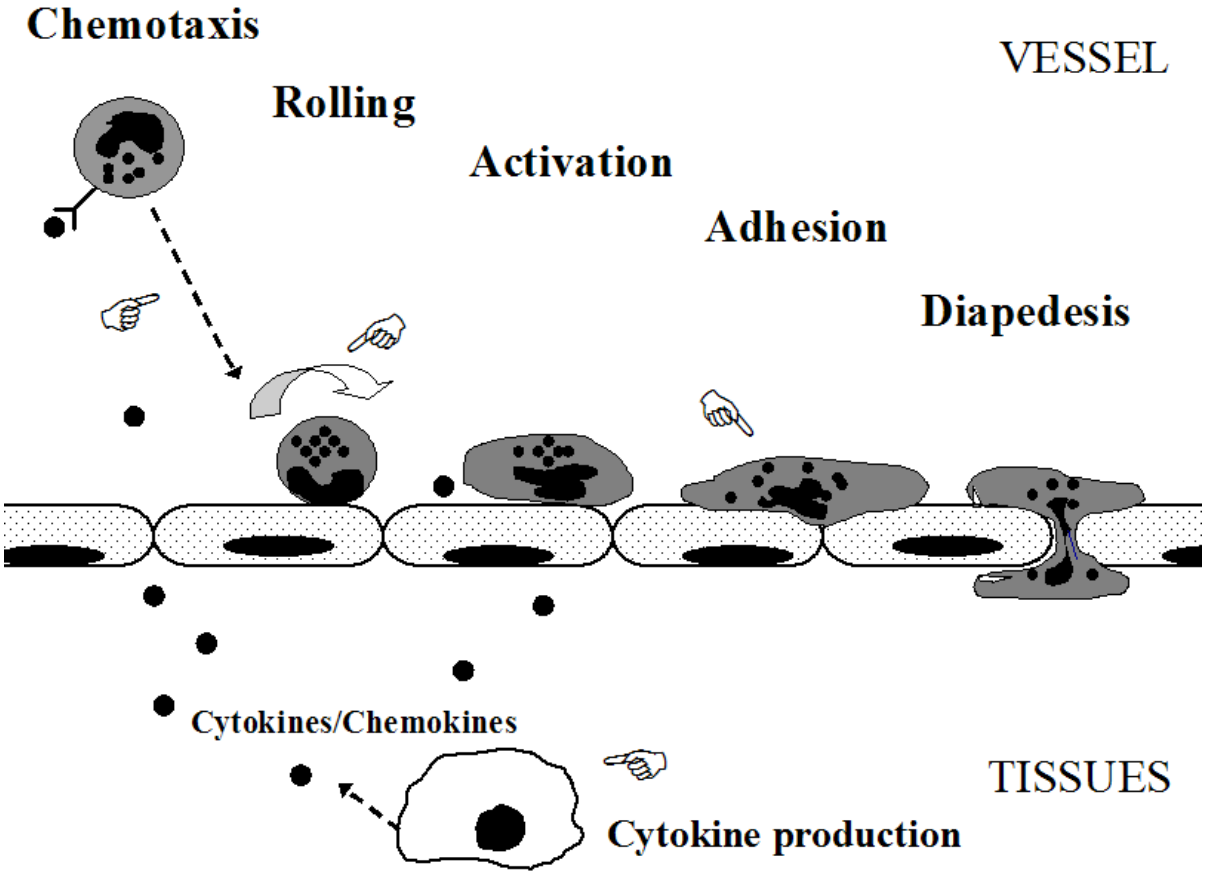


Figure 2. Cryptococcal polysaccharides interfere with leukocyte migration at different stages.
The different phases of leukocyte migration are shown. Circulating leukocytes are attracted to sites of inflammation by the local production of chemokines, which form chemotactic gradients. Leukocyte adhesion is initiated by activation of the endothelium by cytokines, leading to endothelial cytokine production and upregulation of surface adhesion molecules. The first step of adhesion is the margination and rolling of leukocytes on the activated endothelium, which depends on weak "on and off" interactions between selectins (E-selectin on endothelium, L-selectin on leukocytes, and P-selectin on platelets and endothelium) and their ligands. The leukocytes are then activated by cytokines, which results in the shedding of L-selectin from the leukocyte surface and the increased expression of integrins (e.g., CD11b/CD18 and VLA-4) on the surface. The integrins are involved in the subsequent firm binding of leukocytes to the ligands expressed on the endothelium (i.e., the ICAMs and VCAMs), after which the cells finally transmigrate. The "hands" indicate the different processes involved in leukocyte migration and adhesion that are affected by cryptococcal polysaccharides. In this example, grey cells represent migrating PMN, dotted cells are endothelial cells, the white cell a macrophage.

(b) Capsular polysaccharides and cytokine production

The formation of a cellular inflammatory response in the early stage of infection depends on the production of cytokines by mononuclear cells (macrophages and monocytes). These mediators enable the recruitment and activation of other inflammatory cells[122] and induce the expression of adhesion molecules on the endothelium that are involved in leukocyte transmigration[97,100].

Several studies have demonstrated cytokine responses in lung fluid, blood, and cerebrospinal fluid during cryptococcosis in patients and murine models[24,127,158,159]. The magnitude of *in vivo* cytokine responses depends highly on strain virulence (see[110] for further reading). The

effects of purified cryptococcal components, however, have mainly been assessed *in vitro*. Due to the variability of the experimental conditions as well as the complexity of cytokine production, which depends partly on auto regulation, the results are difficult to interpret and extrapolate to the *in vivo* situation. Chaka *et al* demonstrated that different experimental conditions, such as the fungus-to-effector ratio, the intensity of contact between effector and target cells, and the working volume, affected the kinetics of TNFα release and thus the detected levels [25]. Table 1 represents an overview of the pro-inflammatory and anti-inflammatory effects related to cytokine production.

Virtually all *in vitro* studies have demonstrated that whole cryptococci as well as the isolated polysaccharides (GXM, GalXM, and MPs) stimulate human monocytes to various degrees to produce the proinflammatory cytokines IL-12, TNFα, IFN-γ, IL-1β, and IL-6 in the presence of active serum (Table 1;[25,27,47,48,148,151,153,214,216,222,254,255]). Nearly all of the studies demonstrated that the production of proinflammatory cytokines by monocytes is mainly induced by acapsular and thinly encapsulated cryptococci rather than by heavily encapsulated fungi (Table 1**;**[47,48,151,254]). The observation by some reports that MPs are more potent stimulators of monocyte cytokine production than GXM and GalXM[27,47,48] is in line with the stronger cytokine response to acapsular cryptococci, which might be explained by a better exposure of MPs present in the cell wall[248]. However, in conflict with these data, our research group showed that GXM, GalXM, and MPs evoke quite similar patterns of cytokine production by monocytes[255]. These results might be explained by a non-differentiated activation of the cytokine cascade by all of these polysaccharides via a common pathway, probably complement activation in the presence of serum. Interestingly, whereas proinflammatory cytokine production by monocytes is predominantly stimulated by acapsular strains, heavily encapsulated strains and GXM are stronger stimulators of cytokine release by PMN when comparing to acapsular cryptococci[222]. This indicates that GXM, which is absent in acapsular cryptococci, is most likely responsible for PMN stimulation. These results imply a different mechanism or pathway by which the different cryptococcal polysaccharides stimulate either PMN or monocytes.

Cryptococcal polysaccharides also induce anti-inflammatory cytokine responses (Table 1). GXM, GalXM, and MPs induce the production of the anti-inflammatory molecule IL-10 [148,152,253,255], which is capable of down regulating TNFα and IL-1β responses[45,152]. In contrast to pro-inflammatory cytokine responses, Vecchiarelli *et al*[253] showed that the release of the anti-inflammatory cytokine IL-10 by monocytes is mainly provoked by heavily encapsulated cryptococci rather than by acapsular variants and that GXM is responsible for this response. Vecchiarelli *et al* further demonstrated that the addition of GXM to mixtures of acapsular cryptococci and monocytes induced IL-10 production, which in turn inhibited TNFα and IL-1β release in a dose-dependent manner[253,254]. Strikingly, they also showed that GXM inhibited LPS-induced secretion of TNFα, but not of IL-1β[254]. Retini *et al* recently demonstrated that the addition of GXM to acapsular cryptococci inhibited IL-12 production by monocytes, which coincided with enhanced IL-10 production[220]. Thus, polysaccharide-induced IL-10 production might depress proinflammatory responses.

Another possible depressing effect on cytokine responses is the observation that GXM and MP-4 induce shedding of the TNFα receptors p55 and p75 from the surface of PMN[41,60], possibly leading to TNF scavenging in the circulation. This evidence suggests that cryptococcal polysaccharides could have important depressing effects on the host-response by various mechanisms.

	Pro-inflammatory						**Anti-inflammatory**	
	TNFα	**IL-1β**	**IFNγ**	**IL-12**	**IL-6**	**IL-8**	**IL-10**	**TNFαR**
GXM	↑	↑	↑	=	↑	↑	↑	↓
Gal XM	↑	↑	↑		↑	↑	↑	
MPs	↑	↑	↑	↑	↑	↑	↑	↓

Table 1. *In vitro* cytokine induction by purified capsular polysaccharides.

Most studies show that the stimulation of cytokine production by whole cryptococci and isolated cryptococcal polysaccharides depends on the presence of a heat-labile factor in the serum. This factor is most likely complement, which indicates that cytokine production is secondary to complement activation. This was confirmed by the complete abrogation of GXM-induced IL-8 secretion by antibodies to C3 and C5[251]. However, Chaka *et al* demonstrated that MP-1 and MP-2 stimulate TNFα production not only in the presence of active serum (i.e., active complement), but also in the presence of a heat-stable serum factor[27] which appeared to be human mannose-binding protein (hMBP)[26]. Serum hMBP not only activates the complement cascade, but also acts as an opsonin in the presence or absence of complement[76], thereby facilitating antigen binding and uptake by phagocytes.

In conclusion, GXM, GalXM and MPs induce proinflammatory cytokine production by monocytes and PMN *in vitro* in the presence of active complement or serum hMBP. However, depressing effects on cytokines and their receptors have also been reported, such as GXM-induced Th2-related cytokine responses and IL-10 production, the inhibition of LPS-induced cytokine production by GXM, and the shedding of TNF-receptors from the surface of neutrophils by GXM and MP-4. The net outcome of these effects *in vivo* is still unclear. Most likely, the results also depend on time and spatial differences. The application of GXM and MPs in models of bacterial infection or ischemia could provide valuable knowledge about the net effects on cytokine production and their anti-inflammatory properties.

(c) The effects of cryptococcal polysaccharides on chemokine production and receptors

Chemokines mediate the recruitment of effector cells during inflammation, by forming chemotactic gradients, which enable the attraction of leukocytes towards the inflamed tissues.

In vivo, increased levels of MCP-1, MIP-1α have been detected in murine models of pulmonary cryptococcal infection[114,115,241]. Il-8 is another potent chemoattractant of leukocytes and increased levels are present in cerebrospinal fluids of patients during disseminated cryptococcosis[158].

In vitro, whole cryptococci have been shown to provoke the production of MCP-1, MIP-1α, MIP-1β, RANTES and Il-8 by peripheral blood monocytes[53,107,251], and MIP-1α, MIP-1β, MCP-1, and IL-8 in human brain microglial cells[84]. Interestingly, Mozaffarian *et al*[184] showed that both encapsulated and acapsular cryptococci inhibit TNFα-stimulated MCP-1 and IL-8 production by endothelial cells, which is possibly related to the production of fungal prostaglandins[205,206]. Purified GXM, GalXM and MPs adequately induce IL-8 production by human monocytes[251,255], PMN[222] and microglia[154].

Recently, Monari *et al*[180] showed that encapsulated cryptococci down regulate the C5a receptor (C5aR) on the surface of neutrophils, while acapsular strains increase C5aR expression. Pretreatment of these acapsular mutants with GXM decreased binding of C5a to PMN as well as PMN migration towards C5a. Purified GXM slightly inhibited C5aR expression.

Thus, although whole cryptococci induce chemokine production, relatively little is known about the role of the polysaccharides, with the exception of IL-8 and C5a, which are clearly upregulated by all polysaccharides. Furthermore, encapsulated cryptococci reduce the receptor of C5a on the surface of PMN, and GXM might be the responsible compound.

(d) Chemotactic activities of *C. neoformans* and its capsular components

A number of *in vivo* studies demonstrated an influx of PMN and monocytes in response to an intraperitoneal injection of *C. neoformans*[81,105,124]. Dong *et al*[58] implanted gelatin sponges in mice and subsequently injecting the sponges with either buffer or cryptococcal culture filtrate antigens (CneF; a crude mixture of cryptococcal polysaccharides). The sponges were retrieved six hours later and the CneF-injected sponges contained a significantly larger number of leukocytes than sponges injected with buffer alone, indicating that cryptococcal polysaccharides are able to attract leukocytes.

In vitro cryptococci and their capsular polysaccharides also display both direct and indirect chemotactic properties. Cryptococci and their capsular polysaccharides generate a heat-labile chemotactic factor in serum (indirect chemotactic activity)[52,57,58,142,210]. This phenomenon is most likely the result of complement activation and the subsequent generation of chemotactic factors, such as C5a. Diamond *et al*[52] supported this hypothesis by showing that antibodies to C5 but not to C3 were able to eliminate the chemotactic response to *C. neoformans*-activated sera.

Reports on the direct chemotactic properties of cryptococci *in vitro* reveal conflicting data. Two studies by Laxalt and Perfect[142,210] were unable to demonstrate the direct movement of PMN and monocytes in response to cryptococci and a crude mixture of polysaccharides. Diamond *et al*[52] observed that an unrefined mixture of polysaccharides isolated from capsular CneF had minimal direct chemotactic capacities. In contrast, Dong *et al*[57,58] showed that both encapsulated cryptococci and their culture supernatants are substantially chemotactic for PMN in the absence of a serum factor. An acapsular strain, however, was not. The chemotactic abilities of the capsular polysaccharides might be structure-dependent; i.e., cryptococci, culture supernatants and GXM of *C. neoformans* var. *grubii* and var. *neoformans* (former serotypes A and D) directly attracted PMN, while those of *C. gattii* (former serotypes B and C) did not[57,58,154].
Dong and coworkers failed to demonstrate any chemotactic activity in a mixture of MPs at a concentration of 50 μg/ml[57], while we recently showed mannoprotein 4 (MP-4) to be a very potent chemoattractant for PMN[41], at concentrations between 3 and 30 μg/ml. Explaining this controversy, the MP mixture used by Dong *et al* presumably contained only MP-1 and MP-2, and not MP-4, which can be deduced from comparison of the isolation procedures[41,57]. The observation by Dong's group[57] that acapsular cryptococci and their culture supernatant failed to attract leukocytes while MP-4 (present in the cell wall of acapsular mutants) did attract leukocytes, might also be explained by differences in experimental procedures and used concentration.
In conclusion, the cryptococcal polysaccharides GXM and MP-4 have shown to attract leukocytes serum-independently; however, all cryptococcal polysaccharides induce a chemoattracting factor in serum, presumably by complement activation.

(e) Inhibition of leukocyte migration towards chemotactic stimuli

Drouhet *et al*[68] observed as early as 1951 that a crude mixture of cryptococcal polysaccharides inhibited *in vitro* leukocyte migration towards chemotactic stimuli. Over the years, other investigators confirmed this observation[41,52,58,154]. Importantly, all of the studies showed that neither CneF nor isolated polysaccharides affect the viability of leukocytes. Diamond *et al*[52] showed that the addition of high concentrations (>1 mg/ml) of a mixture of cryptococcal polysaccharides inhibited leukocyte migration towards cryptococci-activated serum. Dong *et al*[58] further demonstrated that incubation of PMN with CneF of *C. neoformans* inhibited migration towards fMLP by 30%, but not that towards fresh human serum. In their study, CneF of *C. gattii* completely abolished PMN migration towards both fMLP and fresh human serum containing C5a[58]. These results indicate that the polysaccharides of different strains might affect immune responses differently. Lipovsky *et al*[154] revealed that the incubation of PMN with GXM of *C. neoformans* almost completely blocked the migration of PMN towards IL-8. Finally, and more recently, Coenjaerts *et al* identified MP4 as a potent inhibitor of leukocyte migration toward chemoattractants, such as fMLP and IL8[41]. In these experiments, all other purified capsular components displayed, to various degrees, inhibitive activity on PMN migration towards the used chemoattractants[41]. This study also demonstrated that MP-4 inhibits Ca^{2+} transients in PMN caused by the binding of fMLP, IL-8, and C5a to their G-protein-coupled receptors and vice versa. This suggests that MP-4 inhibits the migration of PMN towards chemoattractants by cross desensitization of the involved receptors.

In conclusion, mainly GXM and MP-4 (and GalXM and MP-2 to a lesser extent) are able to inhibit leukocyte migration towards chemoattractants. The interference is presumably based on the polysaccharide's own chemoattracting properties, resulting in cross-desensitization of chemokine receptors, and additionally by down-regulation of chemokine receptors (i.e., C5a receptor). If the polysaccharide concentrations in the blood are higher than in the infected tissues, an inverse chemotactic gradient will exist between the circulation and the infected tissues, which prevents leukocytes from leaving the circulation and migrating towards the inflamed tissues.

(f) Cryptococcal polysaccharides and leukocyte adhesion to the endothelium

Another mechanism for the impairment of leukocyte migration to inflammatory sites in cryptococcosis could be the interference with leukocyte adhesion to the endothelium (Figure 2). In short, leukocyte adhesion is initiated by the activation of endothelium by cytokines (e.g., IL-1β and TNFα), which leads to endothelial cytokine production and the upregulation of adhesion molecules. The first step of adhesion is the margination and rolling of leukocytes on the activated endothelium, which depends on interactions between selectins (E-selectin on endothelium, L-selectin on leukocytes, and P-selectin on platelets and endothelium) and their ligands. The leukocytes are then activated by cytokines, which results in the shedding of L-selectin from the leukocyte surface and the increased expression of integrins (e.g., CD11b/CD18 and VLA-4) on the surface. The integrins are involved in the subsequent firm binding of leukocytes to the ligands expressed on the endothelium (i.e., the intercellular adhesion molecules [ICAM] and vascular cell adhesion molecule-1 [VCAM-1]), after which the cells finally transmigrate[234].

As described earlier, cryptococcal polysaccharides adequately stimulate cytokine production by mononuclear cells, which is a prerequisite for the upregulation of adhesion molecule expression on the endothelium. In contrast, whole cryptococci have shown to inhibit the cytokine production by endothelial cells[184], possibly due to the production of fungal prostaglandins[205,206]. The effects of the separate cryptococcal polysaccharides, however, on cytokine and chemokine production by the endothelium remain to be investigated.

Although data on the effects of cryptococcal products on leukocyte adhesion are limited, the results indicate that both GXM and MP-4 affect adhesion molecules on the surface of the leukocytes. Dong *et al*[56,60] were the first to note that GXM, and not MPs or GalXM, induced L-selectin loss from the surface of isolated T-lymphocytes and PMN in the absence of serum. In the presence of serum, however, they observed that GalXM and a mixture of MPs were also capable of inducing L-selectin shedding and enhanced GXM-related L-selectin loss, probably via complement activation. By using inhibitors of the signaling-pathways, they also showed that GXM mediates L-selectin loss from lymphocytes via herbimycin A-sensitive pathways not involving protein kinase C[56]. Coenjaerts *et al* only demonstrated modest L-selectin shedding from the surface of GXM-treated PMN in whole blood, and minor L-selectin loss from isolated PMN in the absence of complement[41]. Differences in experimental procedures or the use of structural dissimilar GXM from distinct strains might explain the quantitative discrepancies in L-selectin shedding between these reports. Further

evidence provided by Coenjaerts *et al* indicates that MP-4 is more potent than the other polysaccharides in causing L-selectin shedding[41].
GXM- and MP-4–related L-selectin shedding coincides with an increased expression of CD11b/CD18 integrins on the surface of PMN[41,60], which are involved in the subsequent firm binding of leukocytes to counterligands on the endothelium. Dong and Murphy[61], however, showed that GXM also binds to CD18, which could then theoretically interfere with adhesion.
Little is known about the expression of adhesion molecules on the surface of endothelial cells during cryptococcosis. Earlier, Mozaffarian *et al*[184] had failed to demonstrate an effect of whole cryptococci on the expression of ICAM-1 on endothelial cells. Merkel *et al*[168] showed that a culture-medium extract from an encapsulated as well as an acapsular mutant strain decreased TNFα-related ICAM-1 protein expression on human lung epithelial cells in culture. To date the effect of the individual cryptococcal polysaccharides on the expression of endothelial adhesion molecules remains unknown.

In conclusion, both MP-4 and GXM induce L-selectin shedding which might lead to diminished leukocyte rolling. A premature shedding of L-selectin could lead to the inhibition of leukocyte rolling, the first step in leukocyte binding to endothelium, and thus interfere with leukocyte migration[90,231]. Additionally, binding of GXM to CD18 might reduce firm adhesion. Until recently, however, the actual impact of cryptococcal polysaccharides on leukocyte rolling and subsequent firm adhesion was unknown. Experiments described later in this thesis (Chapters 2 and 3) have broadened our insights in this area.

(g) Conclusions

Cryptococcal capsular polysaccharides are able to interfere with the migration of phagocytes despite adequate stimulation of chemokine production, and their concerted action might account for the mild inflammatory response often observed in cryptococcosis. This has been clearly demonstrated for GXM and unrefined mixtures of polysaccharides by *in vitro* as well as *in vivo* data. As of yet only *in vitro* data are available for GalXM, MP-2 and MP-4. Multiple mechanisms are involved (Figure 2; Table 2).
First, although the capsular polysaccharides effectively induce pro-inflammatory cytokine production, they also enhance the production of anti-inflammatory interleukin-10 (IL-10) and induce tumor necrosis factor-alpha (TNFα) receptor loss from the surface of neutrophils. The net outcome of these stimulating and inhibiting effects of GXM and MP has yet to be established in models of inflammation.
Second, these polysaccharides interfere with leukocyte chemokinesis. When circulating in the bloodstream, their indirect and direct chemoattracting properties can prevent leukocytes from leaving the bloodstream and migrating towards inflammatory sites. Repressive effects on the C5a receptor might also contribute. As shown for MP-4, cross-desensitization of the receptors for host- and bacteria-derived chemoattractants may also play a role in inhibition.
Third, polysaccharides might interfere with leukocyte adhesion to and migration through the endothelium. Both GXM and MP-4 induce L-selectin shedding from the surface of leukocytes, which presumably inhibits leukocyte rolling. Furthermore, GXM binds CD18, which in turn might affect subsequent firm cell adhesion.

	Mechanism
Dysregulation of cytokine production	Induction of IL-10 and Th2 type responses [4,253,220,255] Downregulation of TNFα-receptors [41,60]
Interference with chemokinesis toward chemokines or bacterial products [41,59,154]	Direct chemotactic properties [57,58,154,41] Indirect chemotactic activity (complement activation) [142,57,58] Downregulation of the C5a receptor [180] Cross-desensitization of chemokine receptors [41]
Interference with adhesion	L-selectin shedding [41,60,56] Binding of CD18 [61]

Table 2. Possible mechanisms of interference with leukocyte migration by capsular polysaccharides

VI. Cellular mechanism: receptors for GXM and structure-function studies

Some of the receptors on effector cells, through which cryptococcal polysaccharides stimulate cytokine production, have been identified. The mannose receptor expressed on macrophages, for example, definitely plays a role in the binding to and phagocytosis of cryptococci[43]. In addition, data from Cross *et al*[43] indicate that mannose receptors mediate the phagocytosis of acapsular mutants by macrophages and that this process subsequently induces cytokine production. Furthermore, MPs are recognized by mannose receptors[163]. Therefore, mannose receptors might also be involved in the binding and phagocytosis of cryptococcal polysaccharides, resulting in cytokine production.

Another group of receptors involved in pattern recognition is the family of toll-like receptors (TLR) whose role in cryptococcal infection was recently investigated. TLRs are expressed on a variety of cells and are important mediators of proinflammatory cytokine release. These receptors instigate a pattern of intracellular signaling that ultimately leads to the activation of NFκB and results in production and release of TNFα and other proinflammatory cytokines[167]. A number of bacterial and fungal products, such as lipopolysaccharide (LPS), has been shown to mediate cytokine production through TLR-2 or -4[167]. Shoham *et al*[230] demonstrated that GXM binds to TLR-2- and TLR-4-transfected cells; however, activation of NFκB was observed only after GXM binding to both TLR-4 and CD14. Strikingly, this event did not result in the activation of mitogen-activated protein kinase pathways or TNFα secretion. These results indicate that GXM stimulates cells via CD14 and TLR-4, which then possibly results in the incomplete activation of signaling pathways. Other receptors, therefore, might be involved in GXM-related cytokine release. Additionally, the pattern-

recognition receptor CD14 is probably involved in MP-2-related TNFα induction since Chaka *et al* demonstrated that antibodies against CD14 abrogated MP-2 related TNFα release[26,27].
Another candidate receptor for GXM is CD18. Dong *et al* showed that GXM binds to CD18 on PMN[61] and Taborda demonstrated that CD11b/CD18 and CD11c/CD18 are involved in the phagocytosis of cryptococci[240]. A more recent study demonstrated that GXM binds to and is subsequently taken up by neutrophils and monocytes, and this process could be partially prevented by blocking CD14 and CD18[181]. Thus, although potential receptors have been described for GXM and MP, the precise mechanism by which they exert their effects on the immune system has not been clarified.

Limited studies have described structure-function relations of cryptococcal polysaccharides and the available studies have all focused on GXM. Most studies have described differences between GXM of the former serotypes and spontaneously occurring variants [37,141,164,188,232,233,261].
For instance, it has been recognized for long, that the arrangement of 6-*O*-acetyl substituents, which discerned the former serotypes, and the degree of xylose substitution determine the antigenic activity[7,40] and that the *O*-acetyl substituents are the major binding epitopes for antibodies[7,37,70,133]. However, it appeared that even within one serotype functional differences existed, which could be related to variation in side-chain substitution[39,233]). For one, this has been demonstrated for the anti-phagocytic activity of cryptococci[232]. Washburn demonstrated that the degree of complement binding to the cryptococcal surface depends on the degree of xylose substitution in GXM; i.e., C3 assembly decreased with increasing xylose and glucuronyl substitution[256]. Lastly, in naturally occurring variants of similar serotypes, Sahu *et al* demonstrated a correlation between augmenting xylose substitution in GXM to increased binding of C3 to the surface of cryptococci[225].
The chemotactic abilities of the capsular polysaccharides might be structure-dependent as well; i.e., cryptococci, culture supernatants and GXM of *C. neoformans* var. *grubii* and var. *neoformans* (former serotypes A and D) directly attracted PMN, while those of *C. gattii* (former serotypes B and C) did not[57,58,154]. Moreover, Dong *et al* showed that GXM of *C. neoformans* var. *gatti* interfered with chemokinesis of neutrophils toward chemoattractants, whereas GXM of *C. neoformans* var. *grubii* and var. *neoformans* (former serotypes A and D, respectively) did not[58]. At the same time, Coenjaerts *et al* demonstrated that GXM of another C. neoformans var. *grubii* (serotype A) strain did in fact inhibit chemokinesis[41]. These observations indicate that the effects of GXM on chemokinesis are also dependent on its structure; however, it has been unclear which part of the molecule is involved.
Chemical modification of GXM has provided another tool to relate structure to function. For instance, Young *et al*[261] showed that chemical de-*O*-acetylation weakly increased the accumulation of C3 on the surface of whole cryptococci. By chemically removing *O*-acetyl, Kozel *et al*[133] underlined the importance of *O*-acetyl for antigenic activity, and further demonstrated that it is not involved in the inhibition of phagocytosis. Glucuronyl side-chains provide a negative cellular charge to GXM, and thus far, modification by de-carboxylation has not revealed other functions[133].

More recently, genetic modification of GXM has provided more insight into structure-function relations. Structure mutant strains were described in which either *UXS1* (encoding for UDP-xylose synthase) or *CAS1* (encoding for a glycosyl transferase) were deleted, which resulted in capsules containing GXM deficient in xylose or *O*-acetyl, respectively[121,183]. GXM isolated from a xylose negative mutant strain accelerated the binding kinetics of C3 to cryptococci, which confirmed that xylose is important for the binding of C3[135]. Furthermore, xylose negative GXM was accumulated at a higher rate in the spleen when compared to xylose positive GXM. *O*-acetyl negative GXM from a mutant strain proved less potent in binding monoclonal antibodies and was cleared more rapidly from serum, confirming its importance as a major antibody binding epitope and its role in clearance. In the same study, neither xylose nor *O*-acetyl appeared to play a role in the inhibition of phagocytosis by GXM[135].

In summary, the cellular mechanisms by which cryptococcal polysaccharides operate remain to be clarified. Although potential receptors have been described for GXM and MP, the precise intracellular sequences leading to the effects on the immune system are unknown. Structure-function studies have demonstrated the importance of *O*-acetylation for the antigenic activity, antibody recognition and clearance of GXM and the role of xylose for complement deposition and clearance of GXM. Until recently, no clear structure-function data have been available regarding the effects on leukocyte migration.

VII. Aims and scope of this thesis

The principal aim of this thesis was to further explore the effects of GXM on neutrophil migration *in vitro* as well as *in vivo*.
Neutrophil migration toward inflammatory sites depends on the processes of chemotaxis by chemoattractants and on transmigration through the endothelium. The ability of GXM to interfere with the chemokinesis of neutrophils has been well established. However, its effects on the adhesion of neutrophils to the endothelium and the subsequent transmigration remain to be clarified. Although GXM has been shown to modulate adhesion molecules on the surface of neutrophils, such as L-selectin and CD18, its actual effect on adhesion to the endothelium has not been demonstrated. Moreover, GXM might also affect other adhesion molecules on the surface of neutrophils or endothelial cells. We investigated if and by which mechanism GXM affects neutrophil adhesion to the endothelium, i.e., both phases of initial rolling and subsequent firm binding (**Chapters 2 and 3**).

Limited studies have demonstrated the *in vivo* capacity of GXM to reduce neutrophil recruitment in the inflamed tissues. The intravenous administration of purified GXM in a model of bacterial infection and inflammation has been shown to reduce the influx of inflammatory cells. However, the ability of GXM to inhibit leukocyte migration has not yet been investigated in models of ischemia. We explored the capacity of GXM to interfere with neutrophil migration in a model of post-ischemic myocardial reperfusion injury (**Chapter 4**).

Next, we set out to clarify structure/function relations of GXM and assessed the importance of the building blocks *O*-acetyl and xylose for the GXM-related interference with neutrophil migration. We used chemical modified GXM as well as GXM from mutant strains and compared the effects of GXM deficient in either *O*-acetyl or xylose to that of intact GXM on chemotaxis and endothelial adhesion of neutrophils (**Chapter 5**). Finally, the effect of de-*O*-acetylation on neutrophil migration was verified in the animal model of ischemia (**Chapter 6**).

List of abbreviations

Acapsular cryptococci ≡ cryptococci containing no GXM

CneF = Cryptococcal culture filtrate

fMLP = N-formyl-methionyl-leucyl-phenylalanine

GalXM = Galactoxylomannan

GXM = Glucuronoxylomannan

hMBP = Human mannose binding protein

ICAM-1 = Intercellular adhesion molecule-1

IL = Interleukin

IFNγ = Interferon-gamma

LPS = Lipopolysaccharide

MP = Mannoprotein

MCP-1 = Monocyte chemotactic protein-1

MIP-1α = Macrophage inflammatory protein-1alpha

MIP-1β = Macrophage inflammatory protein-1beta

PAF = Platelet activating factor

PMN = Polymorphonuclear cells (neutrophils)

TLR = Toll-like receptor

TNFα = Tumor necrosis factor-alpha

TNFαR = Tumor necrosis factor-alpha receptor

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CHAPTER 2

Cryptococcal glucuronoxylomannan inhibits *in vitro* adhesion of neutrophils to stimulated endothelium by affecting both neutrophils and endothelial cells

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Summary

Cryptococcal infections are often characterized by a paucity of leukocytes in the infected tissues. Previous research has shown that the capsular polysaccharide glucuronoxylomannan (GXM) inhibits leukocyte migration. In this study, we investigated whether the capsular polysaccharide GXM affects the migration of neutrophils (PMN) through the endothelium by interfering with firm adhesion in a static adhesion model. Treatment of PMN with GXM inhibited PMN adhesion to TNFα-stimulated endothelium by 44%. Treatment of TNFα-stimulated endothelium with GXM led to a 27% decrease in PMN adhesion. GXM-treatment of both PMN and endothelium did not have an additive inhibitory effect.

We demonstrated that GXM-induced L-selectin shedding does not play an important role in the detected inhibition of adhesion. L-selectin was still present on PMN in sufficient amounts after GXM treatment, since it could be further inhibited by blocking antibodies. Furthermore, blocking of GXM-related L-selectin shedding did not abolish the GXM-related inhibition of adhesion.
GXM most likely exerts its effect on PMN by interfering with E-selectin mediated binding.
The use of blocking mAbs against E-selectin – shown to decrease adhesion in the absence of GXM – did not cause additive inhibition of PMN adhesion after GXM pretreatment. The use of blocking antibodies also demonstrated that the inhibiting effect found after GXM treatment of endothelium probably involves interference with both ICAM-1 and E-selectin binding.

Introduction

Cryptococcus neoformans is an encapsulated yeast that can cause life-threatening meningitis in immunocompromised patients. Compared to bacterial meningitis, cryptococcal meningitis is usually characterized by a relative paucity of leukocytes in the CSF and the infected tissues[3,4], despite adequate stimulation of cytokine production[4,38,51,59]. Previous research has shown that the immunomodulatory effects of cryptococcal capsular polysaccharides contribute to the scant leukocyte infiltrates often observed. Cryptococcal culture filtrate, the isolated capsular polysaccharide glucuronoxylomannan (GXM), and mannoprotein 4 (MP-4) all inhibit the influx of leukocytes into inflammation sites[7,13,42]. Since GXM and MP-4 have intrinsic chemoattracting properties[7,11,12] and high titers of both are found in the bloodstream during infections[7,9,19], it has been hypothesized that these compounds - by cross-desensitization - prevent leukocytes from properly responding to chemoattractants, thereby contributing to the scant infiltrate often reported in cryptococcal infections. This has already been demonstrated for GXM in humans as well as in experimental infections in animal models[13,40-42]. Another mechanism for the diminished leukocyte transendothelial migration – one not yet analyzed in detail in the literature - could be the interference with leukocyte adhesion to the endothelium at the site of infection. This has been the subject of the present study.

Leukocyte adhesion to the endothelium is initiated by the activation of endothelium by cytokines already present at the site (e.g., IL-1, TNFα), leading to endothelial cytokine production and the upregulation of adhesion molecules. The first step of adhesion is the margination and rolling of leukocytes on the activated endothelium and depends on interactions between selectins (E-selectin on endothelium, P-selectin on platelets and endothelium, L-selectin on leukocytes) and their counterligands. The leukocytes are then activated by cytokines, which results in the shedding of L-selectin from the leukocyte surface and the increased expression of integrins (e.g., CD11b/CD18, VLA-4) thereon. The integrins are involved in the subsequent firm binding of the leukocytes to the ligands (e.g., the ICAM's, VCAM) expressed on endothelium, after which the cells finally transmigrate[53]. Interference with these binding processes might lead to a diminished efflux of leukocytes in the direction of the inflamed tissues and might involve inhibition of endothelial cytokine production, the blocking or shedding of adhesion molecules from the surface of leukocytes or endothelium, or the inhibition of protein expression.

Although various research groups have shown that cryptococcal culture filtrate and GXM adequately stimulate cytokine production by monocytes and neutrophils (PMN) *in vitro* (IL1β, IL6, IL8, TNFα, IFNγ[38,51,59]), whole cryptococci appear to have an inhibiting effect on the production of chemokines by the endothelium[48]. Impairment of this trigger leading to the extravasation of leukocytes could hypothetically lead to interference with the adhesion process. However, since both capsular and acapsular cryptococci demonstrate this inhibiting effect[48] and GXM is part of the capsule, GXM is probably not responsible for the interference.
The effects of GXM on endothelial adhesion molecules have not yet been studied. Much better documented are its effects on neutrophils (PMN). GXM has been shown to induce L-

selectin shedding from the surface of PMN and lymphocytes[10,14] and, since this shedding occurs prematurely, an inhibiting effect on leukocyte rolling can be expected. L-selectin shedding caused by GXM coincides with the upregulation of the integrin CD11b/CD18[14], which might favor the subsequent firm binding of PMN to endothelium. Dong et al., however, showed that GXM also binds to CD18[15], which in turn might have a negative effect on firm binding. Thus, despite the current knowledge of the effects of GXM on leukocyte adhesion molecules, the actual effects of GXM on the process of firm leukocyte binding are not known.

In this study, we investigated the effect of GXM on the process of PMN adhesion to TNFα-stimulated endothelium using a static adhesion assay. Stimulation of endothelium by TNFα leads to an increase in the expression of adhesion molecules and thus to increased binding of leukocytes[25,36,54]. The static adhesion assay mainly involves binding processes that contribute to firm leukocyte adhesion. It has been extensively demonstrated that neutrophil CD11/CD18 binding to ICAM on the endothelium and E-selectin binding to its counterligands on PMN play comparable roles in this adhesion assay[27,35,46,47]. Their roles are partly co-dependent since E-selectin binding mediates CD18 activation and thus promotes CD11b/18 binding to ICAM[35,39]. The role of L-selectin in the static adhesion assay is probably restricted to serving as one of the ligands of E-selectin[33,60] since L-selectin mainly participates in leukocyte rolling and a threshold dynamic shear force is required for its binding[22].

The objectives of this study were (1) to demonstrate whether GXM inhibits PMN adhesion to endothelium and (2) to establish whether GXM exerts this action by affecting PMN, endothelial cells, or both. Furthermore, we wanted to establish which adhesion molecules are affected by GXM.

Materials and methods

Materials and monoclonal antibodies

TNFα was purchased from Roche (Almere, The Netherlands). The tissue inhibitor TAPI-0 (KD-IX-73-4; HO-NH-CO-CH_2-CH(CH_2-CH(CH_3)$_2$)-CO-Nal-Ala-NH- CH_2-CH_2-NH_2), an inhibitor of L-selectin shedding[20] from Peptides International (Louisville, KY, USA) was dissolved at 25 mg/ml in DMSO and further diluted with PBS to 1 mg/ml. Fibronectin from human plasma was obtained from Micronic (Lelystad, The Netherlands) and Polymyxin B sulfate from Sigma (St.Louis, MO, USA). A Superose 6 HR gelfiltration column was purchased from Pharmacia (Uppsala, Sweden). The fluorescent probe 2',7'-bis-(2-carboxyethyl)-5-(6)-carboxyfluorescin acetoxymethyl ester (BC-ECF AM) was purchased from Molecular Probes Europe (Leiden, The Netherlands). Human pooled serum, obtained from a pool of healthy donors, was heat-inactivated at 56°C for 30 minutes and subsequently filtered. Glucuronoxylomannan (GXM), purified from *Cryptococcus neoformans* (Serotype A, Chemotype 5, ATCC 62066), was a generous gift from Dr. Cherniak, Georgia State University (Atlanta, GA, USA)[6]. Specific primer pairs for E-selectin and ICAM-1 were synthesized by Amersham Pharmacia Biotech (Uppsala, Sweden).

The following blocking monoclonal antibodies (mAbs) were used: DREG 56 (anti-L-selectin, IgG1[33]) from ATCC, BBA2 (anti-E-selectin, IgG1) from R&D systems (Oxon, UK), and RR1/1 (anti-ICAM-1, IgG1[27,36]) from the Fourth Leukocyte Typing Workshop. MAb IB4 (anti-CD18, IgG2a[52,56]) and the control mAb W6/32 (anti-HLA A/B/C/, IgG2a[47]) were raised in our own laboratory from mouse hybridomas obtained from ATCC (HB-10164 and HB-95, respectively). Except for mAb DREG 56 (anti-L selectin), which was used at a concentration of 20 μg/ml[33], all blocking antibodies were used at a concentration of 10 μg/ml. FITC-labeled anti-E-selectin (BBA21) and anti-ICAM-1 (BBA20) were from R&D systems (Oxon, UK) and used at a concentration of 50 μg/ml.

Isolation and culture of endothelial cells from umbilical veins (HUVEC)
Primary HUVEC were obtained according to the method described by Jaffe et al.[30] and cultured in sterile RPMI-1640 (RPMI) supplemented with 10 μg/ml gentamycin and 20% inactivated pooled human serum. Only passages 2-4 were used for the experiments.

Isolation, labeling and GXM treatment of human neutrophils
Human neutrophils (PMN) were isolated as described before[55]. Briefly, blood from healthy volunteers was collected in Vacuette tubes containing sodium heparin as anticoagulant (Greiner), diluted with an equal volume of pyrogen-free PBS, and layered on top of a Ficoll (Pharmacia)/ Histopaque (Sigma) gradient. After centrifugation for 20 minutes at 230 x g, PMN were collected from the Histopaque phase. They were subsequently subjected to a brief hypotonic shock with mQ water, washed, and suspended at 2 x 10^6 cells/ml in sterile RPMI supplemented with 0.1% human serum albumin (RPMI-HSA). The neutrophils were then labeled with 3.3 μM BC-ECF AM for 20 minutes at 4°C. After washing, the cells were incubated with either GXM dissolved in RPMI-HSA, or RPMI-HSA alone at 37°C for one hour.

Pyrogen-free conditions
To ensure lipopolysaccharide-free conditions, all of the materials were kept under pyrogen-free conditions. GXM was tested for the presence of LPS using a Limulus Amoebocyte Lysate assay (Coatest Endotoxin Diagnostica, Mölndal, Sweden) with a sensitivity of 25 pg/ml E.coli LPS. Although the LPS concentration was always less than 0.6 ng/ml, all experiments were carried out in the presence of 10 μg/ml Polymyxin B sulphate to neutralize any undetected LPS contamination[16]. Polymyxin B sulphate had neither a stimulating nor an inhibiting effect on the adhesion of PMN to the endothelium when tested in the static adhesion assay (as described below).

Static adhesion assay and GXM treatment of HUVEC[44]
HUVEC were plated on fibronectin-coated NUNC 72-well microtiter plates (Life Technologies, Breda, The Netherlands) and grown to confluence in 1-2 days. The cells were washed twice with RPMI and stimulated with 10 ng/ml TNFα (corresponding with 200-300 IE/ml) in culture medium for 6 hours at 37°C[25,44]. In some experiments HUVEC were treated with GXM suspended in culture medium for 1 hour at 37°C prior to, simultaneously or after stimulation with TNFα. After 3 washes with RPMI, donor PMN were added to the wells in a concentration of 10^6/ml (PMN:endothelial cell ratio = 5:1) and left to adhere for 15 minutes

at 37°C. After carefully washing the endothelium, the adherent cells were fixed with 2% paraformaldehyde (pH=7.4) and counted within a fixed frame of 1 mm^2 in the center of each well by fluorescence microscopy (Leitz Fluovert inverted microscope). Measurements were performed in quadruple wells for each variable. All tests were repeated using different batches of donor cells. Following GXM incubation, in some experiments, PMN and endothelial cells were also incubated with blocking mAbs at room temperature for 15 minutes. This was then followed by the adhesion experiment.

sL-selectin ELISA

The quantification of L-selectin levels in the supernatants of PMN treated with GXM was performed with a commercial ELISA kit (R&D systems) according to the manufacturer's guidelines. The sensitivity of the ELISA was less than 0.3 ng/ml L-selectin.

Reverse transcription PCR (RT-PCR)

RNA expression of endothelial adhesion molecules was measured by RT-PCR. Briefly, HUVEC in 25 ml culture flasks were exposed to either GXM or culture medium during 2 hours followed by stimulation with TNFα for another 6 hours. After washing, total endothelial RNA was extracted using guanidium isothiocyanate followed by reverse transcription of 1 μg of RNA per sample using oligo dT priming. Amplification by PCR of the target cDNA was conducted using specific primer pairs for E-selectin, sense primer 5'-GCTACAATTCTTCCTGCTC-3' and antisense primer 5'-GGAGAGTCCAGCAGCAGAA AGTC-3'; or for ICAM-1, sense primer 5'-CAAGAGGAAGGAGCAAGACT-3' and antisense primer 5'-ACAAGAGGACAAGGCATAGC-3'. A primer pair for the household protein GAPDH was used to equalize the amounts of RNA present in each sample, sense primer 5'- CACCATGGAGAAGGCTGGGG-3' and antisense primer 5'-ACCAAAGTTGT CATGGATGACC-3'. PCR amplified products were separated by agarose gel electrophoresis.

Preparation of FITC-labeled GXM and detection of GXM binding to HUVEC

Briefly, FITC-labeled GXM was prepared by activation of GXM with cyanogen bromide and subsequent reaction with fluorescein iosothiocyanate[26]. HUVEC monolayers were stimulated with TNFα for 6 hours where after the cells were detached by 1.5 mM EDTA in a calcium-free buffer. After washing, cells were incubated with FITC-labeled GXM in calcium containing HBSS medium for one hour followed by flowcytometric analysis of the immunofluorescently stained cells.

Gelfiltration chromatography and cryptococcal antigen ELISA

GXM samples (in 0.5 ml PBS) of two different concentrations (2 mg/ml and 2 μg/ml) were applied to a Superose 6.0 column (1.5 by 30 cm; Pharmacia) and eluted with PBS at a flow rate of 12 ml/hr. Dextran blue 2000 kDa, apoferritin 440 kDa and HSA 67 kDa were used as molecular mass markers and detected at 280 nm. Fractions of 0.5 ml were collected and assayed for GXM using a commercial ELISA (Premier Cryptococcal Antigen ELISA, Meridian Bioscience, Boxtel, The Netherlands) according to the manufacturer's guidelines. The sensitivity of the ELISA was 0.1 ng/ml.

Statistics

All adhesion experiments were repeated using different donors of both PMN and endothelial cells. Per experiment, each variable was tested in quadruple wells and the counts of adherent PMN were averaged. The average cell counts of multiple experiments were statistically analyzed. Since the level of PMN binding usually varies considerably from experiment to experiment due to donor-to-donor variability, all data were analyzed by a univariate analysis of variance test using a two factor ANOVA, analyzing only main effects (in which the donor was set as a blocking factor). To compare multiple treatment groups to each other or to the control, the ANOVA was then followed by a post hoc analysis by Bonferroni's test. P values < 0.05 were considered significant. In Results, the adherence data are expressed as adherence percentages followed by a 95% confidence interval (95% C.I.).

Results

GXM treatment of PMN inhibits the adhesion of neutrophils to stimulated HUVEC

Neutrophils (PMN) were incubated with GXM at different concentrations at 37°C for one hour after which a static adhesion assay was performed using confluent monolayers of TNFα-stimulated HUVEC. Figure 1A shows an example of the variation in the level of PMN binding from experiment to experiment due to donor-to-donor variability. The results of two representative experiments are presented. Despite the variation, the results of all experiments were averaged and analyzed by a variance test using a two factor ANOVA. Figure 1B represents the averaged adhesion of 7 experiments expressed as adhesion percentages. When GXM was not washed off before the adhesion process, PMN adhesion was inhibited by 39% (Figure 1B, $p<0.01$, 95% C.I. 19-52%). Significant inhibition was detected at GXM concentrations between 10 ng/ml and 100 μg/ml. The optimal inhibiting effect was reached at a concentration of 100 ng/ml where after it gradually diminished at higher concentrations.

Washing the PMN twice after incubation with GXM and prior to the adhesion process did not affect the level of inhibition (Figure 1C, inhibition 44% after washing, $p=0.005$), indicating that the effect is not merely due to an non-specific mechanical impairment of adhesion by the presence of large polysaccharide molecules. When we varied the incubation time of PMN with GXM between 15 minutes and 2 hours, the inhibiting effect did not change. In contrast, when GXM was added simultaneously with PMN to the endothelium, no significant inhibiting effect was noted (data not shown). The latter finding supports the view that the inhibiting effect of GXM in these experiments is due to the effect of GXM on the PMN during the incubation period and not to its effect on the endothelium during the subsequent short adhesion period. Moreover, the generation of an inhibitory effect after 15 minutes of preincubation suggests that the mechanism of action by which GXM affects neutrophils does not involve de-novo protein synthesis.

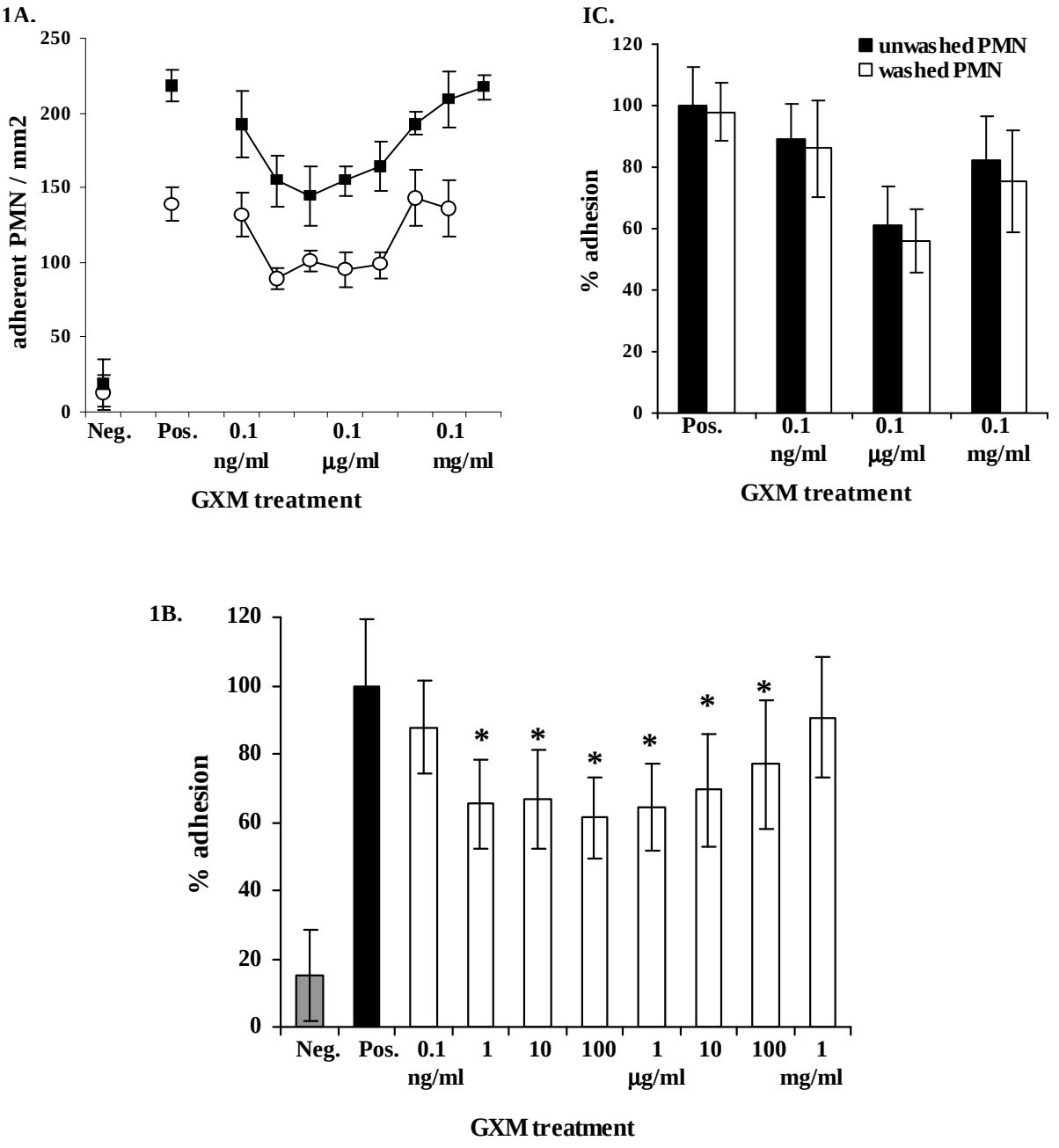

Figure 1. Treatment of PMN with GXM inhibits adhesion to TNFα stimulated HUVEC. BC-ECF-labeled PMN were incubated with GXM (37°C, one hour) followed by adhesion to TNFα stimulated HUVEC monolayers. After washing and fixation, adherent PMN were counted by fluorescence microscopy and observations were made in quadruple wells. The negative control consisted of the adhesion of untreated PMN to unstimulated endothelium; the positive control represents the adhesion of untreated PMN to TNFα-stimulated endothelium. **A.** Results of two separate representative experiments performed with cells from different healthy donors are presented (by squares and circles respectively). The data are presented as the mean absolute counts of adherent PMN. **B.** The results of seven experiments were averaged and subsequently expressed as the percentage of adhesion compared to the positive control (i.e. adhesion of untreated PMN to stimulated endothelium = 100%). **C.** To evaluate the effect of washing, PMN were washed twice with medium after PMN/GXM incubation. Adhesion is expressed as the percentage of adhesion of the positive control (either washed or unwashed PMN). The results of seven experiments were averaged. * = $p<0.05$. The error bars represent the standard deviations.

Effect of GXM treatment of endothelium on PMN adhesion

To investigate the effect of GXM on endothelial adhesiveness, non-stimulated endothelium was incubated with GXM at different concentrations and for different lengths of time. We found that GXM treatment did not affect the adhesiveness of resting endothelium (data not shown).The effect of GXM on the adhesiveness of TNFα-stimulated endothelium was then investigated. The incubation of HUVEC monolayers with GXM prior to or simultaneously with TNFα inhibited PMN adhesion by 27% when compared to endothelium treated with TNFα alone (Figure 2A, $p<0.05$, 95% C.I. 12-42%). The inhibitory effects on endothelium were detected at lower GXM concentrations (1 pg/ml-1 μg/ml) than when PMN were incubated with GXM.

To demonstrate that GXM is able to bind to and affect the endothelium, both unstimulated and stimulated HUVEC were incubated with FITC-labeled GXM followed by FACS analysis of the fluorescently stained cells. Binding of FITC labeled GXM to HUVEC could be demonstrated at concentrations of 0.1 mg/ml and 1 μg/ml, albeit at low levels (Figure 2B). GXM was found to bind more readily to stimulated HUVEC.

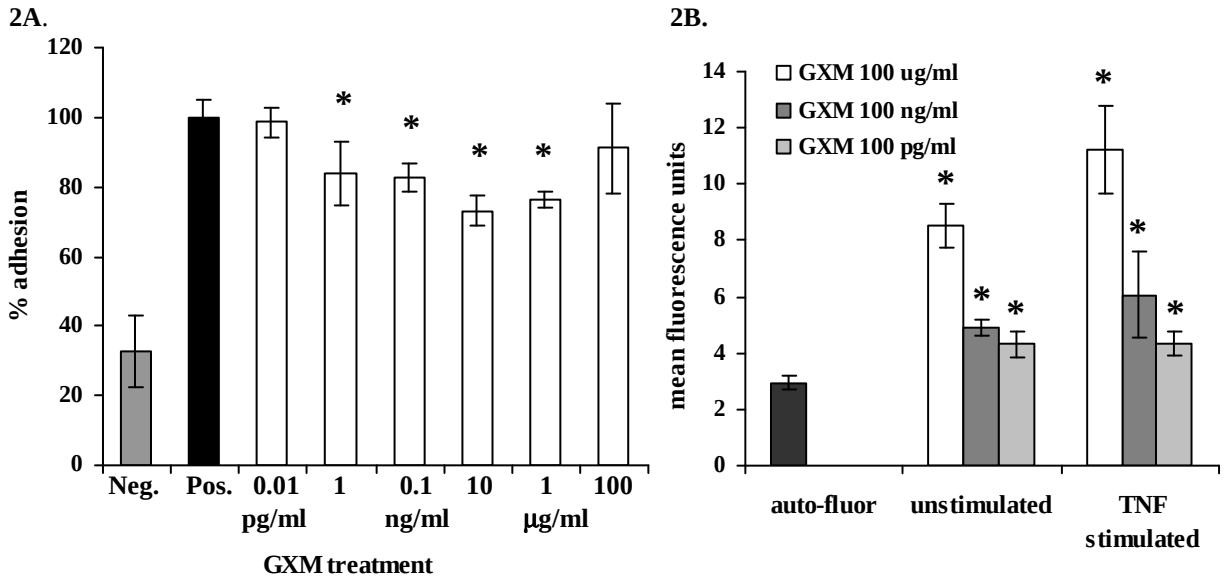

Figure 2. A. Preincubation of endothelium with GXM prior to TNF-α stimulation leads to a decrease in PMN adhesion. HUVEC monolayers were incubated with GXM for one hour at 37°C after which TNFα was added to a final concentration of 10 ng/ml for another six hours. After three washes, fluorescent PMN were added for 15 minutes at 37°C. After washing adherent cells were counted by means of fluorescence microscopy. The results of three experiments were averaged and expressed as percentages of the positive control (i.e. adhesion of PMN to HUVEC treated with TNFα alone = 100%). * = $p<0.05$. The error bars represent the standard deviations.
B. GXM is able to bind to HUVEC. Unstimulated and TNFα-stimulated HUVEC cells were incubated with FITC-labeled GXM for 20 minutes. After washing, fluorescently stained cells were evaluated by FACS analysis. The Y-axis represents the mean fluorescence units; the results of five experiments were averaged. * = $p<0.05$. Error bars present the standard deviations.

Since the same degree of inhibition was also observed when GXM was added after TNFα stimulation, inhibition of protein synthesis does not seem to be the underlying mechanism. We evaluated the effect of GXM on TNFα-related upregulation of endothelial adhesion molecules by RNA analysis. HUVEC were incubated with GXM at various time intervals followed by stimulation with TNFα. Untreated HUVEC expressed ICAM-1 but no E-selectin. TNFα stimulation led to an increase in both ICAM-1 and E-selectin transcription, which was not influenced by GXM (results not shown). Moreover, GXM did not alter the binding of fluorescent-labeled antibodies against ICAM-1 and E-selectin on endothelial cells, indicating that their surface expression was not affected by GXM (results not shown).
Previous research has already demonstrated that GXM does not decrease the viability of the endothelium[29]. Nevertheless, to ensure that the effect of GXM on adhesiveness was not due to a decrease in the viability of endothelial cells, HUVEC monolayers were incubated with GXM for different lengths of time. The viability of the endothelium proved to be >95% as evaluated by trypan blue exclusion and light microscopic examination.

Combined treatment of PMN and endothelium does not lead to additive inhibition
To mimic the *in vivo* situation during cryptococcal infections, as well as to look at possible additive effects, we investigated the effect that the preincubation of both endothelium and PMN with GXM has on adhesion. GXM treatment of both the endothelium and PMN (as described in Material and Methods) led to a decrease in adhesion of at most 38% (results not shown, $p < 0.05$, 95% C.I. 27-49%): a 27-38% inhibition was found at GXM concentrations between 10 pg/ml and 1 μg/ml, diminishing at higher concentrations. Compared to the treatment of PMN or endothelium alone, no statistical significant additive inhibiting effect was found, not even at those concentrations where both treatments separately had exerted an effect in earlier experiments.

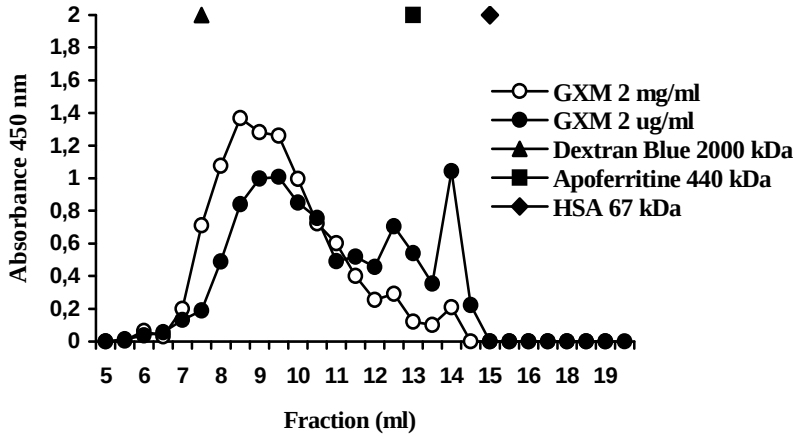

Figure 3. GXM size chromatography. Samples of GXM (2 mg/ml or 2 μg/ml, 0.5 ml) were applied to a Superose 6 gelfitration column. The eluted fractions from the 2 mg/ml sample were diluted 1,000-fold, followed by GXM detection using a commercial cryptococcal antigen ELISA. The eluted fractions from the 2 μg/ml sample were tested undiluted. The Y-axis represents the spectophotometric absorbance measured at 450/630 nm. The results are the averages of two ELISA's. The molecular weight markers Dextran blue 2000 kDa, apoferritin 440 kDa and human serum albumin 67 kDa were directly detected during chromatography by spectophotometry at 280 nm.

GXM aggregation

In the experiments described above, the effects of GXM on both PMN and endothelium diminished at higher GXM concentrations. Earlier, Dong et al described this phenomenon for other effects of GXM[15]. A possible mechanism might be aggregation of the polysaccharide molecules at higher concentrations, thereby lowering the actual numbers of reactive molecules. To support this hypothesis, we analyzed GXM preparations in two concentrations (2 mg/ml and 2 μg/ml, respectively) on a Superose 6 HR chromatography column. Elution was followed by detection of GXM using a commercial cryptococcal antigen ELISA. The eluted fractions from the highest concentration were diluted 1,000-fold in order to reach the detection range of the ELISA. Figure 3 shows that the elution curve of the 2 mg/ml sample is shifted towards the higher molecular weight range when compared to the 2 μg/ml sample, thereby supporting the theory that GXM tends to aggregate at higher concentrations. In addition, low molecular GXM (in the higher fraction numbers) is overrepresented in the lower-concentration curve.

Blocking L-selectin shedding does not affect GXM-related inhibition of PMN adhesion

Since GXM is known to shed the adhesion molecule L-selectin from the surface of PMN[14], we investigated whether this mechanism could account for the observed effect of GXM on PMN, i.e. the inhibition of PMN adhesion to the endothelium. We evaluated the effect of an inhibitor of L-selectin shedding, TAPI-0[20], on the GXM-related inhibition of PMN adhesion. First, to verify the effect of TAPI-0 on L-selectin shedding, we incubated 10^6 PMN in HBSS with 50 μg/ml TAPI-0 at 37°C for 15 minutes and then added GXM to a final concentration of 0.1 mg/ml. The supernatants were subsequently analyzed for L-selectin using a commercial L-selectin ELISA kit and the results of 3 experiments performed in duplicate were averaged. The mean L-selectin shedding caused by GXM (corrected for the L-selectin level present in the supernatants of untreated PMN) was 8.1 ng/ml (S.D. 2.9 ng/ml). Pretreatment of the PMN with TAPI-0 inhibited GXM-related L-selectin shedding by 80% (mean L-selectin concentration 1.5 ng/ml, S.D. 0.9 ng/ml).

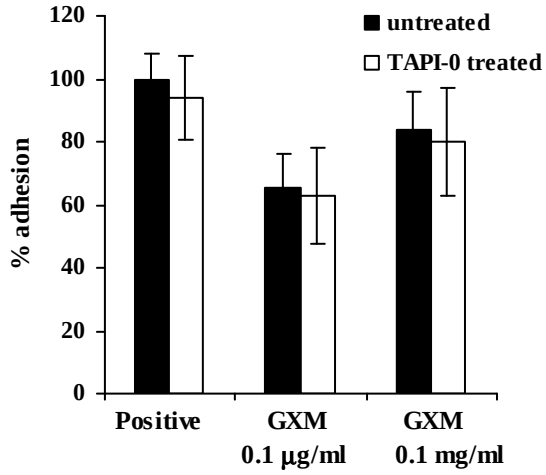

Figure 4. TAPI-0, a blocker of L-selectin shedding, does not affect GXM-related inhibition of PMN adhesion. PMN were incubated with either buffer or 50 μg/ml TAPI-0 at 37°C for 15 minutes after which GXM was added for one hour. Adhesion to TNFα-stimulated HUVEC occurred in the presence of both GXM and TAPI-0. The results are the averages of three experiments. Adhesion is expressed as the percentage of the positive control (i.e. adhesion of untreated PMN to stimulated endothelium = 100%). The standard deviations are presented as error bars.

Secondly, we compared the adhesion of GXM-treated and TAPI-0/GXM-treated PMN to the endothelium. As Figure 4 shows, the inhibition of adhesion caused by GXM (inhibition 35%) persisted in the presence of the blocker of L-selectin shedding TAPI-0 (37%, no significant difference, p=0.4). Thus, L-selectin shedding does not seem to be the mechanism underlying the inhibition of PMN adhesion to the endothelium after PMN preincubation with GXM.

GXM treatment of PMN affects E-selectin-mediated adhesion rather than CD11b/18-ICAM-1- or L-selectin-mediated adhesion

Several investigators have shown that, together, CD11b/CD18 and E-selectin mediated binding effectuate the adhesion in static adhesion models[27,35,43,46]. The relative contribution of these adhesion molecules to binding, however, depends on the expression of ICAM-1 and E-selectin on the endothelium, the amount of which differs over time. The expression of E-selectin has reached its maximum after a 6-hour stimulation of the endothelium by cytokines (TNFα, IL1β), whereas ICAM-1 expression is only 70%[36,54]. In this situation, both E-selectin and CD11b/CD18-ICAM binding play a quantitatively comparable and partly independent role in adhesion[43,46]. After a 24 hour stimulation, nearly all of the E-selectin has been shed from the endothelium and ICAM-1 expression has reached its maximum, conferring a major role to CD11/CD18-ICAM binding[43,46].

To discriminate between the interference of GXM with E-selectin binding to its ligand on neutrophils and GXM interference with CD11b/CD18-ICAM binding, we studied the adhesion of GXM-treated neutrophils to HUVEC stimulated for either 6 or 24 hours with TNFα. The blocking mAb IB4 (anti-CD18) was used as a control to quantify the role of CD11/CD18-ICAM binding to endothelium stimulated with TNFα for 6 or 24 hours. Since the level of PMN binding was usually considerably lower using 24-hour stimulated endothelium as compared to 6 hour stimulated endothelium, the results of the two time points could not be averaged and were thus statistically analyzed separately.

In our experiments, the mAb against CD18 caused a 28% inhibition of adhesion to endothelium stimulated with TNFα for 6 hours (Figure 5, 95% C.I. 18-39%) and a 74% inhibition of adhesion to endothelium stimulated for 24 hours (95% C.I. 56-93%). The mAb against E-selectin caused a 44% inhibition (95% C.I. 33-55%) after a 6-hour stimulation and no statistically significant inhibition after a 24 hours stimulation. These results confirm the earlier findings of researchers[43,46].

We observed that GXM-related inhibition of adhesion was more pronounced using endothelium stimulated for 6 hours than that stimulated for 24 hours. After a 6 hour stimulation the maximal detected inhibition was 35% (p=0.001, 95% C.I. 20-43%), whereas after a 24 hour stimulation no significant inhibition was found (Figure 5, mean inhibition 15%, p=1), suggesting that the mechanism of inhibition might be based on interference with E-selectin binding, not CD11/18-ICAM binding.

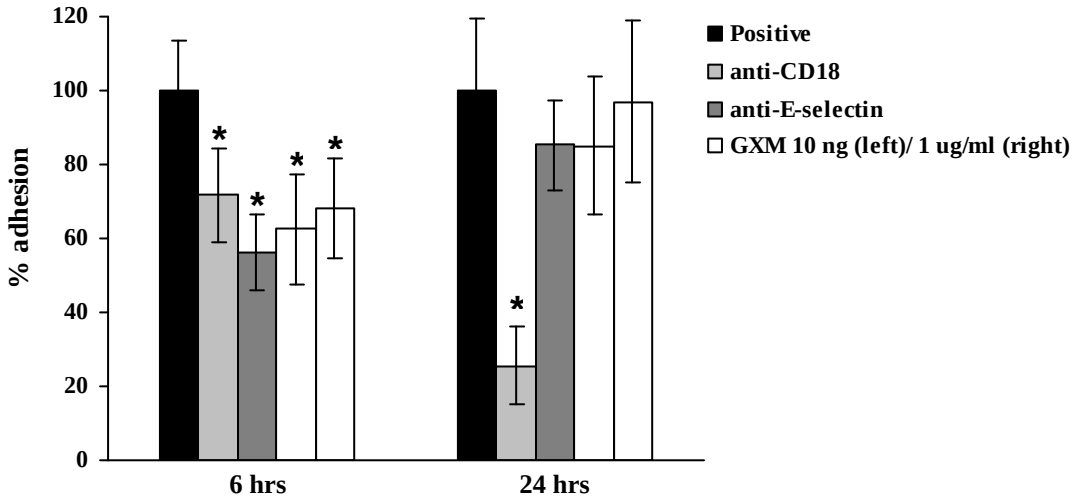

Figure 5. Comparing the reduction of PMN adhesion to 6 or 24 hour stimulated HUVEC. HUVEC monolayers were stimulated with TNFα for either 6 or 24 hours and subsequently washed. Untreated or GXM-treated PMN were added for 15 minutes. To evaluate the relevance of both E-selectin- and CD18-mediated binding at either time point, PMN adhesion to HUVEC was performed after incubation of PMN with mAb IB4 (anti-CD18) or after incubation of stimulated HUVEC with mAb BBA2 (anti-E-selectin). Adhesion is expressed as the percentage when comparing to adhesion of control PMN to 6-hour- or 24-hour-stimulated endothelium (100%). The results are the averages of four experiments. *= $p<0.05$ (comparing to adhesion of the untreated PMN to 6-hour- or 24-hour-stimulated HUVEC, respectively). Error bars present standard deviations.

To further investigate whether GXM interferes specifically with the binding of one of the involved adhesion molecules, we used blocking antibodies against each adhesion molecule. After GXM treatment the PMN were incubated with the designated mAbs (directed against L-selectin (DREG56), CD18 (IB4), or a control mAb against HLA A/B/C). TNFα-stimulated HUVEC were incubated with either a control mAb or a blocking mAb against E-selectin (BBA2) or ICAM-1 (RR1/1). The control mAb against HLA did not affect adhesion (results not shown). Figure 6A shows that blocking mAbs against the adhesion molecules CD18 (IB4), ICAM (RR1/1), L-selectin (DREG56), and E-selectin (BBA2) inhibited the adhesion of control PMN to stimulated endothelium by, respectively, 31% (95% C.I. 14-46%), 20 % (95% C.I. 2-37%), 21% (95% C.I. 10-38%), and 42% (95% C.I. 32-52%) as compared to the adhesion of control PMN in the absence of mAb.

Treatment of PMN with GXM caused a 41% inhibition of adhesion (95% C.I. 32-48%). The mAbs against CD18), ICAM-1, and L-selectin increased the inhibition of GXM-treated PMN to 60% (95% C.I. 42-77%), 56% (95% C.I. 38-74%), and 66% (95% C.I. 59-73%), respectively. This further decrease in adhesion was statistically significant when compared to the GXM-related inhibition (adhesion of GXM-treated PMN to stimulated endothelium; p-values 0.05, 0.003 and 0.005, respectively) and antibody-related inhibition alone (adhesion of non-GXM-treated PMN to stimulated endothelium in the presence of the antibodies; p values 0.03, 0.02 and 0.005, respectively).

In contrast, the blocking mAb against E-selectin (BBA2) was not capable of further decreasing the adhesion of GXM-treated PMN (inhibition caused by GXM: 40%; inhibition when BBA2 was added: 41%, 95% C.I. 37-46%, p=0.65 comparing both). Similarly, the inhibition of adhesion caused by BBA2 and GXM together did not differ from treatment with the antibody alone (41% versus 42%, p=0.9). Thus, GXM most likely interferes with the binding of PMN to endothelial E-selectin.

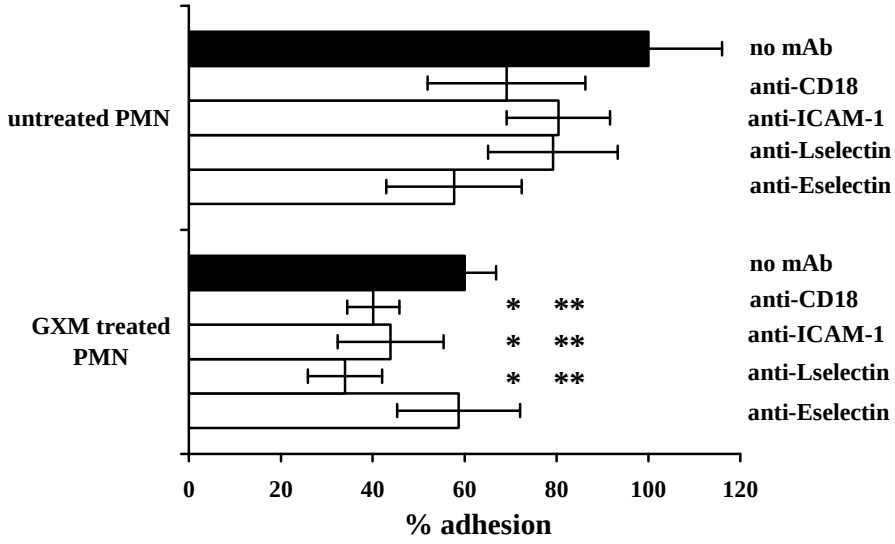

Figure 6A. The impact of blocking adhesion molecules on the GXM-related reduction of PMN adhesion. Medium or GXM-treated PMN (0.1 μg/ml, 37°C, 1 hr) were incubated with medium (black bars) or mAbs (white bars) against L selectin (DREG 56) or CD18 (IB4). TNFα-stimulated HUVEC monolayers (6 hrs) were treated with medium (black bars) or blocking mAb against E-selectin (BBA2) or ICAM-1 (RR1/1). Adhesion of PMN to HUVEC occurred in the presence of the antibodies. Adhesion is expressed as a percentage of the positive control (adhesion of untreated PMN to stimulated endothelium; 100%). The results are averages of four experiments. * = $p<0.05$ compared to the inhibiting effect of GXM treatment only. **= $p<0.05$ compared to the effect of mAb on adhesion of untreated PMN. The standard deviations are presented as error bars.

GXM treatment of the endothelium presumably inhibits PMN adhesion by interfering with both ICAM-1 and E-selectin binding.

The mechanism underlying the decrease in adhesion caused by GXM treatment of the endothelium was evaluated using blocking antibodies against adhesion molecules. Confluent HUVEC monolayers were treated with either medium or GXM for one hour, followed by TNFα stimulation for an additional 6 hours. After washing, the endothelium and PMN were either incubated with blocking antibodies, control HLA antibodies, or medium. Again, the HLA-directed control mAbs did not affect adhesion (results not shown).

Figure 6B shows that GXM treatment of the endothelium yielded a 25% inhibition (95% C.I. 19-31%). The combined use of GXM and either mAb IB4 (anti-CD18) or DREG56 (anti-L-selectin) led to a stronger inhibition of adhesion (50% and 42%, respectively, $p<0.05$) compared to the inhibition caused by either the mAbs (25% and 26%, respectively) or GXM (25%). These results indicate that the CD18 and L-selectin binding pathways are probably

not affected by GXM. In contrast, the combined use of GXM and mAb RR1/1 (against ICAM-1) did not lead to a stronger inhibition of adhesion compared to treatment with either one separately (30% versus 25% and 28%, respectively, p values 0.5 and 0.8 respectively). This suggests that GXM might affect the endothelium by interfering with ICAM-1 binding pathways. Additionally, mAb BBA2 (anti-E-selectin) in combination with GXM inhibited PMN adhesion comparably to that by GXM or the mAb alone (32% versus 25%, p=0.1 and 32%, p=0.4, respectively). These results suggest that GXM treatment of endothelium also interferes with E-selectin binding.

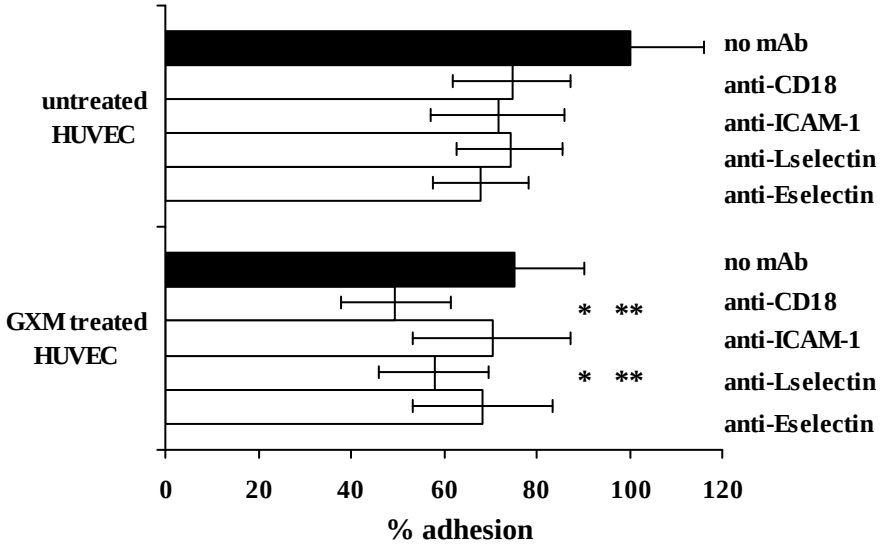

Figure 6B. The effect of blocking adhesion molecules on PMN adhesion to GXM-treated endothelium. HUVEC were treated with culture medium or GXM prior to stimulation with TNFα (for another 6 hours). After washing, the endothelium and PMN were incubated with various blocking mAbs for 20 minutes. Adhesion is expressed as the percentage of the positive control (adhesion of PMN to TNFα-stimulated endothelium). The results are the averages of five experiments. * = $p<0.05$ compared to the inhibiting effect of GXM only. ** = $p<0.05$ as compared to the effect of mAb only. The error bars represent the standard deviations.

Discussion

The biological properties of the polysaccharides present in the capsule of *Cryptococcus neoformans* that contribute to the pathogenesis of cryptococcosis include the inhibition of phagocytosis, the induction of immune unresponsiveness, cross-desensitization, the alteration of cytokine production, the enhanced infectivity of the HIV virus, and the inhibition of neutrophil influx into potential sites of inflammation[3,12,50]. Although capsular GXM might not be the sole causative compound of the impaired leukocyte migration - other cryptococcus derived products such as prostaglandins and mannoproteins may also contribute[7,49] - it is certainly an important virulence factor[3]. It has been hypothesized that the intrinsic chemoattracting properties of GXM, which circulates in the bloodstream of infected individuals, might be responsible for the observed inhibition of leukocyte migration towards infected tissues[13,41]. In this paper, we propose an additional mechanism leading to impaired transmigration; namely, the inhibition of neutrophil adhesion to the endothelium by GXM.

Although many microorganisms are capable of promoting adhesion by stimulating cytokine production and the expression of adhesion molecules[17,21,28,31,34] and some even use adhesion molecules on leukocytes and the endothelium for invasion [32], few examples exist of microorganisms that negatively affect the process of leukocyte adhesion to the endothelium (e.g., Leishmania, *Porphyromonas gingivalis*, *Escherichia coli*, *Staphylococcus aureus*[8,18,43,58]).
In this paper, we show that cryptococcal GXM is able to inhibit adhesion of PMN to stimulated endothelium. All of our experiments showed that these inhibitory effects gradually diminish at higher concentrations, which has also been documented for other immunological effects of GXM[15]. We have two possible explanations for this paradoxical result. First, we were able to demonstrate that this molecule tends to aggregate at higher concentrations, thereby possibly lowering the actual numbers of reactive molecules. Secondly, the phenomenon might be explained by the ability of GXM to activate neutrophils at higher concentrations (i.e., to cause L-selectin shedding and upregulation of CD11b/CD18[7,14]). The latter explanation, however, could only explain the effect of GXM on PMN, not on endothelium.

We also demonstrated that GXM inhibits the adhesion of neutrophils to the endothelium by affecting both types of cells. Notably, the range of concentrations of GXM at which these effects were found is much lower (1 pg/ml-10 μg/ml) than that described in the literature for other GXM effects (25 μg/ml-1 mg/ml)[11,14,40,57,59]. The lower concentration range is, however, more in line with physiological GXM levels. Although levels up to several mg/ml have been measured in serum of AIDS patients, they are usually 100-fold lower in non-HIV-infected individuals[9,19]. Interestingly, the optimal effect of GXM on PMN occurred at concentrations of 10-100 ng/ml, while such an effect on endothelium was found at much lower concentrations (1 pg/ml-10 ng/ml). This suggests different mechanisms underlying the inhibiting effects of GXM on either PMN or endothelial cells. The reported molecular weight of GXM varies between 10 and 1400 kDa depending on the method of isolation and the degree of sonication[2,5]. Assuming - for calculation purposes - a molecular weight of 500 kDa for GXM, the average number of GXM molecules per endothelial cell would be three at a concentration of 1 pg/ml. This might indicate that GXM affects endothelial cells via some sort of signaling pathway rather than a receptor- or ligand blockade. We demonstrated that FITC-labeled GXM is able to bind the endothelium, endorsing the proposed mechanism. The absence of a cumulative inhibitory effect when treating both PMN and the endothelium with GXM suggests interference with the same binding pathway. Alternatively, it might also be explained by redundancy; the participation of certain adhesion pathways might increase when other pathways are blocked. Further research should allow discrimination between both possible explanations.
Since GXM is known to cause L-selectin shedding from the surface of PMN[14], it seemed likely that this mechanism contributes to the inhibition of PMN adhesion to endothelium. We were able to prove, however, that the observed effects of GXM on PMN in the static adhesion model are not based on L-selectin shedding. First, we showed that blocking GXM-related L-selectin shedding (by TAPI-0) does not abolish the GXM-related inhibition of PMN adhesion. This indicates that surface release of L-selectin is not involved in the GXM-related inhibition of adhesion. Second, we showed that blocking antibodies against L-selectin

were still able to inhibit adhesion of GXM treated PMN considerably. Both observations make it unlikely that L-selectin shedding is the principal mechanism underlying the GXM-related inhibition of adhesion. In our experiments the average detected level of L-selectin shed from PMN by GXM was lower (8 ng/ml in the supernatant of 10^6PMN) than previously described. Dong et al. detected 20 ng/ml L-selectin, which accounted for 42% L-selectin loss[14]. The ability of blocking anti-L-selectin antibodies to inhibit adhesion further after GXM treatment of PMN might therefore be explained by a higher percentage of L-selectin remaining on the surface of PMN after GXM treatment. Furthermore, although in the static adhesion assay L-selectin might serve as one of the several ligands of E-selectin, L-selectin binding is more important for the initial rolling of neutrophils on the endothelium rather than for the later phase of firm binding and transmigration[22,53]. This might be a second explanation for the absence of an effect of GXM-related L-selectin shedding on static adhesion.

To further unravel the mechanism underlying the GXM-related inhibition of adhesion, we analyzed the impact of blocking monoclonal antibodies against different adhesion molecules on the inhibition of adhesion caused by GXM. We were able to show that the effect of GXM on both PMN and the endothelium probably involves interference with the E-selectin binding pathway since blocking antibodies against E-selectin did not cause an increase of the GXM-related inhibition of adhesion after either treatment. It has been shown that E-selectin is expressed on stimulated endothelium[36,54] and that it binds preferentially to sialyl Lewis X (sLe^x) carbohydrate structures expressed on the surface of PMN[23]. A few of these ligands have been identified, namely ESL-1[37], PSGL-1 (the ligand for P-selectin[1]) and L-selectin[33,60]. We also know that E-selectin binding is involved in both the initial phase of leukocyte rolling[53] as well as firm binding, the latter by contributing to leukocyte activation[39,45]. The interference of GXM with these binding pathways might therefore impair both phases of neutrophil adhesion, thus contributing to the observed diminution of leukocyte migration toward the inflamed tissues in cryptococcal infections.
Since we already showed that L-selectin binding is not inhibited by GXM, it remains to be investigated which of the other known ligands of E-selectin is affected by GXM (i.e. ESL-1 or PSGL-1). Since relatively short incubation periods with GXM are required to attain its inhibitory effects, impairment of protein expression seems unlikely. GXM might bind to or shed E-selectin receptors on PMN. For instance, it has previously been demonstrated that GXM actually binds to PMN [15]. As noted earlier, GXM causes PMN to shed L-selectin. Our experiments, however, show that this phenomenon does not underlie the observed effects of GXM on PMN. Dong et al showed that GXM does not affect the expression of CD15, a Lewis X carbohydrate antigen, negatively on the surface of PMN[14]. The effects on other E-selectin specific ligands remain to be investigated. Interestingly, Dong et al, showed that GXM is able to bind to PMN and even binds specifically to CD18 on PMN, not to L-selectin, CD11a or CD11b[15]. In our adhesion experiments, however, CD18-related binding seems not to be impaired by GXM.

Furthermore, by using blocking antibodies, we showed that GXM probably also inhibits E-selectin-mediated adhesion by affecting the endothelium, thus possibly affecting E-selectin itself. We further demonstrated that treatment of the endothelium with GXM might also

affect ICAM-1, a ligand of CD11/CD18, expressed on the endothelium. Interference with CD18 binding might therefore be expected. Our experiments, however, did not show that GXM interferes with the binding of CD11/CD18 to the endothelium, since antibodies against CD18 were still able to significantly inhibit adhesion to GXM-treated endothelium. CD11/CD18, which usually binds preferentially to ICAM-1, might bind to other ligands (e.g., ICAM-2) when binding to ICAM-1 is impaired, since much overlap and redundancy exists in the binding of integrins to a large variety of ligands[24]. Regarding the effect of GXM on endothelium, GXM did not decrease the surface expression or transcription of E-selectin and ICAM-1 on endothelial cells.

Thus, although GXM clearly interferes with PMN binding to the endothelium, the underlying mechanism remains to be resolved.
We are currently studying the effects of GXM on the separate ligands on PMN and its effects on the initial stage of PMN adhesion, i.e., rolling.

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CHAPTER 3

Cryptococcal glucuronoxylomannan interferes with neutrophil rolling on the endothelium

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Summary

The major capsular polysaccharide glucuronoxylomannan (GXM) of the pathogenic fungus *Cryptococcus neoformans* has been associated with the depression of a variety of immunological host responses. For one, GXM has been shown to interfere with the migration of phagocytes to sites of inflammation by interference with both chemokinesis and leukocyte adhesion to the endothelium. We previously reported that GXM blocks the firm adhesion of neutrophils (PMN) to endothelium in a static adhesion model, most likely by interfering with E-selectin binding pathways.

Using a flow model we now demonstrate that GXM also interferes with the initial rolling phase of PMN adhesion to endothelium (a 40% decrease) as well as to E-selectin transfected CHO cells (43% inhibition). Furthermore, we show that CD14 and TLR4, which are known receptors for GXM, mediate this interference with PMN rolling. However, thus far we were not able to identify the ligand of E-selectin on the surface of PMN that is specifically affected by GXM.

In conclusion, cryptococcal GXM interferes with both rolling and fixed binding of neutrophils on the endothelium, providing a novel means of contributing to the absence of neutrophil infiltration observed in cryptococcal infections.

Introduction

The yeast-like fungus *Cryptococcus neoformans* can cause life-threatening meningitis, mainly in immunocompromised patients[17]. The cryptococcal capsule has been recognized as an important virulence factor. Several experimental animal studies have demonstrated that capsular strains induce more severe infection than acapsular mutants[21, 32]. Additionally, the identification and subsequent targeted disruption of genes involved in capsule synthesis have emphasized the relevance of the capsule for virulence[5-7].
Capsular virulence is acquired by a number of mechanisms. First, thick capsules are able to mask the fungi from host phagocytes[31, 49]. Second, the capsular polysaccharides are shed abundantly into the patient's body fluids during infection[10, 42] and have been shown to affect the host response in several ways.

Glucuronoxylomannan (GXM) is the major polysaccharide component of the capsule and the presence of high serum titers of GXM has been associated with progressive disease[2, 10, 47]. The effects of GXM include suppression of T-cell mediated immunity, modulation of cytokine secretion, enhancement of HIV replication[4], and interference with leukocyte migration[14, 35-37]. The precise cellular mechanism by which GXM exerts its effects has not been clarified although potential receptors have been described for GXM (Toll-like receptor 2 and 4, CD14 and CD18)[16, 45, 54]. A recent study demonstrated that GXM binds to and is subsequently taken up by neutrophils and monocytes, and this process could be partially prevented by blocking CD14 and CD18[45].

A number of mechanisms contribute to the interference with leukocyte migration into inflammatory sites. First, GXM has shown to interfere with chemokinesis *in vitro*[9, 13, 18, 35]. The intrinsic chemoattracting properties of GXM[12, 13], cross-desensitization of chemokine receptors[12, 13, 35] and down-regulation of chemokine receptors[44] might prevent leukocytes from properly responding to chemoattractants. We recently demonstrated that interference of GXM with leukocyte adhesion to the endothelium at the site of infection is a second important mechanism for the diminished leukocyte transendothelial migration[19].

Interactions of leukocytes with the endothelium during an inflammatory response occur through a series of steps, progressing from rolling to firm adhesion and finally migration into tissues[55]. The first step of adhesion is the margination and rolling of leukocytes on the cytokine-activated endothelium and depends on interactions between selectins (E-selectin on endothelium, P-selectin on platelets and endothelium, L-selectin on leukocytes) and their counterligands. The leukocytes are then activated by the process of selectin binding itself as well as by cytokines, which results in the shedding of L-selectin from the leukocyte surface and the increased expression and activity of integrins (e.g., CD11b/CD18, VLA-4) thereon. The integrins mediate in the subsequent firm binding of the leukocytes to their ligands (e.g., the ICAMs, VCAMs) expressed on endothelium, after which the cells finally transmigrate.

Previous studies have demonstrated that GXM affects the expression of adhesion molecules on the surface of neutrophils and lymphocytes (Table 2). GXM has been shown to induce shedding of L-selectin from the surface of neutrophils (PMN) and lymphocytes, which

coincides with the upregulation of CR3 (CD11b/CD18)[9, 11, 15]. Furthermore, GXM has been shown to bind to CD18[16, 45]. We previously showed that GXM actually interferes with the firm binding of neutrophils to the endothelium in a static adhesion assay by affecting both neutrophils and endothelial cells[19]. We excluded that GXM-related inhibition of adhesion was based on interference with CD18 binding or L-selectin shedding, but rather relied on interference with E-selectin binding pathways. E-selectin is expressed on stimulated endothelium and it binds preferentially to sialyl Lewis X carbohydrate structures (sLe^x; CD15s), which are presented on ligands expressed on the surface of PMN. A few of these ligands have been identified, namely ESL-1 on murine neutrophils, PSGL-1 (P-selectin glycoprotein ligand-1) and L-selectin, which are constitutively expressed on leukocytes[59]. The function of $sLewis^x$ and PSGL-1 depends highly on their modification by glycosylation (by fucosyltransferases), by cleavage of propeptides or by sulfation. The expression of L-selectin is rapidly down regulated upon cell activation by a variety of chemotactic factors and activating substances (e.g., C5a, FMLP, IL-8, TNF, leukotriene B4)[29, 59] through proteolysis at a membrane-proximal site[43].

Since GXM has been shown to induce premature L-selectin shedding from the surface of PMN and lymphocytes and affect E-selectin binding pathways, we investigated the actual effect of GXM on the process of neutrophil rolling. Neutrophil rolling was studied using TNFα-stimulated endothelium as well as E-selectin transfected Chinese Hamster Ovary (CHO) cells in a dynamic flow system. Furthermore, we set out to identify the E-selectin ligands (ESL-1, PSGL-1, L-selectin and sLe^x) on the surface of PMN that are affected by GXM. Finally, we assessed which candidate receptors for GXM are involved in interference with neutrophil rolling.

Materials and Methods

Reagents

Human pooled serum (HPS) was obtained from a pool of healthy donors, was heat-inactivated at 56°C for 30 minutes and subsequently filtered. HEPES buffer contained 20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4 supplemented with 5 mM glucose, 1.0 mM $CaCl_2$, and 0.5% (w/v) human serum albumin (HSA). TNFα was purchased from Roche (Almere, The Netherlands) and Phorbol 12-Myristate 13-Acetate (PMA) from Sigma. Glucuronoxylomannan (GXM), purified from *Cryptococcus neoformans* (ATCC 62066, Serotype A, Chemotype 5) according to methods described previously[8] was a generous gift from Dr. Cherniak, Georgia State University (Atlanta, GA, USA).

Antibodies

The blocking monoclonal antibodies (mAbs) to IB4 (anti human CD18, IgG2a[50]) and DREG56 (anti human L-selectin, IgG1[30]) were isolated from the supernatant of hybridomas obtained from the American type Culture Collection (Rockville, MD, USA). The blocking mAb BBA2 and FITC-labeled mAb BBA21 (anti human E-selectin, IgG1) were purchased from R&D systems (Oxon, UK). The blocking mAb PL-1 (anti human PSGL-1, IgG1[46]) was obtained from Immunotech (Marseille, France). The blocking mAbs anti-human CD14 (clone

UCHM1, IgG2[45]) and anti-TLR4 (clone HTA125, IgG2[53]) were obtained from Serotec (Raleigh, NC, USA). All blocking antibodies were used at a concentration of 10 μg/ml. The FITC-conjugated mouse antibodies against human L-selectin (IgG2a), CD15 (IgM), isotype IgG antibodies and FITC labeled Goat F(ab') anti-Mouse were purchased from BD (Franklin Lakes, NY, USA).

Endotoxin-free conditions

To ensure lipopolysaccharide-free conditions, all of the materials were kept under pyrogen-free conditions. GXM was repeatedly tested for the presence of LPS using a Limulus Amoebocyte Lysate assay (Coatest Endotoxin Diagnostica, Mölndal, Sweden) with a sensitivity of 25 pg/ml E.coli LPS. The LPS concentration was always less than 0.6 ng/ml. Furthermore, experiments were randomly performed in the presence of 10 μg/ml polymyxin B, which did not affect the results at any time.

Isolation and GXM treatment of human neutrophils (PMN)[19]

PMN were isolated from healthy volunteers. Blood was collected in Vacuette tubes containing sodium heparin as anticoagulant (Greiner), diluted with an equal volume of pyrogen-free PBS, and layered on top of a Ficoll (Pharmacia)/ Histopaque (Sigma) gradient. After centrifugation for 20 minutes at 230 x g, PMN fractions were collected from the Histopaque phase and washed with RPMI-1640 (GIBCO) supplemented with 0.1% human serum albumin (RPMI/HSA). To clear erythrocytes the PMN fractions were subjected to a brief hypotonic shock with mQ water, washed with RPMI/HSA, and suspended in sterile HEPES buffer at 2 x 10^6 cells/ml.

Cells were incubated with GXM in HEPES buffer for 30 minutes at different temperatures. To achieve optimal rolling capacity, this was always followed by incubation at 37 °C for another 5 minutes. Incubations with the designated blocking antibodies were performed at a concentration of 10 μg/ml for 15 minutes at 4°C. Samples of untreated and GXM treated PMN were evaluated for viability by trypan blue staining and cells were counted before each flow experiment to ensure that comparable amounts of PMN were flown over the endothelium.

Cells and cell lines

Primary human umbilical vein endothelial cells (HUVEC) were isolated from donor umbilical veins according to the method described by Jaffe et al.[27] and cultured in sterile RPMI supplemented with 10 μg/ml gentamycin and 20% inactivated pooled human serum. Only passages 2-4 were used for the experiments. Cell monolayers were grown to confluence on gelatin-coated glass coverslips. Before perfusion experiments the HUVEC monolayers were stimulated with 5 ng/ml TNFα for 2 hours after which E-selectin expression is sufficiently upregulated to support rolling as opposed to the adhesion molecules that support subsequent firm binding of PMN (e.g.; ICAM-1)[1, 40].

Chinese Hamster Ovary (CHO) stably transfected with human E-selectin and control CHO cells[28, 39] were kindly provided by R.C. Fuhlbrigge (Department of Dermatology, Brigham and Women's Hospital, Boston, MA, USA) and originated from R. Lobb (Biogen, Cambridge, MA, USA). The cells were cultured in Modified Eagles Medium (MEM, GIBCO, Life

Technologies, Breda) containing 10% fetal calf serum (vol./vol.) and penicillin/streptomycin. CHO cell monolayers were grown to confluence in 3-4 days on plastic cover slips.

Immunostaining and FACS® analysis of PMN antigen expression

To evaluate the expression of PMN surface antigens, PMN (5.10^6/ml) were either incubated with GXM (10 μg/ml to 1,000 μg/ml) or with PMA 100 ng/ml (a control for L-selectin shedding[29]) in RPMI/HSA at 37°C for 45 minutes on a shaker. Then mixtures were placed on ice and FITC-conjugated antibodies directed against L-selectin or CD15 were added to a final concentration of 10 μg/ml for 30 minutes on ice. Incubation with unconjugated antibodies against PSGL-1 (PL-1) was followed by incubation with FITC conjugated goat F(ab') anti-mouse IgG before analysis. Cells were washed and analyzed by flowcytometry (FACS®).

Preparation of FITC-labeled GXM and detection of GXM binding to E-selectin transfectants

Briefly, FITC-labeled GXM was prepared by activation of GXM with cyanogen bromide and subsequent reaction with fluorescein iosothiocyanate[23]. E-selectin expressing CHO-cells were lifted by EDTA (2 mM in PBS), subsequently washed and incubated with either FITC-labeled antibodies against E-selectin (BBA21) or FITC-labeled GXM in Hanks/HSA at different concentrations for 30 minutes at a shaking rotator. Cells were analyzed by flowcytometry (FACS®).

Perfusion experiment and analysis

Perfusions under steady flow conditions were performed in a modified form of a transparent parallel plate perfusion chamber[52] as described by van Zanten et al.[58]. The perfusion chamber has a slit height of 0.2 mm and a width of 2 mm and contains a circular plug on which cell coated coverslips (18x18 mm) were placed and exposed to PMN suspensions. For each individual perfusion 1.2 ml of PMN suspension (2.10^6/ml in HEPES buffer) was aspirated from a reservoir through plastic tubing through the perfusion chamber with a Harvard syringe pump (Harvard apparatus, South Natic, MA) by which the flow-rate could be precisely controlled. The wall shear stress (t) was calculated from the equation $t = (6Q.\eta)/(w.h^2)$, in which Q is the flow rate, η is the suspending medium viscosity, w is the slit width, and h is the slit height. The shear stress was calculated as dynes/cm^2. In individual experiments the PMN were perfused through the chamber at 37°C and at a wall shear stress of 3.0 dynes/cm^2.
During perfusions the flow chamber was mounted on a microscope stage (DM RXE, Leica, Weitzlar, Germany), which was equipped with a B/W CCD-video-camera (Sanyo, Osaka, Japan), coupled to a VHS video recorder. Perfusions were recorded on videotape. Video images (Figure 1) were evaluated for the number of cells in close contact with the endothelium (= adherent cells), the rolling velocity per cell and the cluster index, using dedicated routines made in the image-analysis software Optimas 6.1 (Media Cybernetics systems, Silverspring, MD, USA). PMN in contact with the surface, which consisted of both rolling cells and firmly adhered cells (from now on together referred to as "adherent cells") appeared as bright white-centered cells after proper adjustment of the microscope during recording (Figure 1). The adherent cells were detected by the image analyzer. The number of adherent PMN was measured after 5 minutes perfusion at a minimum of 25 high power fields (total surface of at least 1 mm^2). Rolling velocities were measured using custom-made

software in Optimas 6.1. A sequence of 50 frames representing an adjustable time interval (δt) was digitally captured. At each frame, the position of each cell was detected and for all subsequent frames, the distance traveled by each cell and the number of images in which a cell appears in focus was measured. The velocity of a cell (v) in micrometers per sec was calculated from the equation: $v = L/\delta t(x-1)$, in which L is the covered distance (μm), δt is the time interval between images (seconds), and x the number of images in which a cell appears. The cut-off value to distinguish between rolling and static adherent cells was set at 1 μm/sec. The cluster index was set to be the difference between the measured and the expected number of cells inside an arbitrary area around an adherent cell. In equation: Cluster index per cell = m-(X*a/A-1), in which m is the measured number of cells in the rectangle area, X is the total number of cells in the image, A is the size of the total image, and *a* is the size of a rectangular cell-surrounding area. For each experiment, the mean cluster index was calculated for a minimum of 500 cells.

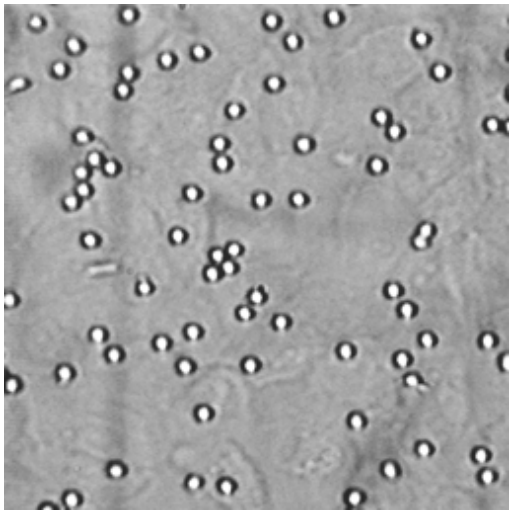

Figure 1.
Capture of a video frame following recording of PMN rolling on endothelial cells. PMN in close contact with the endothelial surface that appear as bright white-centered cells during recording were counted in 25 frames (total surface 1 mm^2).
For video fragment see the online version of this paper which will appear on the website of Cellular Microbiology in the near future (www.blackwellpublishing.com).

Statistics

The average cell counts of multiple experiments were statistically analyzed. Since the level of PMN binding usually varies from experiment to experiment due to donor-to-donor variability, all data were analyzed by a univariate analysis of variance test using a two factor ANOVA, analyzing only main effects (in which the donor was set as a blocking factor). To compare multiple treatment groups to each other or to the control, the ANOVA was then followed by a post hoc analysis by Bonferroni's test. P values < 0.05 were considered significant. In the results section the adherence data are expressed as adherence percentages.

Results

GXM reduces neutrophil rolling on the endothelium by affecting neutrophils

PMN were incubated with different concentrations of GXM, which did not affect the viability as evaluated by trypan blue staining nor the total counts of PMN present in the test tubes before perfusion. Since we were primarily interested in analyzing the effect of GXM on rolling, we prevented the transition from rolling to firm adhesion by adding blocking mAbs against CD18 to the PMN mixtures before the flow experiments. This resulted in an average ratio of rolling cells to static adhered cells of 9:1 under our experimental conditions (90.8 % versus 9.2 %, respectively).

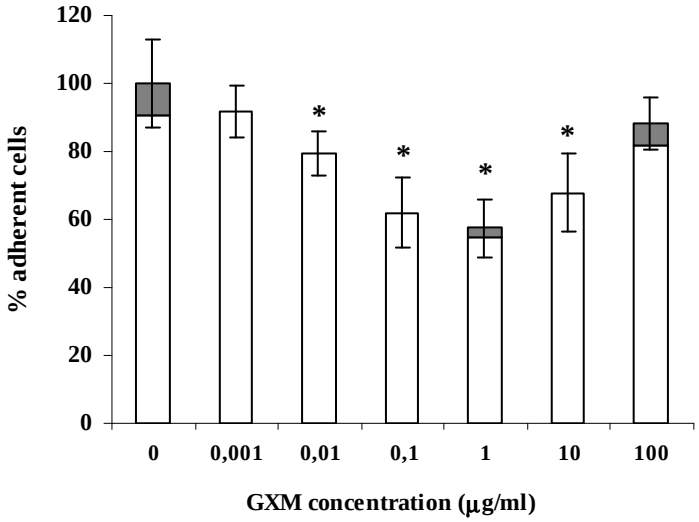

Figure 2. Effect of PMN pretreatment with GXM on PMN rolling on the endothelium.
PMN were incubated with buffer or GXM (room temperature, 30 minutes), followed by the addition of anti-CD18 mAb. For each individual perfusion 1.2 ml of PMN suspension (2.10^6 cells/ml) was flown over TNFα-stimulated HUVEC monolayers at 3 dynes/cm^2. Cells were allowed to interact with the endothelium for 5 minutes followed by video capturing of images. For each flow, the numbers of rolling (adherent) cells were counted in 25 recorded video images and expressed per mm^2. The adherence counts of nine experiments were averaged. The bars express the percentage of adherent cells when compared to the positive control (i.e. adhesion of untreated PMN to stimulated endothelium = 100%). * = $p<0.05$. The white colored parts of the bars represent the cells that are rolling whereas the gray colored parts of bars represent the cells that are firmly adhered (determined for 0.1 and 100 µg/ml GXM). The error bars represent the standard deviations.

GXM-pretreatment of PMN at either room temperature or 37°C reduced the number of adherent PMN (= rolling and static adherent cells together) in a dose-dependant dependant fashion when compared to untreated control cells (Figure 2; results of incubation at room temperature). Optimal inhibition was 42% and was reached at a GXM concentration of 1 µg/ml ($p<0.05$). GXM treatment did not affect the rolling velocities nor did it change the ratio of the number of rolling cells to the number of static adherent cells (90.8 % of control cells were rolling versus 95.1 % of the GXM-treated cells; Figure 2; $p=0.8$). Washing the PMN after preincubation with GXM did not change the inhibitive effect or the shape of the curve. Preincubation with GXM at 4°C clearly reduced the degree of inhibition from 42% to 19% ($p<0.01$).

The incubation of HUVEC with GXM before or during stimulation with TNFα did not significantly affect neutrophil rolling in this flow model (data not shown, adherence 86% when compared to that of control cells, p=0.5).

GXM interferes with neutrophil rolling on E-selectin transfected cells

E-selectin is expressed on the endothelium and binding of PMN to E-selectin is involved in the initial phase of leukocyte rolling[59]. Additionally, PMN binding to E-selectin plays a role in the process of firm binding by contributing to PMN activation[34, 38]. We previously reported that GXM inhibited firm PMN binding in a static adhesion assay by interfering with binding to E-selectin on the endothelium[19]. Here, we assessed whether GXM also affects PMN rolling on E-selectin**.** PMN were flown over monolayers of CHO cells expressing E-selectin. The specificity of the transfected cells was confirmed by preincubation of the monolayers with anti E-selectin antibodies (BBA2), which completely blocked PMN rolling (inhibition 97% ± 6.2 %).

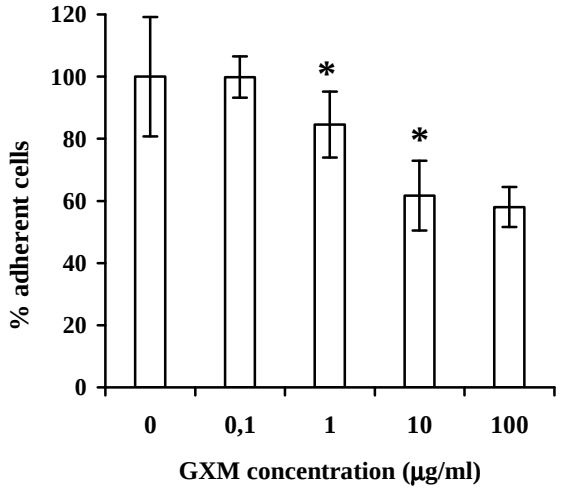

Figure 3. Effect of GXM on PMN rolling on E-selectin transfectants. PMN were incubated with GXM (37°C, 30 minutes). Thereafter samples were individually flown over monolayers of CHO cells expressing E-selectin. The adherence counts of eight experiments were averaged. Results are expressed as the percentage of adhesion compared to the positive control (i.e. adhesion of untreated PMN to stimulated endothelium = 100%). * = $p<0.05$. The error bars represent the standard deviations.

GXM pretreatment of PMN decreased PMN rolling on E-selectin transfectants by 43 % ($p<0.01$) in a concentration dependent fashion when compared to the rolling of untreated PMN (Figure 3). Higher concentrations of GXM were required to inhibit PMN rolling on E-selectin when compared to that of PMN rolling on HUVEC.

Similar to preincubation of HUVEC with GXM, the preincubation of E-selectin transfectants monolayers with GXM did not affect PMN rolling (data not shown). Moreover, FITC-labeled GXM did not specifically bind E-selectin expressing CHO cells when compared to control CHO cells in suspension (Figure 4). We therefore ruled out that binding of free GXM to E-selectin during flow with GXM-treated PMN caused the observed inhibition.

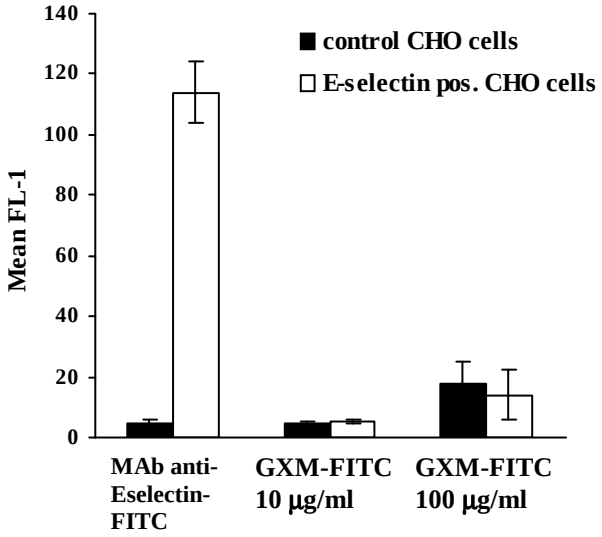

Figure 4. Binding of FITC-labeled GXM to E-selectin expressing CHO cells.
E-selectin transfected CHO cells and control CHO cells were incubated with FITC-labeled GXM (37°C, 30 minutes). After washing, fluorescent cells were evaluated by FACS® analysis. The expression of E-selectin on the CHO cells was verified by FITC-labeled mAb against E-selectin (BBA21). The Y-axis represents the mean fluorescence units; the results of three different experiments were averaged. * = $p<0.05$. Error bars present the standard deviations.

Effects of GXM on the ligands of E-selectin

We studied the effects of preincubation with GXM on the expression of the known E-selectin ligands L-selectin, PSGL-1 and sLe^x (CD15s) on the surface of isolated PMN (Figure 5). Thus far, ESL-1 has been identified on murine PMN only[33] and consequently we were not able to study the expression of this specific ligand on human PMN.

Treatment of PMN with PMA (control) caused a 68% reduction of L-selectin expression. At a concentration of 1,000 µg/ml, GXM reduced the expression of L-selectin by 18%. However, L-selectin expression was not significantly reduced at the lower GXM concentration range where inhibition was detected in the rolling experiments (i.e.; 10-100 µg/ml). The expression of PSGL-1 and CD15 (sLe^x) were not significantly affected by GXM (Fig. 5).

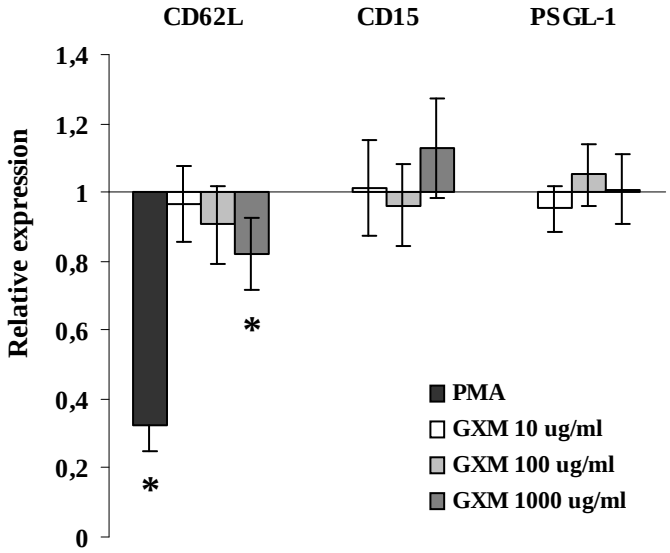

Figure 5. Surface expression of E-selectin ligands on PMN. The surface expression of L-selectin, CD15 and PSGL-1 after incubation with GXM (10-1,000 µg/ml, 37°C, 30 minutes) or PMA (100 ng/ml) was compared. The subsequent binding of FITC-labeled antibodies directed against L-selectin, CD15 and PSGL-1 was analyzed by flowcytometry (FACS®). The Y-axis depicts the expression of the adhesion molecules relative to the expression after incubation with RPMI/HSA (=1). The results of five experiments were averaged. * = $p<0.05$. The error bars represent the standard deviations.

GXM might affect the interaction between E-selectin and its ligands by other means than decreasing the expression of adhesion proteins on PMN. Therefore, rolling experiments were conducted with PMN in the presence of blocking antibodies directed against human PSGL-1 and L-selectin. PL-1 (anti- PSGL-1[46]) did not significantly affect the rolling of control PMN on E-selectin (Figure 6; inhibition 8%, p=0.6). This absence of inhibition might be explained by a redundancy in E-selectin binding to a large variety of ligands: when adhesion to a specific adhesion molecule is blocked, the adherence to other ligands might become more important. Alternatively, PSGL-1 binding to E-selectin might not play an important role in this specific model. Importantly, in the presence of both anti-PSGL-1 and GXM, the GXM-induced reduction in PMN rolling was not affected (Figure 6), indicating that GXM does not affect PSGL-1.

In contrast, mAb DREG56 against L-selectin blocked the rolling of control PMN on E-selectin by 51% ($p<0.05$). When L-selectin blocking was followed by GXM treatment, PMN rolling could be further decreased to 24% when compared to the adherence of untreated cells (Figure 6; 76% inhibition, $p<0.05$). These results confirm that L-selectin is a ligand for E-selectin in this model and indicate that GXM is able to inhibit neutrophil rolling despite blocking L-selectin.

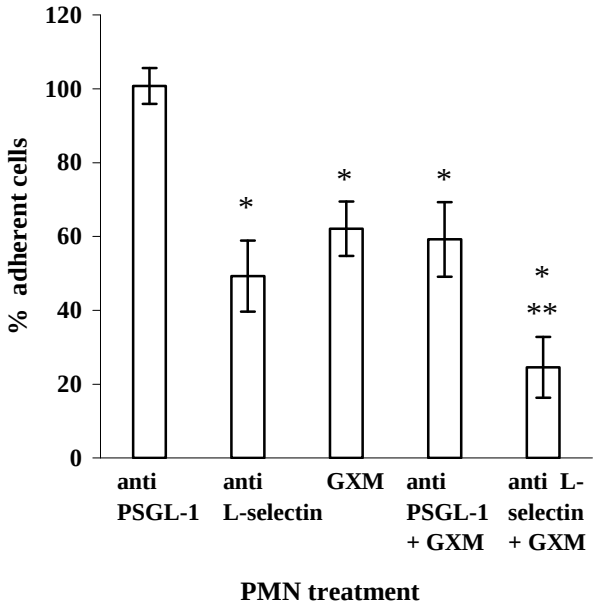

Figure 6. The effect of antibodies against L-selectin and PSGL-1 on GXM-related inhibition of rolling. PMN were incubated with blocking mAb against PSGL-1 (PL-1) or L-selectin (DREG-56) followed by the addition of GXM or buffer (37°C, 30 minutes). Cell suspensions were flown over E-selectin expressing CHO cells. The adherence numbers of five experiments were averaged and expressed as a percentage of the adherence of control PMN (=100%). * = $p<0.05$, when compared to the rolling of control PMN. ** = $p<0.05$, when compared to treatment with antibodies alone. The error bars present the standard deviations.

Next, we evaluated the effect of GXM on the clustering of neutrophils. The binding of L-selectin on PMN to other sialylated and fucosylated ligands on adjacent PMN causes the interaction of neutrophils in flow with adherent neutrophils. This interaction causes the PMN to roll over the surface in clusters (i.e., tethering)[22]. In our experiments mAb DREG56 (anti L-selectin) decreased the clustering of PMN on E-selectin by 41 % (Table 1). In contrast, GXM treatment decreased the cluster index by only 14%, which suggests that, also in this respect, the function of L-selectin is minimally affected by GXM. These findings as well as the observation that GXM causes no L-selectin shedding at the range of GXM concentrations used in the flow-model led us to conclude that the effects of GXM on L-selectin play a minor role in the interference with PMN rolling.

Treatment of PMN	Cluster index	p value compared to untreated cells	p value compared to mAb anti-L-selectin
No treatment	0.98 ± 0.05		
mAb anti-L-selectin	0.59 ± 0.09	< 0.01	
GXM 10 µg/ml	0.86 ± 0.11	0.05	< 0.01
GXM + mAb anti-L-selectin	0.69 ± 0.10	< 0.01	0.21

Table 1. Clustering of PMN on E-selectin transfectants. PMN were treated with GXM or buffer, followed by the incubation with mAb DREG 56 directed against L-selectin. Cells were then flown over E-selectin transfectants. The cluster index represents the interaction of neutrophils in flow with adherent neutrophils, which causes the PMN to roll over the surface in clusters. The clustering of cells to adjacent cells (i.e.; tethering) was analyzed by computer software from recorded images (see Experimental procedures for equations).

The role of the receptors for GXM in the inhibition of PMN rolling

Since GXM might affect the ligands of selectins on the surface of PMN, the obvious step would be to investigate whether GXM modulates receptors that regulate the expression or function of selectin ligands. However, as stated before, thus far no specific receptor on the surface of PMN has been identified that regulates the expression and function of the ligands of E-selectin other than for L-selectin. We therefore chose to study whether known receptors for GXM are involved in the GXM related effects on neutrophil rolling.

The cell surface receptors CD18, CD14 and Toll-like receptor 4 (TLR-4) have been identified as candidate receptors for GXM[16, 45, 54] and subsequent internalization is mediated by CD18 and CD14[45]. To investigate if binding and uptake of GXM by CD14, TLR-4 and CD18 is involved in the effects of GXM on PMN rolling, we assessed the impact of blocking antibodies against these receptors in our flow model. The optimal blocking concentration of the mAbs was derived from previous research[45, 50, 53] as well as from dose-response experiments (1-50 µg/ml).

In these set of experiments GXM treatment inhibited PMN rolling by 37% and this effect could be virtually abolished by pretreatment of the PMN with antibodies against CD14 or TLR4 (Figure 7). In contrast, antibodies against CD18 (IB4) did not affect the inhibition caused by GXM. Importantly, the rolling of control PMN was not affected by mAb against CD14 or TLR-4. However, the mAb against CD18 slightly inhibited the adherence of control PMN by 11%, and therefore the adherence numbers of PMN treated with both anti-CD18 and GXM were set as a percentage of treatment with anti-CD18 only (Figure 7). From these experiments, we conclude that CD14 and TLR-4 mediate the inhibition of PMN rolling by GXM, while CD18 is not involved.

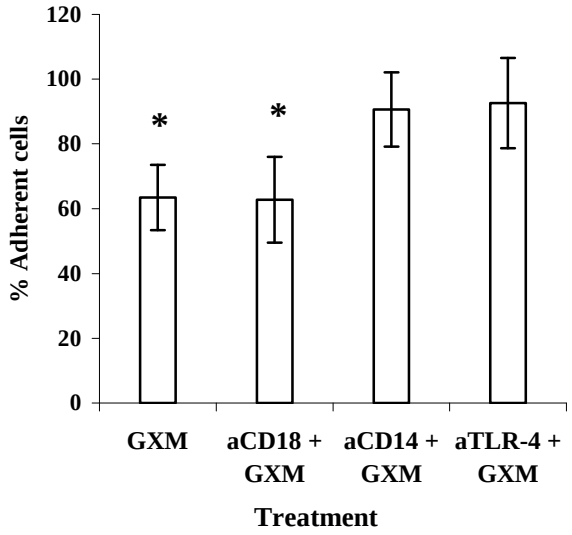

Figure 7. The effect of blocking receptors for GXM. PMN were incubated with blocking mAb against CD14, TLR-4 or CD18 for 15 minutes on ice followed by the addition of GXM or buffer at 37°C for another 30 minutes. The mixtures were then flown over E-selectin expressing CHO cells. The results are averages of 5 experiments. PMN adherence is expressed as a percentage of the adherence of control cells that received the same antibody-treatment (=100%). * = $p<0.05$, when compared to the rolling of PMN which were not treated with GXM. The error bars represent the standard deviations.

Discussion

More than three decades of research have produced accumulating evidence that the cryptococcal capsule[21, 32] and in particular the capsular polysaccharide GXM[14, 35-37] contribute to the limited inflammatory infiltrates observed during cryptococcal infections[25]. Presently, two mechanisms have been proposed by which circulating GXM prevents leukocytes to cross the blood-tissue barrier towards sites of inflammation, namely interference with chemotaxis[9, 9, 13, 35] as well as with leukocyte binding to the endothelium. Regarding adhesion, GXM has been shown to modulate the adhesion molecules L-selectin and CD18 on the surface of leukocytes (summarized in Table 2[11, 15, 16, 35]). We recently showed that GXM actually interferes with the firm neutrophil adhesion to endothelium in a static adhesion model presumably by interference with E-selectin binding pathways and not by CD18 binding or L-selectin shedding[19].

In this paper, we demonstrate that GXM also interferes with the crucial initial step in the capture of PMN from the circulation, which is mediated by the selectins. We show that pretreatment of PMN with GXM partially inhibits the rolling of PMN on the endothelium as well as on E-selectin transfected cells. Since the GXM-related interference with both firm adhesion[19] and rolling of PMN (this paper) is partial, these mechanisms will not solely be responsible for the GXM-related interference with leukocyte migration, but will add to the blocking of chemokinesis.

In the case of PMN rolling on HUVEC the magnitude of inhibition gradually diminished at higher GXM concentrations, which has also been documented for other effects of GXM such as the binding to CD18[16] and the inhibition of firm PMN adhesion to endothelium[19]. The phenomenon was explained by the tendency of the large GXM molecules to aggregate at higher concentrations, thereby possibly lowering the actual numbers of reactive molecules[19]. We showed that - using size chromatography- the elution curve of a 2 mg/ml GXM dilution was shifted towards the higher molecular weight range when compared to that of a 2 μg/ml

sample. In addition, low molecular GXM was over-represented in the lower concentration sample[19].
However, in the flow-experiments conducted on E-selectin transfectants, GXM was nonetheless able to inhibit PMN rolling at higher concentrations, although the extent of inhibition stabilized. Hence, polymerization might not entirely explain the discrepancy in the inhibiting capacity of GXM at higher concentrations between rolling on HUVEC and that on E-selectin transfectants. Additionally, due to the qualitative and quantitative differences in the expression of ligands for PMN between HUVEC and E-selectin transfectants a different dose might be required for GXM to inhibit PMN rolling on each of these cell types. First, the E-selectin transfectants only express E-selectin whereas HUVEC expresses a range of different ligands for PMN binding. We further established that the expression of E-selectin is twice as high on the E-selectin transfectants as that on HUVEC as measured by FACS (results not shown). Thus, since GXM inhibits rolling primarily through inhibition of E-selectin mediated pathways, a higher concentration would be required to inhibit rolling on E-selectin transfectants.
Another possibility would be that GXM-induced upregulation of the integrins CD11b/CD18 at higher concentrations[9, 15] leads to an increase of firm PMN binding to the ICAMs expressed on HUVEC and not on the E-selectin transfectants. However, this explanation seems unlikely since we blocked CD18 in all experiments that were conducted on HUVEC. Additionally, the ratio of static adhered cells to rolling cells did not increase at higher GXM concentrations.

We previously showed that GXM treatment of endothelium reduced the firm binding of PMN in static adhesion model by 27% and suggested that this effect was based on interference with E-selectin binding pathways[19]. However, we were unable to substantiate this effect in the flow-model. This might be explained by specificity in the mode of action of GXM on PMN when comparing rolling and firm adhesion as two different stages in neutrophil migration.

We were not able to identify a specific ligand of E-selectin on the surface of PMN that is affected by GXM. Theoretically, GXM might interfere with adhesion by specifically blocking or shedding adhesion molecules. Table 2 summarizes the known effects of GXM on leukocyte adhesion. Since GXM has been shown to cause L-selectin shedding from the surface of PMN[9, 15], it seemed likely beforehand that this mechanism contributes to the inhibition of PMN rolling on endothelium and on E-selectin. We were able to prove, however, that the observed effects of GXM on PMN rolling are not based on L-selectin shedding. First, we were not able to demonstrate any significant L-selectin shedding at the lower GXM concentration range (i.e.; below 100 μg/ml) where inhibition of PMN rolling was demonstrated, although we did detect a significant 18 % loss of L-selectin from the surface PMN at higher GXM concentrations (i.e.; 1,000 μg/ml). In addition, the ability of GXM to further inhibit PMN rolling after treatment with antibodies against L-selectin suggests that L-selectin shedding is not the principal mechanism underlying the effects of GXM. The fact that other researchers have detected a more pronounced L-selectin shedding by GXM[15] might be explained by the use of chemotypically different GXM. Furthermore, the expression of PSGL-1, which is another known ligand of E-selectin on PMN was unaffected by GXM. In addition, interference with the glycosylation of selectin ligands[41] seems unlikely, since GXM did not change the surface expression of sLewis^x, (i.e., CD15), which is one of the

carbohydrate structures expressed on the ligands of selectins. Although the possibility remains that not all of the candidate ligands for selectins on human PMN have been recognized yet, our results indicate that GXM does not directly alter the expression of E-selectin ligands**.**

Selectins/ ligands	**Cell type**	**Expression**	**Reference**
L-selectin	PMN	↓ 50% (w.o. serum (GXM>500 μg/ml)	(15)
	PMN	↓ 30% (with serum, GXM>500 μg/ml)	(9)
	PMN	↓ 18% (w.o. serum, GXM 1,000 μg/ml)	This paper
PSGL-1	PMN	No effect	This paper
sLewis^x (CD15)	PMN	Slightly ↑ or =	(15, this paper)
E-selectin	HUVEC	No effect	(19, Chapter 2)
Integrins/ ligands			
CD11b/18	PMN	↑	(15,9)
	PMN	binding of CD18 (GXM ≥125 μg/ml)	(16,44)
ICAM-1	HUVEC	No effect	(19)
Adhesion to endothelium		**Effect**	
Rolling	PMN	↓ 42 % on HUVEC and E-selectin	This paper
Firm adhesion	PMN	↓ 44%; via ↓ binding to E-selectin	(19, Chapter 2)

Table 2. Reported effects of GXM on PMN adhesion.

The lack of knowledge on the molecular changes induced by GXM at the surface of PMN that cause a decrease in rolling, makes it difficult to identify a receptor that is involved. We therefore approached the problem by studying the role of receptors previously recognized for GXM. CD14, TLR4 and CD18 are expressed on the surface of PMN[51, 55] and have been described as cellular receptors for GXM[16, 45, 54]. A recent study demonstrated that GXM binds to and is subsequently taken up by PMN[45], which occurred at room temperature and 37°C but not at 4°C. Indeed, in our experiments GXM incubation at 4°C reduced, but did not abolish the ability of GXM to interfere with PMN rolling. This might be explained by the additional incubation at 37°C, which was performed prior to each perfusion to optimize PMN rolling.
In the same study, GXM internalization appeared to be mediated by CD14 and CD18, which was supported by the fact that blocking antibodies against these receptors abrogated the binding and uptake of GXM. In our current study blocking antibodies against CD14 and TLR4, but not against CD18, abrogated the GXM-induced reduction in PMN rolling. First, these findings support the existing concept of CD14 and TLR4 as receptors for GXM. Second, although based on indirect evidence, the effect of GXM on PMN rolling seems to be mediated by GXM binding to CD14 and TLR4, and not to CD18.

TLR4 has been recognized as the signal-transducer receptor for LPS and CD14 generally functions as a co-receptor for LPS[57]. The binding of LPS to these receptors on leukocytes is associated with the induction of cytokine release[3, 26], L-selectin shedding[51], an increased adhesive capacity of the integrins CD11b/CD18[60] and priming of neutrophils for enhanced production of oxygen radicals[48]. Binding of GXM to CD14 and TLR4 has been shown to induce intracellular signaling in TLR4 and CD14 transfected CHO cells[54], but this did not result in cytokine production in these particular transfectants. The fact that GXM has been shown to modify the expression of L-selectin (shedding) and CD11b and CD18 (upregulation) on the surface PMN is consistent with CD14/TLR4 related effects. However, in our experiments GXM only induced moderate L-selectin shedding at higher concentrations (1 mg/ml). At the lower concentration range where we observed a reduction of PMN rolling (10-100 μg/ml) no significant L-selectin shedding was detected. Therefore, it seems unlikely that binding of GXM to CD14 and TLR4 will directly affect adhesion by activating signaling pathways that lead to activation of PMN and premature L-selectin shedding. Rather, CD14 and TLR4 might merely mediate the phagocytosis of GXM by PMN.

The fact that neutrophils, monocytes and tissue macrophages are able to internalize GXM [24, 45] might be an important clue to lead us to one (or more) cellular processes that are involved. For instance, uptake and subsequent metabolization of intracellular localized GXM might affect other cellular processes or lead to a temporary shape-change, ultimately affecting cell adhesion. For example, it has already been demonstrated that the internalization of GXM by PMN reduced their fungicidal capacity [45]. Furthermore, it has been shown that changes in cell morphology (e.g., by cytochalasin B or hypo-osmotic swelling) can reduce tethering and rolling [20]. By changing the shape of cells, GXM might affect the distribution of adhesion receptors on the microvilli of neutrophils, which is crucial for the initial contact between leukocytes and endothelial cells [56, 59]. However, in that case GXM would indiscriminately affect several adhesion molecules and not E-selectin binding only. These remain important issues to be addressed in future research.

Acknowledgements
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CHAPTER 4

Capsular glucuronoxylomannan of *Cryptococcus neoformans* reduces neutrophil influx in ischemic injury

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Summary

The capsular polysaccharide glucuronoxylomannan (GXM) of *Cryptococcus neoformans* has been shown to interfere with the chemotaxis and endothelial adhesion of neutrophils. Previously, intravenous administration of isolated GXM in a model of bacterial infection reduced the influx of inflammatory cells. Here we show that GXM is also able to interfere with neutrophil migration in a model that is not related to infection. We assessed the effects of intravenous GXM on neutrophil infiltration in a rat model of myocardial ischemia, where neutrophil influx contributes to post-ischemic reperfusion injury. Additionally, we investigated whether GXM-related complement activation and generation of C5a in the circulation plays a role in preventing neutrophils to migrate toward inflammatory tissues.

Rats were subjected to coronary artery ligation followed by a 3-hour reperfusion period. Intravenous administration of GXM markedly reduced the influx of neutrophils in the ischemic myocardium as measured by a reduction of 65% of tissue MPO activity. This reduction of MPO activity was clearly correlated to the serum concentration of GXM. Since complement activation by GXM was minimal at the doses applied *in vivo*, it is unlikely that generation of chemotactic C5a in the circulation by GXM caused the observed reduction in leukocyte migration. Despite the interference with neutrophil influx, GXM did not significantly reduce infarct size.

In conclusion, cryptococcal GXM has the ability to reduce neutrophil influx in models other than infection.

Introduction

The lack of inflammatory response during infections with the pathogenic fungus *Cryptococcus neoformans*[6,29] has been mainly ascribed to the repressive effects of the cryptococcal capsular polysaccharides on the host immune response.
The main capsular polysaccharide glucuronoxylomannan (GXM) is abundantly shed into the body fluids of the host during cryptococcosis[10,20,43] from where it exerts different effects on the inflammatory response.

In vitro, GXM interferes with the process of chemokinesis towards chemoattractants[8,13,39] despite adequate stimulation of cytokine and chemokine production[6,39,49]. It has been hypothesized that the presence of GXM in the bloodstream prevents leukocytes from properly responding to chemoattractants by a combination of its own intrinsic chemoattracting properties[12,13] and modulation of chemokine receptors[47]. Furthermore, since GXM is able to induce complement activation[37], the generation of massive amounts of chemotactic C5a in the circulation could hypothetically prevent neutrophils to respond to chemotactic stimuli in the inflamed tissues.
A second major mechanism underlying the reduction in leukocyte migration is the interference with the process of attachment to and the subsequent migration through the endothelium at the site of inflammation. At fairly high concentrations (above 0.5 mg/ml) GXM modulates adhesion molecules at the surface of leukocytes, such as shedding of L-selectin[11,15] and binding of CD18[16]. Moreover, we have recently demonstrated that GXM actually blocks both neutrophil rolling (Ellerbroek et al, 2004, submitted) as well as firm neutrophil adhesion to the endothelium at a lower concentration range (100 ng/ml to 100 μg/ml), most likely by interference with E-selectin binding pathways[18].

Limited *in vivo* data support the hypothesis that GXM interferes with leukocyte migration. We previously demonstrated an inverse correlation between GXM titers in serum and leukocyte counts in the cerebrospinal fluids during human cryptococcal meningitis[41]. *In vivo* studies that demonstrate the impact of purified GXM on neutrophil migration are limited to a model of gelatin sponge implantation[14] and pneumococcal menigitis[40]. We therefore investigated the ability of GXM to inhibit leukocyte migration outside the scope of infection in a model of ischemia.

During myocardial ischemia, the restoration of the blood supply is a dual edged sword. On the one hand, further infarction and cell necrosis will be prevented, but on the other hand, the reintroduction of blood flow has been associated with aggravation of tissue damage by eliciting a spectrum of pathologic changes[42]. Reperfusion injury has been associated with arrhythmias, (transient) ventricular wall dysfunction and excess mortality during the first day after thrombolysis[42]. Neutrophil infiltration has been shown to be one of several mechanisms contributing to reperfusion injury[25]. Whereas an inflammatory response normally benefits the host by eliminating host invaders, in the case of ischemia it is directed against the host-tissue and will contribute to further tissue injury[30] by plugging of capillaries [22] and by the release of proteases[30] and oxygen-derived free radicals[3]. The early influx of neutrophils reaches its peak 2-6 hours after restoration of the blood flow of ischemic myocardium[17,54]

and has been directly correlated to infarct size[54]. In addition, it has been demonstrated in animal models that neutrophil depletion and several types of anti-neutrophil therapy can reduce infarct size in post-ischemic reperfusion injury[21,32,36,38,50].

Here, we investigated the effects of intravenously administered GXM on neutrophil infiltration and infarct size in a rat model of myocardial ischemia/reperfusion. We also assessed whether complement activation by GXM and subsequent generation of C5a plays a role in the GXM-related inhibition of leukocyte migration.

Materials and methods

Materials

Hexadecyltrimethylammonium bromide (HTAB), Nitroblue tetrazolium, *O*-dianisidine hydrochloride, human recombinant C5a and zymosan were purchased from Sigma (Louisville, MO, USA). Sephadex G-75 was obtained from Pharmacia Biotech (Uppsala, Sweden). Human serum albumin (HSA) was obtained from a pool of healthy donors. Glucuronoxylomannan (GXM) purified from *Cryptococcus neoformans* (Serotype A, Chemotype 5, ATCC 62066), was a generous gift from Dr. Cherniak, Georgia State University (Atlanta, GA, USA)[7]. To ensure LPS-free conditions GXM was tested for the presence of LPS using a Limulus Amoebocyte Lysate assay (Coatest Endotoxin Diagnostica, Mölndal, Sweden) with a sensitivity of 25 pg/ml E.coli LPS. The LPS concentration was always less than 0.6 ng/ml.

Experimental protocol

Rats were prepared for coronary artery ligation. After a stabilization period of 15 minutes, the coronary artery ligature was closed; in sham animals the suture was not closed. After a one-hour period of occlusion the circulation of the coronary artery was restored by opening the suture. A bolus of 250 µl normal saline (placebo) or 0.25-2.5 mg GXM dissolved in 250 µl normal saline was administered intravenously to rats via a central venous catheter in the vena femoralis either 15 minutes after coronary occlusion or 5 minutes after the start of the reperfusion period.

After a three hour period of reperfusion the rats were deeply anesthetized with pentobarbital and the hearts were quickly excised, placed on ice, and processed for determination of the ischemic area.

Blood was collected from the vena cava in plastic tubes without anticoagulant as well as in tubes containing sodium heparin as anticoagulant just before the heart was isolated. The blood was centrifuged immediately in a refrigerated centrifuge at 2100 x *g*. Plasma and serum were collected and immediately frozen and stored at -70°C.

Animals

Male Wistar rats (Harlan Zeist, The Netherlands) weighing 280-320 gram were housed in groups of two, on a 12/12h light/dark cycle, with standard chow and water ad libitum. The experimental procedures were approved by the Ethical committee for the use of experimental animals within the Erasmus University Medical Centre, Rotterdam.

Preparation for coronary artery ligation

Animals were either subjected to coronary artery ligation or sham operation, according to the method of Fishbein *et al*[24] with slight modifications[52]. Briefly, animals were anesthetized with 60 mg/kg pentobarbital intraperitoneally and the trachea was intubated (PE-240). The skin overlying the fourth intercostals space was cut and the underlying muscles were separated and kept aside. The thorax was opened after positive pressure ventilation was started and the heart was carefully pushed to the left by applying pressure to the right side of the thorax. A silk (6-0) suture was looped under the left descending coronary artery near the origin of the pulmonary artery. Then the heart was returned to its normal position.

Determination of the ischemic area

At the termination of experiments, the coronary arteries of the excised hearts were immediately perfused with cold phosphate buffered saline by infusion of the aorta. To determine the non-perfused area (ischemic region; area at risk) the suture was closed again and the isolated heart was perfused with trypan blue to stain the perfused area, whereas the area at risk remained unstained. This area was separated and frozen at –70°C for later MPO measurements.

The anatomy of the coronary vasculature in rats is such that accidental occlusion of a side branch leads to a small infarction (<20%), and therefore exclusion from further analysis. Proper occlusion of the coronary artery resulted in an extensive transmural infarction comprising a major part of the left ventricular free wall (45-55%), with small variations in size[33].

Quantification of neutrophil infiltration

Myeloperoxidase (MPO) activity was used as a marker of tissue infiltration by neutrophils [4,28,54,56]. MPO was extracted from the cardiac tissues according to the method described by Bradley *et al*[4,28]. Briefly, the dissected ischemic areas (non-perfused areas) were rapidly frozen in liquid nitrogen, pulverized and then weighed. The material was then homogenized in 0.5% hexadecyltrimethylammonium bromide (HTAB) in 50 mM potassium phosphate buffer, pH 6.0 to provide a 10% homogenate (w/v). Samples were then briefly sonicated on ice, freeze-thawed 3 times after which sonication was repeated. Suspensions were then centrifuged at 25.000 x *g*. The resulting supernatants were chromatographed on small 1 ml Sephadex G-75 columns to separate MPO from tissue myoglobin and vascular hemoglobin that have been described to affect the MPO assay[56]. The second effluent with the size of the void volume was collected and assayed. The detection of MPO was based on its ability to degrade hydrogen peroxide. The MPO activity was determined by mixing 50 µl sample with 1.45 ml of 50 mM potassium phosphate buffer containing 0.167 mg/ml *o*-dianisidine hydrochloride. The reaction was started by adding hydrogen peroxide to a final concentration of 0.0005% and measured spectrophotometrically. The change in absorbance at 460 nm was

measured every 15 s for 5 min using a Genesys 10 UV spectrophotometer from Thermo Spectronic (Rochester, NY, USA). The MPO activity was expressed as the change in absorbance per minute per 100 mg of heart tissue.

Infarct size[53]

After termination of the experiment, the hearts were carefully cut out and placed on ice. In order to determine the non-perfused area (area at risk) the suture was closed and the isolated heart was perfused with trypan blue to stain the perfused area blue, whereas the area at risk remained unstained. The heart was placed at –80°C for 10 min before it was cut in 1 mm slices from apex to base. From each slice, the right ventricle was removed and the left ventricle was divided into the non-perfused area (area at risk; ischemic region) and the perfused area. The area at risk was then cut into slices and incubated with nitro blue tetrazolium at 37 °C for 10 min (Sigma Chemical; 1 mg/ml, pH 7.4), which stains vital tissue purple but leaves irreversibly damaged tissue unstained (the infarct area). After the infarct area was separated from the non-infarct area, the areas were dried and weighed separately. The infarct size was calculated by dividing the weight of the non-viable area by the weight of the area at risk (i.e., the non-perfused region).

GXM detection in sera

GXM was determined in serum of selected animals by the Premier Cryptococcal Antigen ELISA (Meridian Bioscience Europe, Belgium). To create a standard curve, a dilution series of GXM was prepared in serum from untreated rats. The standard concentration curve was created by reducing the absorbance data from the dilution series of GXM by computer software capable of generating a four-parameter logistic curve fit. The lower level of detection was 1 ng GXM/ml.

Activation of complement in rat plasma

Complement activation by GXM was achieved by mixing 45 µl plasma with 5 µl of GXM diluted in PBS to achieve final GXM concentrations ranging between 10 µg/ml to 1 mg/ml. The positive control consisted of rat plasma stimulated with zymosan, which is a yeast-derived polysaccharide and a potent inducer of the alternative pathway of complement [867]. This was achieved by incubating plasma with zymosan to a final concentration of 15 mg/ml at 37 °C for one hour. After centrifugation, the supernatants were aspirated from the zymosan pellets and assayed for the presence of complement factor C5a.

Detection of C5a in plasma

Undifferentiated control U937 cells and undifferentiated U937 cells stably transfected with the human receptor for C5a (U937-C5aR) were a kind gift of E.R. Prossnitz [847]. These cells are capable of a C5a-specific calcium flux[34]. The cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, glucose and gentamycin.
The presence of complement factor C5a in plasma samples was detected by the ability of C5a to induce mobilization of calcium in the U937-C5aR cells. The U937-C5aR cells were loaded with a calcium specific intracellular probe (Fluo-3, acetoxymethyl (AM) ester) in RPMI-1640 containing 0.1% human serum albumin (HSA) for 20 minutes at room temperature[5]. Cells were washed and suspended to a concentration of 10^6/ml in RPMI/HSA.

The U937-C5aR cells were then stimulated with recombinant human C5a (control) or rat plasma at different dilutions. C5a has been shown to induce a rapid and transient increase in free intracellular calcium concentration in the U937-C5aR cells which correlates with an increase in Fluo-3AM fluorescence signal as measured by flowcytometry (FACS®)[5,34]. Each sample of cells (180 µl cells at 1.10^6 cells/ml) was first measured for its fluorescence at 530 nm to determine the basal calcium level. Then 20 µl of rat plasma or 20 µl of a 10-fold concentrated stimulus was added followed by repetitive measurements during 10 seconds. For each determination, the response was taken as the peak fluorescence occurring within 10 seconds of ligand addition. Debris and dead cells were excluded with a gate on forward and sideward scatter (FSC and SSC, respectively).

Isolation, GXM treatment and homogenization of PMN

Human PMN were isolated as described before[18] and suspended at 1 x 10^6 cells/ml in sterile RPMI supplemented with 0.1% human serum albumin (RPMI-HSA). PMN preparations contained $\geq$ 95% neutrophils as identified by Giemsa stain and were found to be 99% viable by trypan blue exclusion. The PMN were incubated with increasing concentrations of GXM at 37°C for 60 minutes. After washing, the cells were homogenized by suspension in 0.5% HTAB in 50 mM potassium phosphate buffer followed by brief sonication on ice. Samples were centrifuged at 25.000 x *g* and the supernatants were assayed for MPO activity as described before.

Statistical analysis

Data were analyzed by a univariate analysis of variance using a two factor ANOVA. To compare multiple treatment groups to each other, the ANOVA was followed by a post hoc analysis by Bonferroni's test. P values < 0.05 were considered significant.

Results

Characteristics of the animals

Of a total of 64 rats, seven rats were subjected to a sham operation (controls) and 57 underwent coronary artery ligation (infarct rats), which was followed by a 3-hour reperfusion period. Of the 57 infarct animals, 19 were treated by normal saline (placebo) and 38 animals by GXM intravenously at different doses (0.25 or 2.5 mg) and administered at two different time points. Six infarct animals were left out of the analysis because of a too small area at risk (<20%) due to accidental ligation of smaller side-branches. These animals were equally distributed over the treatment groups. The hearts of the remaining 58 animals (7 shams and 51 infarct animals) were analyzed either for the MPO content of the ischemic region (42 animals) or for infarct size (16 animals). The mean weight of all animals was 304 gram +/- 15 gram (ranging from 287 gram to 332 gram) and was comparable between treatment groups.

The effect of GXM on the influx of neutrophils in the ischemic myocardium

The neutrophil accumulation in the dissected ischemic myocardium (i.e., the non-perfused area) was quantified by the detection of tissue MPO. I.v. administration of GXM led to a dose-dependant reduction of the MPO activity up to 65% in the ischemic myocardium when compared to that of untreated animals (Figure 1; $p<0.05$). The reduction of tissue MPO activity in animals that had received GXM during the occlusion period did not differ significantly from that caused by a GXM dose applied 5 minutes after start of the reperfusion (MPO activity of 0.53 versus 0.75, respectively; $p=1$).

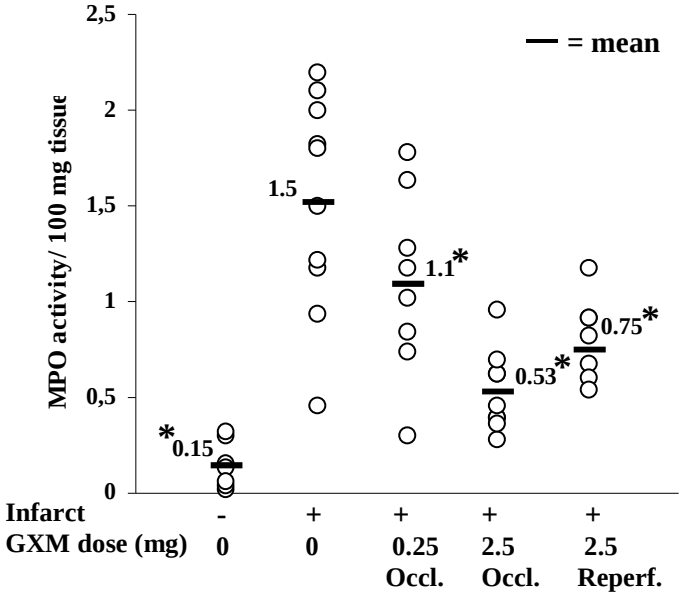

Figure 1. The effect of intravenous GXM on the MPO content of ischemic myocardium.
Rats were subjected to coronary artery ligation (infarct animals) or sham operation (n=7)) during one hour followed by a 3-hour reperfusion period. Infarct animals received a placebo (250 µl normal saline; n=10) or a bolus of GXM intravenously, either during occlusion ("occl.") or at the start of the reperfusion ("reperf."; n=8 or n=9, respectively). The hearts were removed at the termination of experiments and the non-perfused (ischemic) area was determined, cut out and homogenized. MPO in the resulting supernatants was detected by quantifying its ability to degrade hydrogen peroxide in the presence of hydrochloride coupled to a dye. The color development caused by the reaction was detected by spectrophotometry. The Y-axis expresses the MPO activity as the absorbance change per minute per 100 mg tissue; the X-axis depicts the different treatment groups. * = $p < 0.05$, when compared to the MPO activity of the placebo treated infarct animals.

To ensure that the decrease in MPO activity was not caused by a direct effect on the viability of neutrophils or MPO itself, isolated human neutrophils were incubated *in vitro* with increasing concentrations of GXM at 37 °C followed by evaluation of the viability and MPO activity. After incubation with GXM 99% of neutrophils were found to be viable by Trypan blue exclusion. Furthermore, *in vitro* GXM-treatment of isolated neutrophils did not reduce the MPO activity (Figure 2) when compared to that of untreated neutrophils. As a final control, samples of homogenized ischemic myocardium derived from untreated infarct rats

were incubated with GXM and assayed for MPO activity. Again, GXM did not decrease the MPO activity in these samples (results not shown).
We therefore concluded that the GXM-related decrease in MPO-activity in ischemic heart tissue could be ascribed to a decline in neutrophil influx rather than to a direct toxic effect of GXM on either neutrophils or MPO.

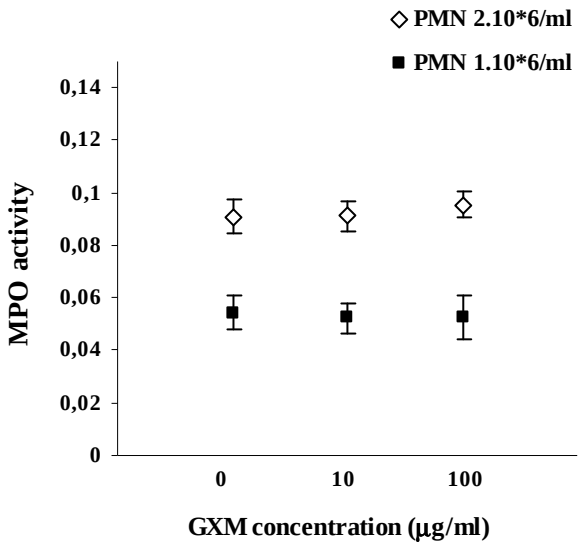

Figure 2. GXM does not affect the MPO activity of isolated neutrophils. Isolated human neutrophils suspended to 1.10^6 or 2.10^6 cells/ml in RPMI/HSA were incubated with rising concentrations of GXM in RPMI/HSA buffer or with buffer alone at 37°C for 60 minutes. After homogenization, samples of 50 µl of the resulting supernatants were assayed for MPO activity. The results of four experiments were averaged. The Y-axis represents the MPO activity per 50 µl sample. The error bars present standard deviations.

Correlation of the serum concentration of GXM with the MPO content of ischemic myocardium

To verify the observed dose-response of GXM on neutrophil influx, the plasma concentration of GXM was measured in serum of GXM-treated rats and compared with the MPO-activity of the corresponding homogenized myocardial tissue samples (Figure 3B).
In the animals treated by a 0.25 mg bolus the serum concentrations of GXM obtained 3- 3.5 hours after administration ranged between 7 and 37 µg/ml (n=6; mean GXM concentration 19 +/- 10 µg/ml), in those treated by a 2.5 mg GXM dose the concentrations ranged between 75 and 175 µg/ml (n=8; mean 118 +/- 30 µg/ml, Figure 3A). Figure 3B demonstrates a significant inverse correlation between the serum concentration of GXM and the MPO content of the ischemic myocardium of rats treated with GXM (correlation coefficient: -0.78, n= 17; two-sided $p<0.05$). These data further underscore the role of GXM in the interference with neutrophil migration.

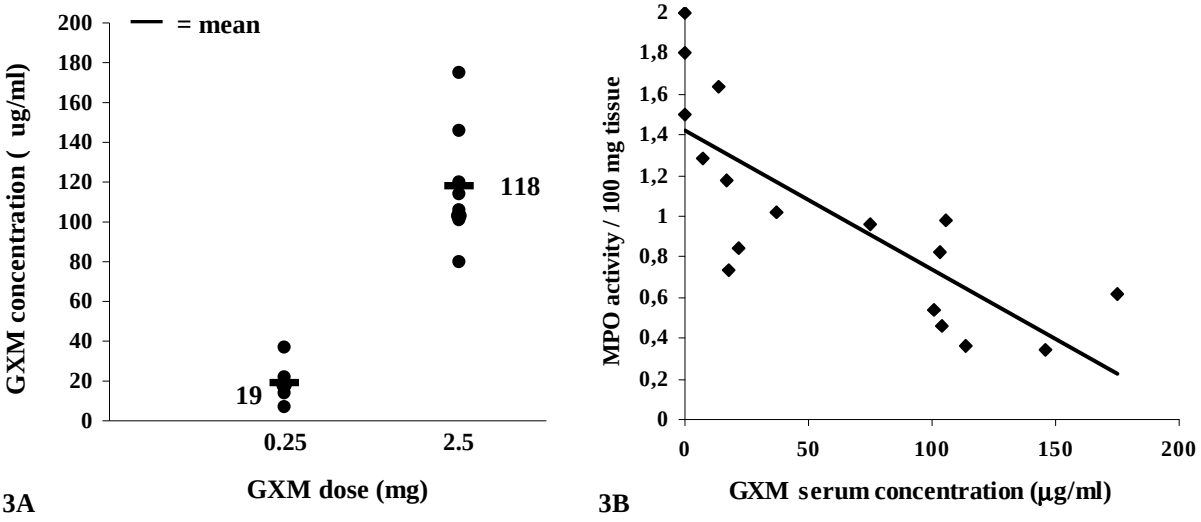

Figure 3. Correlation between the MPO content of ischemic myocardium and serum concentration of GXM. GXM concentrations were measured by ELISA in serum from infarct rats treated with intravenous GXM or normal saline. Concentraions were calculated using a standard absorbance curve of a GXM dilution series. The MPO activity was determined in the ischemic myocardium of corresponding rats. **A.** Comparison between the serum concentrations of GXM from animals that had received either 0.25 mg (n=6) or 2.5 mg GXM (n=8). **B**. Inverse correlation between the serum concentration of GXM and the MPO activity in the ischemic myocardium of corresponding rats. The black dots represent the separate rats (n=17) treated with GXM in different doses or with normal saline. The black line represents the trend line, which was calculated by linear regression (correlation coefficient: –0.77, n=17; two-sided P<0.001).

Complement activation by GXM in rat plasma

In vivo C5a generation.

To investigate any potential contribution of complement activation to GXM-related interference with neutrophil migration, we evaluated the generation of C5a in the rats treated with GXM. The presence of C5a was detected by the ability of C5a to induce calcium mobilization in undifferentiated U937 cells transfected with the human C5a receptor (U937-C5aR)[34]. By this method human recombinant C5a (rC5a) could be detected at concentrations above 10^{-11} M (data not shown).

First, we assessed the toxicity of rat plasma to the U937-C5aR cells. At dilutions up to 1:5 the addition of rat or human plasma induced cell lysis, according to changing forward and side scatters during FACS analysis and trypan blue exclusion, possibly due to toxic factors present in the rat plasma. Therefore, all plasma samples were assayed at a 1:10 dilution for the presence of C5a, at which evidently no cell lysis occurred.

Next, we verified whether rat C5a is actually able to bind to the human C5a receptor (C5aR). Fresh plasma from an untreated rat was stimulated by zymosan, which is a potent activator of the alternative pathway of complement[23]. Zymosan-activated rat plasma induced a strong response in the U937-C5aR cells as opposed to untreated rat plasma (Figure 4A), comparable to that achieved by 10-8 M human rC5a. We therefore concluded that the human C5a receptor on the U937 cells is able to bind rat C5a.

Next, we measured the presence of C5a in the plasma of rats from the different treatment groups. However, no significant amounts of C5a could be detected in plasma samples from rats that had been submitted to coronary artery occlusion with or without GXM treatment (Figure 4A).

The inability to detect C5a in these plasma samples, however, might be a consequence of the detection limit of the used method. We therefore assessed the *in vitro* ability of GXM to induce C5a generation.

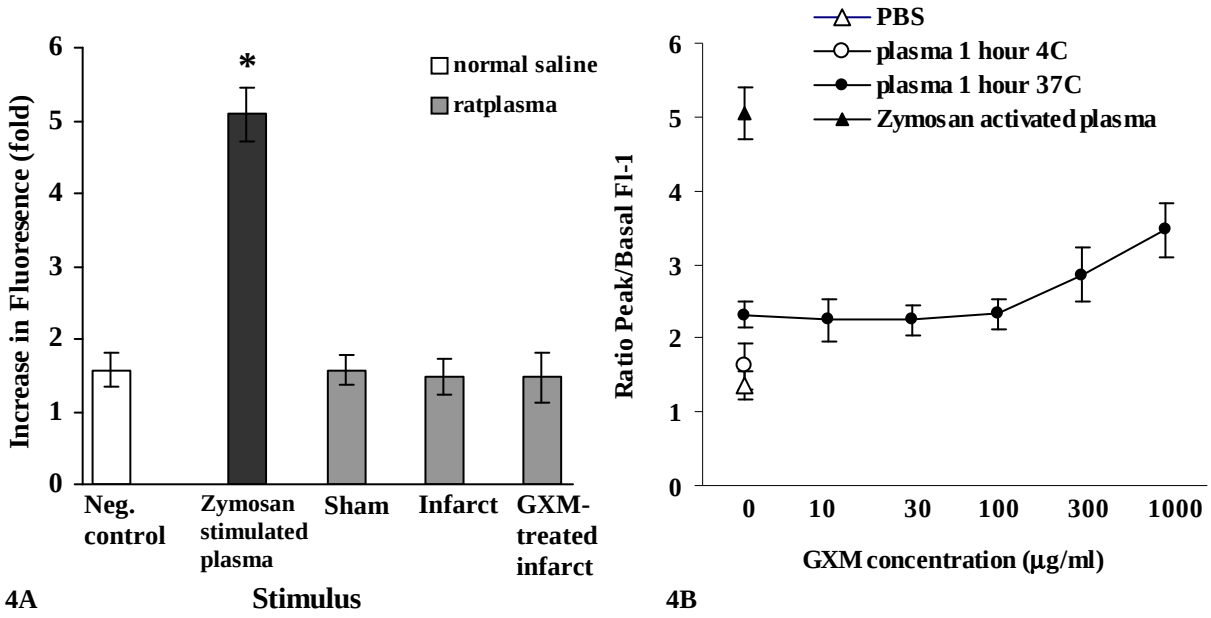

Figure 4. Detection of C5a in plasma samples. C5a in samples was detected by the ability of C5a to induce a rapid mobilization of calcium in U937-C5aR transfectants loaded with a calcium specific intracellular probe. The resulting increase in the fluorescent signal was detected by FACS® by repetitive measurements during 10 seconds. The data are expressed as relative values comparing the peak fluorescence to the basal fluorescence value before stimulation (increase in fluorescence = peak value: basal value). **A**. Detection of C5a in plasma from animals subjected to myocardial ischemia. Plasma of animals from all treatment groups (i.e.; sham animals, infarct rats with or without GXM treatment (n=4, n=5 and n=7, respectively) was assayed for the presence of C5a (grey bars) and compared to the C5a activity in normal saline (negative control, white bar) and Zymosan-stimulated rat plasma (positive control, black bar). The results of the animals within one treatment group were averaged. **B.** *In vitro* generation of C5a in rat plasma stimulated by GXM. Plasma of control rats (n=4) was stimulated *in vitro* at 37°C with Zymosan (positive control) or increasing concentrations of GXM. The samples were assayed for the presence of C5a.

In vitro C5a generation.

Plasma of control rats was stimulated with GXM at 37°C and assayed for the ability to induce calcium mobilization in the U937-C5aR transfectants. Notably, plasma incubation at 37°C in the absence of GXM led to a low-level of C5a generation when compared to plasma that was kept at 4°C (Figure 4B). Incubation of rat plasma with GXM at 37°C led to a dose-dependent generation of C5a when compared to untreated plasma that was kept at 37°C (Figure 4B). However, at GXM-concentrations that were comparable to those detected in animals treated with GXM (7-175 µg/ml), *in vitro* no or little of C5a was generated. We therefore concluded that complement activation by GXM plays little or - presumably - no role in the observed interference with neutrophil migration.

The effect of GXM on myocardial infarct size

We evaluated the effect of the GXM-related depletion of neutrophils in the ischemic myocardium on infarct size. Animals were subjected to the same protocol of coronary artery ligation and reperfusion and received either 2.5 mg GXM or normal saline during the occlusion period. Importantly, the ratios of the size of the area at risk (i.e., the ischemic area) to the size of left ventricle were comparable between groups (Mean ratio 0.49 +/- S.E.M. 0.04 and 0.48 +/- S.E.M. 0.04, respectively).

However, despite the observed reduction in neutrophil influx in the ischemic area, the treatment with GXM did not lead to a significant smaller infarct (Figure 5; detected difference 0.07 (10%); p=0.3).

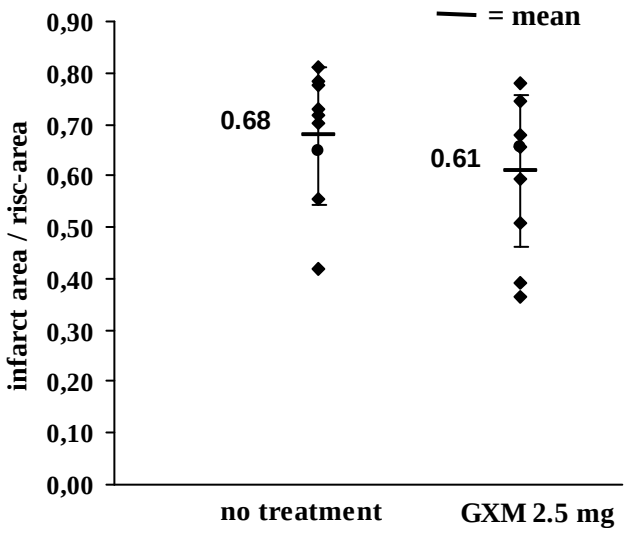

Figure 5. The effect of GXM treatment on myocardial infarct size. Animals were subjected to coronary artery ligation during 1 hour followed by a 3-hour reperfusion period. A bolus of normal saline (n=8) or 2.5 mg GXM (n=8) was administered during the occlusion period. After termination, the hearts were cut out and the different areas were separated, dried and weighed separately. The infarct size is expressed as the relative ratio of the weight of the irreversibly damaged tissue (infarct area) to the weight of the area at risk (the non-perfused or ischemic area). The dots present the infarct sizes of the separate animals. Error bars represent the standard deviation.

Discussion

The ability of the capsular polysaccharide GXM of Cryptococcus neoformans to interfere with leukocyte migration *in vitro*[8,13,18,39] has been supported by limited *in vivo* studies. GXM been shown to contribute to the limited inflammatory infiltrates observed during cryptococcal infections [39,41] and to reduce leukocyte migration when administered in a model of gelatin sponge implantation[14] and bacterial infection[40]. Additionally, CneF (cryptococcal culture filtrate) has been demonstrated to reduce leukocyte migration when administered in diseases such as bacterial infection[44] and aseptic inflammation[45,46]. However, the impact of other capsular components present in CneF, such as mannoproteins and galactoxylomannan (GalXM), cannot be out ruled in the case of CneF treatment.

This paper endorses the capacity of GXM to inhibit neutrophil infiltration in models other than infection. We applied purified GXM in a rat model of myocardial ischemia/reperfusion injury with the objective to inhibit the early influx of neutrophils following ischemia, which has been related to the aggravation of tissue damage[25,54].

We quantified the influx of neutrophils by the enzymatic detection of MPO, a major neutrophil protein[35], which has become a widely accepted method for neutrophil quantification in tissues[28,56]. We have demonstrated that i.v. treatment with GXM markedly reduced the MPO content in ischemic myocardium. Since GXM did not alter the viability of neutrophils *in vitro* and did not directly affect the MPO activity, we concluded that the reduction in MPO activity could be ascribed to a decrease in neutrophil migration rather then to toxicity.

Prior animal studies have demonstrated that purified GXM or mixtures of cryptococcal polysaccharides are well tolerated in doses ranging between 0.25 and 90 mg/kg when administered intravenously[14,40,44-46]. In the current study, we administered GXM at either 0.83 or 8.3 mg/kg to reach estimated serum concentrations of 10 and 100 μg/ml, respectively. These doses were lower than previously applied in animal models of inflammation (30 mg/kg[40]; 0.25-40 mg/kg[44]; 30-90 mg/kg[45,46]. The selection of the doses in our study was based on the concentrations at which GXM *in vitro* inhibits neutrophil chemokinesis (50-1,000 μg/ml;[8] and unpublished data) and adhesion to the endothelium (0.01-100 μg/ml;[18], Ellerbroek et al, Cellular Microbiology, in press). These concentrations correspond with the titers usually detected during cryptococcal infection[10,20,43]. Further, the decision to administer a single dose of GXM was justified by the reported half-life time of GXM that ranges between 14 and 48 hours[19,26,27].
Optimal inhibition of MPO activity was detected after the administration of 2.5 mg GXM (mean serum concentration 118 +/- 30 μg/ml) and a clear correlation could be demonstrated between the serum concentration of GXM and the degree of inhibition. No significant difference was observed when comparing GXM administration during occlusion to that just after the start of reperfusion.

The current view is that GXM blocks leukocyte migration by a combination of inhibition of leukocyte adhesion ([15,16,18], Ellerbroek et al, Cellular Microbiology, in press) and interference with chemokinesis[8,13,39]; the latter by a combination of its own intrinsic chemoattracting properties[12,13] and modulation of chemokine receptors[47]. In those studies, the effects of GXM evolved in the absence of serum and were therefore independent of complement activation. However, since GXM is known to activate the complement system[37], the resulting generation of the chemoattracting factor C5a in the bloodstream might be an additional mechanism underlying the observed interference with leukocyte migration. The presence of massive amounts of C5a in the bloodstream would theoretically prevent neutrophils from responding properly to other chemoattracting substances present in the inflamed tissues. In the current study, we were not able to detect any C5a in the plasma of rats treated by GXM. However, this could be the result of the detection limit of the assay used. Important therefore is our finding that, GXM *in vitro* did not cause excessive complement activation in rat serum at concentrations corresponding with those detected in sera from treated rats. We conclude that the generation of C5a most likely plays no role in the interference with neutrophil migration.

Despite its clear effect on neutrophil influx in the ischemic myocardium, GXM treatment did not lead to a significant reduction in infarct size. In order to prove the observed trend toward a reduction of infarct size (10%), the treatment groups would have to be drastically

expanded. Previously, several methods for experimental PMN depletion have been investigated in animal models of ischemia-reperfusion injury. Although a considerable number of these studies showed a clear effect on infarct size[21,32,36,38,50], some reports, however, failed to show any effect of anti-neutrophil measures on reperfusion injury[2,48,55]. In humans, only limited trials have been performed with anti-adhesive therapy thus far, but none of these studies could demonstrate a reduction of myocardial infarct size[1,51]. Most likely, the inconsistency in the success rates of these studies can partly be explained by differences in the experimental procedures, e.g., the animal species, the duration of the ischemic and reperfusion periods, the size of the non-perfused area, the method of measuring infarct size. For instance, the ratio of irreversible versus reversible myocardial damage will rise with an increasing duration of the ischemic period. Additionally, the different anti-neutrophil therapies display great diversity in the cellular mechanism by which neutrophil migration is inhibited, which will affect the degree of neutrophil inhibition.

Thus, it may be worthwhile to further assess the effect of GXM on neutrophil depletion and infarct size after a shorter ischemic period. Further, GXM-induced inhibition of neutrophil influx might benefit the later stages of infarct healing and remodeling. Moreover, since neutrophil influx has been shown to be an independent factor favoring arrhythmias during reperfusion[9,31], we plan to evaluate the effect of GXM-therapy on the myocardial electro physiology during reperfusion.
In conclusion, GXM reduces neutrophil influx in myocardial reperfusion injury, which is not mediated by the generation of C5a. In this set of experiments GXM treatment did not affect infarct size significantly.

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CHAPTER 5

O-Acetylation of cryptococcal glucuronoxylomannan is essential for interference with neutrophil chemokinesis and adhesion

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Summary

The major capsular polysaccharide glucuronoxylomannan (GXM) of *Cryptococcus neoformans* has been recognized as an important virulence factor that is involved in the down regulation of immune responses in the host, such as interference with neutrophil migration. Although several receptors have been indicated to play a role in this process, the structural perspective is not known. Prominent structural features of GXM are 6-O acetylation of the (1→3)-α-D-mannan backbone and xylose substitution. We assessed the importance of these carbohydrate motives for the interference with *in vitro* neutrophil (PMN) chemokinesis, rolling on E-selectin and firm adhesion to the endothelium. GXM deficient in either acetyl or xylose was isolated from mutant cryptococcal strains and compared to GXM of an isogenic strain (acetyl and xylose-positive). Additionally, GXM from a different strain was chemically deacetylated.

A dramatic reduction of the inhibitory capacity of GXM was observed after de-*O*-acetylation in the *in vitro* assays for PMN chemokinesis, rolling on E-selectin and firm adhesion to the endothelium. In contrast, xylose-deficient GXM showed a marginally reduced capacity to interfere with the reported assays.

Our findings indicate that 6-*O*-acetylated mannose of GXM is a crucial motive for the inhibition of neutrophil recruitment.

Introduction

The pathogenic fungus *Cryptococcus neoformans* mainly causes serious infections in the immunocompromised host, such as AIDS patients. The cryptococcal polysaccharide capsule has been recognized as a crucial virulence factor. Its main component is the polysaccharide glucuronoxylomannan (GXM), which is abundantly shed into its surroundings and can be detected in body fluids of patients during infection[10,18,37]. High serum titers of GXM during cryptococcosis have been associated with progressive disease and subdued inflammatory responses[10,45]. It has previously been recognized that GXM exerts various effects on the immune apparatus of the host, such as interference with phagocytosis[25], activation of the complement system[28], suppression of T-cell mediated immunity[46], upregulation of cytokine production[5,31,47] and induction of immunogenic tolerance[26].

Moreover, cryptococcal culture filtrate and isolated GXM have been shown to interfere with the migration of leukocytes toward inflammatory sites *in vivo*[13,33,34,38-40], and different mechanisms contribute to this phenomenon. First, cryptococcal polysaccharides impair neutrophil migration towards chemoattractants[9,12,32] despite adequate stimulation of chemokine production[5,32,47]. A combination of the intrinsic chemoattracting properties of circulating GXM[11,12] and down regulation of chemokine receptors[41] will prevent neutrophil from leaving the bloodstream and migrating towards inflammatory sites. Second, polysaccharides interfere with leukocyte adhesion to the endothelium. GXM has been demonstrated to induce L-selectin shedding[9,14], to bind CD18[15] and to actually impair both phases of neutrophil adhesion to the endothelium, namely the initial rolling (Chapter 3; Ellerbroek, 2004, Cellular Microbiology, in press) as well as subsequent firm binding to the endothelium[17] by interfering with E-selectin binding pathways.

Although several potential cellular receptors for GXM have been recognized (i.e., CD14, TLR4 and CD18)[15,41,50], the mechanism by which GXM modulated neutrophil migration remains to be elucidated. Studies that relate the function of GXM to its structure enhance the knowledge of the mechanisms that underlie the immunological effects of GXM. From a structural perspective, nothing is known about the epitopes of GXM responsible for its inhibitory capacity on PMN migration. GXM consists of a linear (1→3)-α-D-mannan backbone that is variably *O*-acetylated and substituted with glucuronic acid and (variable amounts of) xylose[7].
The importance of *O*-acetyl and xylose for other immunological phenomena has mainly been studied using different cryptococcal serotypes and naturally prevailing capsule variations [7,29,44,51,52,58]. Additionally, chemical modification of GXM and, more recently, the identification of the genes involved in *O*-acetylation (*CAS1*, encoding a putative glycosyl transferase[23]) and the synthesis of UDP-xylose (*UXS1*, encoding UDP-xylose synthase[1,43]) have provided more knowledge of their relevance for the function of GXM.
For instance, the classification of strains into serotypes (A-D) was based on the number of 6-*O*-acetyl substituents and by the degree of xylose substitution; which determine the antigenic activity[2,7,8] and *O*-acetyl has been recognized as the major epitope for recognition of GXM by antibodies[7,16]. Chemical de-*O*-acetylation of GXM decreased the binding of antibodies[2], and *O*-acetyl-deficient GXM from a *cas1*Δ mutant strain proved less potent in binding

monoclonal antibodies[27]. Additionally, *O*-acetyl deficient GXM was cleared more rapidly from serum[27].
Differences in complement deposition on cryptococci could be related to the degree of xylose substitution in GXM of various strains[48,56]. GXM lacking xylose from a *uxs1*Δ mutant strain changed the binding kinetics of C3 to cryptococci, which confirmed that xylose is important for the binding of C3[27]. Chemical and genetic modification of GXM showed that neither xylose nor *O*-acetyl appeared to play a role in the inhibition of phagocytosis by GXM[25,27]. Glucuronyl side-chains provide a negative cellular charge to GXM, and thus far, modification by decarboxylation has not revealed other functions[25].

In this study, we investigated the importance of 6-*O*-acetylation and xylose substitution of the mannose backbone for the capacity of GXM to interfere with *in vitro* neutrophil migration. To this end, we compared chemically deacetylated GXM and *O*-acetyl- or xylose-deficient GXM from *cas1*Δ and *uxs*Δ strains, respectively to wild-type GXM for their ability to interfere with neutrophil chemokinesis and adhesion to the endothelium.

Materials and methods

Cryptococcal strains

The strains used in this study are described in Table 1. The *C. neoformans* strains lacking xylose (NE178) or *O*-acetylation (NE168)[23,43] were obtained by backcrossing the disrupted strain with the original strain (JEC155, *MAT*α *ura5 ade2*, serotype D and JEC156, MAT**a** *ura5 ade2*[57]). NE167 was used as an isogenic control[23]. *C. neoformans* serotype A (ATCC-62066) was obtained from the ATCC. Pneumococcal polysaccharide (PnPS type 3) was a kind gift of H. Snippe (Eijkman Winkler Institute, University Medical Center, Utrecht, The Netherlands[53]).

Strains	Genotype	Serotype	Capsule modification	Source or Reference
NE167	*MAT*α *CAS1 UXS1*	D	-	(23, 27)
NE168	*MAT*α *cas1*Δ::*ADE2*	D	*O*- acetyl-deficient	(23, 27)
NE178	*MAT*a *uxs1*Δ::*ADE2*	D	Xylose-deficient	(27, 43)
ATCC-62066	unknown	A, chemotype5	-	ATCC

Table 1. Cryptococcal strains used in this study.

Isolation and deacetylation of GXM

GXM was purified from the various strains as previously described[6]. Briefly, *C. neoformans* was grown in a chemically defined broth for 5 days followed by autoclaving. The polysaccharides present in the medium were precipitated with calcium acetate and ethanol.

After dissolving the polysaccharides in sodium chloride and brief sonication by ultrasound, GXM was precipitated by differential complexation with hexadecyltrimethylammonium bromide (HTAB; Sigma, Louisville, MO, USA). Purified GXM was again precipitated with ethanol, dissolved and sonicated for 2 hours, followed by centrifugation and dialysis. Finally, GXM was recovered by lyophilization, dissolved in PBS (5 mg/ml), sterilized by filtration and stored at -20°C.
GXM from strain ATCC-62066 was deacetylated by incubating GXM at 5 mg/ml in aqueous NaOH at pH 11 for 24 hours. After neutralization with aqueous HCl, the solution was dialyzed against bi-distilled water and lyophilized.

Chemical and physical properties of GXM
Acetyl group determination was performed according to Hestrin[21] using acetylcholine as a standard. The acetyl content of GXM preparations is expressed relative to that of GXM derived from ATCC-62066 (serotype A), which contained an average of 2 acetyl groups per 3 mannose residues as determined by ^{1}H NMR spectroscopy at 500 MHz in D_2O. For monosaccharide analysis, samples were subjected to methanolysis (1.0 M methanolic HCl, at 85°C for 24 hrs], followed by trimethylsilylation [5:1:1 pyridine/ chlorotrimethylsilane/ hexamethyldisilazane, at room temperature for 30 min), and analyzed by gas chromatography (GC) and GC- mass spectrometry (GC-MS)[24.] Rheological measurements were performed on a MCR300 modular compact rheometer using a cone-plate geometry of stainless steel with a diameter of 50 mm and a cone angle of 2 degrees. Results were obtained using a rotation test and are expressed as viscosity (η) in mPa·s. Molecular weight distribution of GXM was determined by gelfiltration chromatography of 1 mg/ml GXM samples (Superose 6.0 column, Pharmacia) as previously described (Chapter 2,17).

Maintenance of Endotoxin-free conditions
All materials were kept under pyrogen-free conditions. Preparations of the various types of GXM were tested for the presence of Endotoxin using a Limulus Amoebocyte Lysate assay (Coatest Endotoxin Diagnostica, Mölndal, Sweden). The detected LPS concentration was always less than 0.6 ng/ml.

Cells and cell lines
Primary endothelial cells from human umbilical veins (HUVEC) were isolated from donor umbilical veins according to the method described by Jaffe *et al*[22] and cultured in commercial Endothelial Growth Medium (EGM-2®, Cambrex, Walkersville, USA). Only passages 1 and 2 were used for the experiments.
Chinese Hamster Ovary (CHO) cells stably transfected with human E-selectin[36] were kindly provided by R.C. Fuhlbrigge (Department of Dermatology, Brigham and Women's Hospital, Boston, MA, USA) and originated from R. Lobb (Biogen, Cambridge, MA, USA). The cells were cultured in Modified Eagles Medium (MEM, GIBCO, Life Technologies, Breda) containing 10% fetal calf serum (v/v) and penicillin/streptomycin. CHO cell monolayers were grown to confluence in 3-4 days on plastic cover slips before flow-experiments.

Isolation and GXM treatment of human neutrophils

Human neutrophils (PMN) were isolated from healthy volunteers as previously described[17]. Isolated PMN were suspended in either sterile RPMI/HSA (static adherence assays), HBSS/HSA (Hanks Balanced Salt solution; chemotaxis assay) or HEPES buffer (rolling experiments) at 2.10^6 cells/ml. HEPES buffer contained 20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.2 mM KH_2PO_4 supplemented with 5 mM glucose, 1.0 mM $CaCl_2$, and 0.5% (v/v) human serum albumin (HSA).

Based on the results of previous studies and dose response curves the conditions of GXM treatment (i.e.; duration, GXM concentration and temperature) differed between the various assays (This thesis Chapters 2 and 3; [9,17, Ellerbroek et al, 2004, Cellular Microbiology, in press]). Prior to the static adherence assay, PMN were incubated at 37°C in 0.1 to 1 μg/ml GXM for 45 minutes at which maximal inhibition was detected previously[17]. Before the rolling experiments the PMN were treated with 10 μg/ml GXM at room temperature for 30 minutes. For the chemotaxis assay, a dose-response curve was performed for the two unmodified types of GXM. For GXM derived from ATCC 62066 (GXM-62066) the optimal inhibiting concentration was 250 μg/ml (room temperature, 30 minutes), whereas for GXM derived from NE167 optimal inhibition required a concentration of 100 μg/ml.

Static adhesion assay

Static adhesion assays were performed as previously described[17,35]. HUVEC were plated on fibronectin-coated NUNC 72-well plates (Life Technologies, Breda, The Netherlands) and grown to confluence in 1-2 days. The cells were stimulated with 10 ng/ml TNFα (Roche, Almere, The Netherlands) in culture medium37°C, six hours). After three washes with RPMI-40 medium, the untreated (control) or GXM-treated PMN were added to the wells in a concentration of 10^6/ml (PMN : endothelial cell ratio = 5:1) and left to adhere at 37°C for 15 minutes. After carefully washing the endothelium, the adherent cells were fixed with 2% paraformaldehyde (pH 7.4) and counted within a fixed frame of 1 mm^2 in the center of each well by fluorescence microscopy (Leitz Fluovert inverted microscope). Measurements were performed in quadruple wells for each variable. All tests were repeated using different batches of donor cells.

Chemotaxis assay[19]

PMN were labeled with 5 μg/ml Calcein-AM (Molecular Probes, Eugene, Or, USA) for 30 minutes at room temperature. After washing, the PMN were incubated with the different types of GXM for another 30 minutes. Migration was measured using 96-well micro-chambers with polycarbonate filters (ChemoTx, Neuro Probe) as previously described[19]. To each bottom well 29 μl of HBSS (negative control) or 29 μl of the chemotactic factor fMLP was added (1 nM; Sigma Chem Co, St. Louis, MO, USA). Polycarbonate filters with an 8 μm pore size (ChemoTx, Neuro Probe, MD, USA) were placed on top of the plate and 25 μl of PMN suspension ($2.5x10^6$/ml in HBSS/HSA) was directly placed onto the hydrophobic filter sides. The chamber was then incubated for 45 minutes (37°C, 5% CO_2). The non-migrating cells were then gently flushed off the filter with PBS and the cells that migrated into the filter and the bottom chamber were determined by measuring the fluorescence in a multi-well fluorescence plate reader (excitation 485 nm, emission 530 nm; Flexstation,

Molecular Devices). Control wells containing Calcein-labeled cells were included to obtain the maximum fluorescence value.

Perfusion experiment and analysis

Perfusions under steady flow conditions were performed in a modified form of a transparent parallel plate perfusion chamber[49,54]. The perfusion contains a circular plug on which cover slips (18x18 mm) coated with E-selectin expressing CHO cells were placed and exposed to PMN suspensions in HEPES buffer.

For each individual perfusion 1.2 ml of PMN suspension (2.10^6/ml) was aspirated from a reservoir through plastic tubing through the perfusion chamber with a Harvard syringe pump (Harvard apparatus, South Natic, MA) by which the flow rate could be precisely controlled. In individual experiments the PMN were perfused through the chamber at 37°C at a wall shear stress of 3.0 dynes/cm^2. During perfusions the flow chamber was mounted on a microscope stage (DM RXE, Leica, Weitzlar, Germany), which was equipped with a B/W CCD-video camera (Sanyo, Osaka, Japan), coupled to a VHS-video recorder. Perfusions were recorded on videotape, and video images were evaluated for the number of cells in close contact with the E-selectin CHO cells (= adherent cells), using dedicated routines made in the image-analysis software Optimas 6.1 (Media Cybernetics systems, Silverspring, MD, USA). PMN in contact with the surface, which consisted of both rolling cells and firmly adhered cells (from now on together referred to as "adherent cells") appeared as bright white-centered cells after proper adjustment of the microscope during recording. The adherent cells were detected by the image analyzer. The number of adherent PMN was measured after 5 minutes perfusion at a minimum of 25 high power fields (total surface of at least 1 mm^2).

Statistics

All data were analyzed by a univariate analysis of variance test using a two factor ANOVA, analyzing only main effects (in which the donor was set as a blocking factor). To compare multiple treatment groups to each other or to the control, the ANOVA was then followed by a post hoc analysis by Bonferroni's test. P values < 0.05 were considered significant.

Results

Chemical and physical aspects of GXM

The acetyl content and monosaccharide analysis of unmodified GXM and chemically and genetically modulated GXM are presented in Table 2. Corresponding with earlier reports[3], unmodified GXM of the strain classified as serotype D (NE167) had a lower *O*-acetyl content (60%) when compared to that of the serotype A strain (ATCC-62066).

The acetyl contents of chemically deacetylated GXM of strain ATCC-62066 and the *O*-acetyl-negative *cas1*Δ strain NE168 were < 5% and 9%, respectively, when comparing to *O*-acetyl-positive GXM of the original strains (100%).

	Man	*Xyl*	*GlcA*	*%Ac*
GXM ATCC-62066	3.9	2.2	1.0	100
GXM ATCC-62066: De-*O*-Ac	3.7	2.2	1.0	<5
NE167	3.6	1.1	1.0	60
NE168 (*cas1*Δ)	3.6	1.9	1.0	9
NE178 (*uxs1*Δ)	3.5	-	1.0	81

Table 2. Chemical analysis of GXM. The monosaccharide content of GXM was determined by methanolysis followed by gas chromatography (GC) and GC- mass spectrometry (GC-MS) and expressed as molar ratios. The acetyl content is expressed relative to that of GXM derived from ATCC-62066 (serotype A), which contained an average of 2 acetyl groups per 3 mannose residues as determined by ^{1}H NMR spectroscopy. *Man* = mannose; *Xyl* = xylose; *GlcA* = glucuronic acid; *Ac* = acetyl

In general, the ratio of mannose/xylose/glucuronic acid varies between 3:1:1 and 3:4:1, depending on the strain[3]. Chemical de-*O*-acetylation of GXM of strain ATCC-62066 did not change the monosaccharide ratio. The mannose and glucuronic acid ratios of all types of GXM did not differ significantly.

Strain NE178 lacked the *UXS1* gene encoding for UDP-xylose synthase, and consequently GXM derived from this strain contained no detectable amount of xylose. The isogenic control NE167 strain (serotype D) had a lower xylose content relative to all the other types of GXM (Table 2).

Rheological measurements showed no difference in viscosity between GXM and chemically deacetylated GXM at both concentrations and temperatures tested (Table 3); moreover, the apparent molecular weights were the same as determined by gelfiltration (data not shown).

	20 ℃		*37 ℃*	
	100 μg/ml	**1 mg/ml**	**100 μg/ml**	**1 mg/ml**
GXM	1.03	1.19	0.67	0.81
GXM De-*O*-Ac	1.01	1.19	0.68	0.80
PnPS type 3	1.01	1.39	0.70	0.95
PBS		1.00		

Viscosity (η) in mPa·s

Table 3. Viscosity of GXM. Rheological measurements were performed on a MCR300 modular compact rheometer and expressed as viscosity (η) in mPa·s. Intact GXM was compared to chemically deacetylated GXM (GXM-De-AC). PBS and Pneumococcal polysaccharide type 3 (PnPS type 3) were used negative and positive controls, respectively.

_O_-acetylation of GXM is required for interference with neutrophil chemokinesis

In vitro, GXM treatment of PMN has been shown to inhibit the chemokinesis of PMN toward various chemoattractants such as IL8, PAF, C5a and fMLP[9,12,13,32]. To assess the importance of *O*-acetyl or xylose for this function of GXM, we compared the effect of intact GXM to that of GXM lacking *O*-acetylation or xylose in a chemotaxis assay.

In our chemotaxis assay, preincubation of PMN with unmodified GXM derived from strain ATCC-62066 inhibited the chemokinesis of PMN toward fMLP by 34% (Figure 1, $p<0.05$), whereas chemically deacetylated GXM from this strain did not affect chemokinesis significantly (inhibition 9%, $p=0.24$; $p<0.05$ when compared to unmodified GXM). Similarly, *O*-acetyl-positive GXM derived from strain NE167 blocked PMN migration by 46% ($p<0.05$), whereas GXM deficient in *O*-acetylation (from the isogenic *cas1*Δ strain NE168) was considerably less potent (inhibition 19%, $p<0.05$ when comparing to untreated PMN and to treatment by *O*-acetyl-positive GXM). GXM lacking xylose from *uxs1*Δ strain NE178 blocked PMN migration toward fMLP by 38%, which did not differ significantly from the inhibition caused by xylose-positive GXM from the isogenic strain NE167.
We therefore conclude that *O*-acetylation of GXM, but not xylose, is necessary for its ability to interfere with PMN chemokinesis.

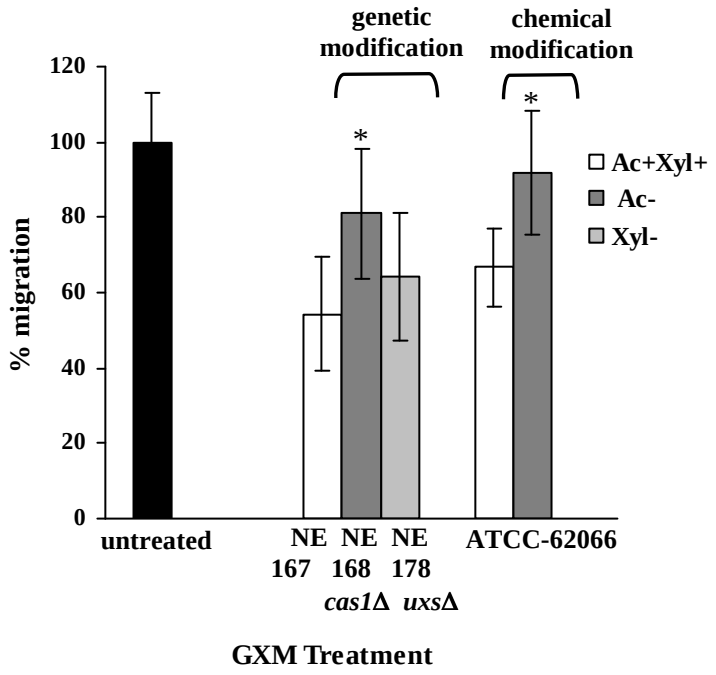

Figure 1. Chemotaxis of GXM-treated PMN toward fMLP. PMN were treated with medium (no treatment) or GXM of the different strains (room temperature, 45 minutes) and their chemokinesis toward fMLP was compared in a chemotaxis assay. The cells that migrated into the filter and the bottom chamber were determined by measuring the fluorescence in a multi-well fluorescence plate reader. Observations were made in triplicate. Results are the average of 11 experiments and migration is expressed as a percentage of the migration of untreated PMN (positive control; 100%). Ac+Xyl+ = acetyl and xylose containing GXM; Ac- or Xyl- = acetyl or xylose deficient GXM. * = $p<0.05$ when comparing the chemokinesis of modified GXM to that of PMN treated with unmodified GXM. The error bars represent the standard deviations.

_O_-acetylation of GXM is essential for its ability to inhibit neutrophil rolling

We previously reported that GXM impairs the rolling of PMN on stimulated endothelium as well as on E-selectin expressing CHO cells (Chapter 3, Ellerbroek et al, 2004, Cellular Microbiology, in press). In the current study, we chose to flow the PMN over monolayers of E-selectin transfected CHO cells rather than over stimulated endothelium, since it provided us with a more selective rolling model in which firm binding between integrins and their ligands is minimized.

Here, both types of intact GXM derived from strains ATCC-62066 and NE167 reduced PMN rolling on E-selectin by 40% and 32%, respectively (Figure 2; $p<0.05$ for both treatments). However, both chemical and genetic de-*O*-acetylation of GXM abolished the ability of GXM to reduce neutrophil rolling (4% and 5% inhibition, respectively; $p=1$ in both cases when comparing to untreated PMN).

In contrast, GXM lacking xylose (from mutant strain NE178) reduced neutrophil rolling to a comparable extent as xylose-positive GXM from NE167 (Figure 2; 26% versus 32% inhibition, respectively, $p=1$).

These results are comparable to those observed in the chemokinesis experiments and indicate that *O*-acetylation, but not xylose, is important for the interference of GXM with neutrophil rolling.

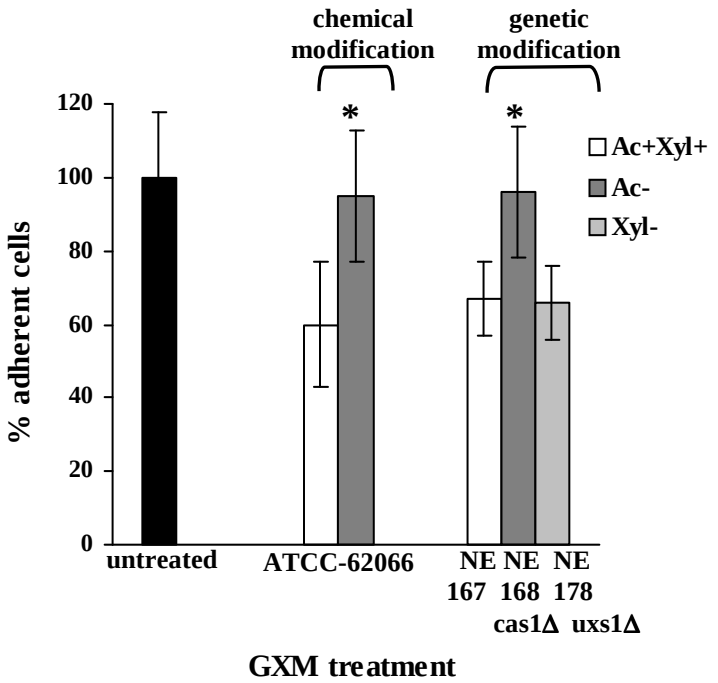

Figure 2. Rolling of GXM-treated PMN on E-selectin transfectants. PMN were incubated with buffer (untreated) or GXM (room temperature, 30 minutes) and subsequently flownover E-selectin expressing CHO cells. Cells were allowed to interact with the endothelium for five minutes followed by video capturing of images. For each flow, the numbers of rolling (adherent) cells were counted in 25 video images recorded and expressed per mm^2. The adherence counts of nine experiments were averaged. The bars express the percentage of adherent cells when compared to the positive control (i.e. adhesion of untreated PMN to stimulated endothelium = 100%). Ac+Xyl+ = acetyl and xylose containing GXM; Ac- or Xyl- = acetyl-or xylose-deficient GXM. * = $p<0.05$ when comparing rolling of PMN treated with genetically or chemically modified GXM to that of unmodified GXM from the isogenic strain. The error bars represent the standard deviations.

Firm adhesion of neutrophils to stimulated endothelium

In a previous study, we reported that preincubation of PMN with GXM led to a considerable reduction in the firm adherence of PMN to TNFα-stimulated endothelium (This thesis Chapter 2;[17]).

In the current study, treatment of PMN with chemically or genetically deacetylated GXM (isolated from strains ATCC-62066 and NE168, respectively) scarcely affected PMN adhesion (Figure 3; 0-9%) when compared to unmodified GXM derived from the original strains (29-34% inhibition).

In addition, xylose-deficient GXM (NE178) appeared less potent than xylose-positive GXM (NE167) in reducing PMN adhesion (inhibition of adhesion 17-18% versus 29-31%, respectively; $p<0.05$ when comparing both), although the difference was less pronounced as observed for deacetylated GXM.

Thus, in addition to *O*-acetyl, xylose appears to play a modest role in the GXM-related interference with firm PMN adhesion.

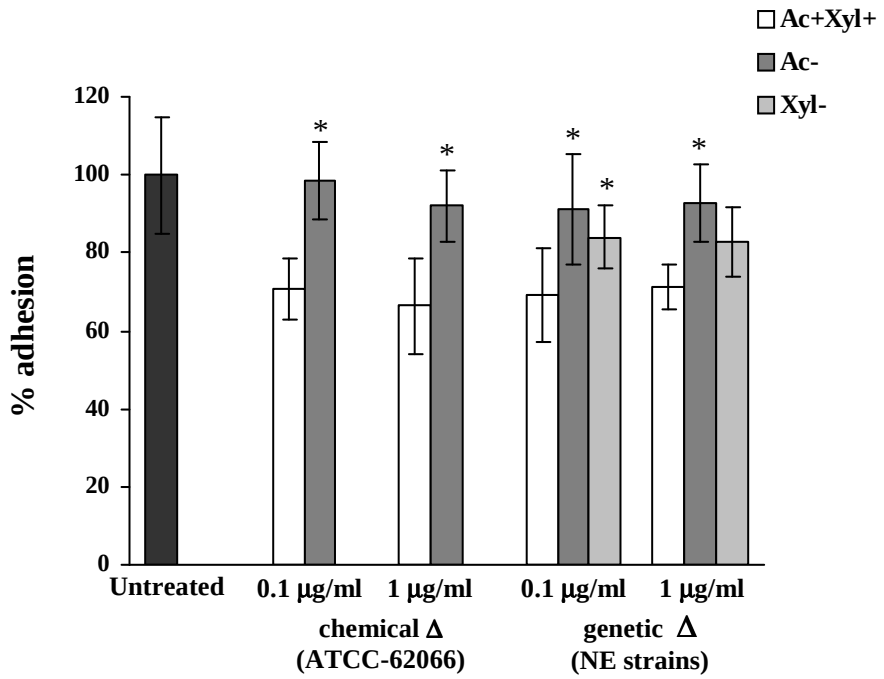

Figure 3. Firm adhesion of GXM-treated PMN to endothelium. BC-ECF-labeled PMN were incubated with GXM (37°C, 45 minutes) followed by adhesion to TNFα-stimulated HUVEC monolayers (see Materials and Methods). Adherent PMN were counted by fluorescence microscopy and observations were made in quadruplicate. The negative control consisted of the adhesion of untreated PMN to unstimulated endothelium; the positive control represents the adhesion of untreated PMN to TNFα-stimulated endothelium. The results of 15 experiments were averaged and subsequently expressed as the percentage of adhesion compared to the positive control (i.e., adhesion of untreated PMN to stimulated endothelium = 100%). Ac+Xyl+ = acetyl and xylose containing GXM; Ac- or Xyl- = acetyl or xylose deficient GXM. * = $p<0.05$ when comparing PMN treatment with modified GXM to that of unmodified GXM from the isogenic strain. The error bars represent the standard deviations.

Discussion

Previous studies from others and our group on the mechanism of action of GXM have focused on the different stages of neutrophil migration and the identification of GXM-binding receptors. Knowing the importance of the structural epitopes of GXM for the process of PMN inhibition will aid the search for specific receptors that are directly involved in the modulating actions of cryptococcal GXM on the host immune response.

Studies that used chemically or genetically modified GXM have revealed the importance of the individual structural components of GXM for its attribution to virulence. The identification of genes involved in *O*-acetylation and xylosylation (*CAS1* and *UXS1*, respectively) and subsequent studies using mutant cryptococcal strains have underscored the importance of *O*-acetylation and xylose for virulence of the organism and their role in the pathogenesis of cryptococcal infection[23,27,43]. These studies showed that *O*-acetylation is crucial for the recognition of GXM by antibodies and for GXM clearance from the host. Furthermore, xylose appears to be important for the accumulation of complement factor C3 on the surface of cryptococci and for accumulation of GXM in the spleen of the host[27].

Here, we investigated the contribution of *O*-acetyl and xylose to the interference of GXM with the chemokinesis of PMN[9,12,32] and the process of PMN adhesion to the endothelium ([17] and Chapter 3, Ellerbroek et al, Cellular Microbiology 2004, in press). We compared chemically deacetylated GXM and GXM derived from *cas1Δ* and *uxsΔ* mutant strains that were deficient in either *O*-acetyl or xylose, respectively, to GXM from the corresponding (isogenic) strains. Deacetylated GXM was considerably less potent than intact GXM in interfering with PMN chemokinesis toward fMLP as well as with both phases of PMN adhesion. This might be explained by a shared component in the cellular process with which GXM interferes with these different stages of migration (e.g., a common GXM receptor or signaling pathway).
GXM of strains NE167 and ATCC-62066, inhibited PMN migration to a comparable extent while differing in acetyl content (Table 2). Apparently, a certain level of *O*-acetylation is sufficient for the inhibitory effects of GXM. Notably, while Janbon *et al* did not detect any *O*-acetylation on GXM of the *cas1*Δ strain (NE168)[23], we detected 9% *O*-acetylation. This discrepancy, which we consider as non-relevant for the interpretation of our data, might be caused by the different assays employed to determine *O*-acetylation or, alternatively, by the slightly different purification strategies.

Since de-*O*-acetylation completely abolished the GXM-related interference with PMN rolling and firm adhesion, 6-*O*-acetyl-D-mannose appears to be a major factor for these functions of GXM. However, deacetylation did not completely abolish GXM-related interference with chemokinesis. Thus, for the interference with chemokinesis additional factors might play a role.

In our experiments, xylosylation of GXM appeared unimportant for the effects on PMN chemotaxis or PMN rolling, but did appear to play a modest role in the interference with the firm adhesion to endothelium. This indicates that specificity also exists in the cellular

pathways following GXM treatment when comparing these different stages in neutrophil migration. Notably, the degree by which xylose-containing GXM inhibited the different migration assays was comparable between GXM from strains ATCC 62066 and NE167 that differed in (relative) xylose-content, which also suggests that the role of xylose will only be modest.

The physico-chemical properties of GXM and its deacetylated analog, such as viscosity and size were similar and therefore do not explain the effect of GXM on chemokinesis and adhesion of PMN. In contrast, de-*O*-acetylation of mannose in xanthamgum, a heteroglycan polysaccharide produced by *Xanthomonas campestris*, resulted in increased viscosity[20]. Thus, the observed effects appear to be specific and apparently a receptor must exist that is crucial for the inhibitory effects of GXM and binds 6-*O*-acetyl-α-D mannose, possibly as part of a larger saccharide structure.

At present, a receptor that binds 6-*O*-acetyl-α-D-mannose, thereby initiating the cellular mechanisms leading to interference with PMN chemokinesis and adhesion remains to be elucidated. *O*-acetyl groups are common substituents of bacterial polysaccharides and generally determine immune reactivity and function as epitopes for antibody binding; other functions have scarcely been described[4,30,55]. Additionally, no neutrophil receptors are known to bind *O*-acetylated glycan.
Since TLR4, CD18 and CD14 are potential receptors for GXM[15,42,50] and we recently demonstrated that both TLR4 and CD14 play a role in the GXM-related interference with PMN rolling (Ellerbroek et al, Cellular Microbiology 2004, in press), additional binding studies with de-*O*-acetylated GXM will reveal whether *O*-acetyl is the major binding epitope for these specific receptors and reveal the importance of these receptors for the GXM-induced inhibition of PMN migration.

In conclusion, we established that *O*-acetyl-mannose is crucial for the effects of GXM on chemokinesis and endothelial adhesion of neutrophils; however, the exact role that *O*-acetyl fulfils in this process remains to be clarified.

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CHAPTER 6

Deacetylation of cryptococcal glucuronoxylomannan reduces its ability to interfere with *in vivo* neutrophil migration

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Summary

The capsular polysaccharide glucuronoxylomannan (GXM) has been demonstrated to interfere with both neutrophil chemokinesis and adhesion to the endothelium and to diminish the influx of inflammatory cells when administered intravenously in models of bacterial infection and ischemia. GXM consists of a polymannose backbone modified by 6-*O*-acetylation and side chains of glucuronic acid and xylose. We previously showed that deacetylation of GXM considerably reduced its ability to interfere with *in vitro* chemokinesis of neutrophils as well as with neutrophil adhesion to endothelium. The purpose of this study was to verify *in vivo* if 6-*O*-acetyl mannose is an essential motive of GXM for its interference with neutrophil migration.

The effect of GXM-treatment on neutrophil influx in ischemic myocardium was assessed in a rat model of ischemia/reperfusion injury and was compared to that of chemically deacetylated GXM. Deacetylation of GXM considerably decreased its capacity to reduce the influx of neutrophils in the ischemic myocardium (a non-significant reduction by de-*O*-acetylated GXM versus 65% reduction of MPO activity by unmodified GXM), while serum levels were similar. Moreover, differences in complement activation cannot explain the observed difference in interference with neutrophil influx since both types of GXM induced complement activation to a similar degree.

In conclusion, *O*-acetylation of GXM appears crucial for the interference of neutrophil migration *in vitro*, as well as *in vivo*.

Introduction

The capsular polysaccharide glucuronoxylomannan (GXM) of *Cryptococcus neoformans* has been recognized as an important virulence factor. GXM is abundantly shed into the blood and other body fluids of the host during cryptococcosis[7,17,30] from where it exerts different effects on the immune response. For one, it has been demonstrated that GXM interferes with leukocyte migration. *In vivo*, intravenous administration of purified GXM reduced leukocyte influx in models of infection[29] and aseptic inflammation[11]. More recently, we established that GXM treatment also diminished neutrophil (PMN) influx in a model of ischemia (This thesis, Chapter 4; submitted).

Several mechanisms account for the reduced leukocyte migration. First, GXM interferes with the process of chemokinesis towards chemoattractants[6,11,28] presumably by a combination of its own chemoattracting properties[9,10] and modulation of chemokine receptors[31]. Furthermore, since GXM is able to induce complement activation[27], the generation of massive amounts of chemotactic C5a in the circulation could hypothetically prevent neutrophils to respond to chemotactic stimuli in the inflamed tissues. Second, we showed that GXM interferes with the process of rolling and firm adhesion of PMN to the endothelium and thus presumably affects subsequent transmigration (This thesis, Chapters 2 and 3; [16], Ellerbroek et al, Cellular Microbiology, in press). Possible mechanisms include the modulation of adhesion molecules such as L-selectin[8,12], CD18[13] and interference with E-selectin binding mechanisms[16].

Structure-function studies will reveal which structural elements of GXM are important for its immunological effects. GXM consists of a (1→3)-α-D-mannan backbone that is variably *O*-acetylated and substituted with glucuronic acid and xylose.
The importance of *O*-acetylation and xylose for the antigenic activity of GXM and for complement deposition were mostly established by studies using spontaneously occurring variants and serotypes[1,5,34,35,40,42]. Additionally, chemical modification of capsule GXM and the production *of mutant cryptococcal strains lacking the genes involved in the synthesis of xylose (*UXS1) or *O*-acetylation (*CAS1*) have further clarified the importance of these building blocks[1,22,25,26,33,42]. A study using *cas1*Δ and *uxs1*Δ mutant strains confirmed that *O*-acetylation and xylose determine the antigenic activity and that *O*-acetyl is the major epitope for antibody recognition[26]. Further, it was concluded that the presence of xylose affects the binding kinetics of complement factor C3 to cryptococci, and both xylose and *O*-acetylation appeared to be involved in the clearance of GXM, but not in the phagocytosis of opsonized cryptococci[26].

We recently established that *O*-acetyl is an essential determinant for the GXM-related interference with leukocyte migration *in vitro* (This thesis, Chapter 5; submitted). We showed that chemically deacetylated GXM and *O*-acetyl-negative GXM derived from a cas1Δ mutant strain proved considerably less potent than wild-type in reducing chemokinesis, rolling and firm adhesion of PMN. We further demonstrated that xylose only plays a modest role in the interference with firm PMN adhesion. The goal of the present study was to confirm the importance of *O*-acetylation for the GXM-associated interference

with *in vivo* PMN migration. We recently demonstrated in a rat model of myocardial ischemia/reperfusion injury that intravenous administration of GXM significantly decreased the influx of PMN in the ischemic myocardium as measured by a reduction in tissue MPO (This thesis, Chapter 4; submitted).

In the current study, we have used the model of ischemia/reperfusion to compare the effect of chemically deacetylated GXM to that of unmodified GXM on PMN infiltration in the injured myocardium. We also assessed whether differences in the interference with PMN migration could be explained by differences in complement activation and subsequent generation of C5a.

Materials and methods

Isolation, chemical deacetylation and analysis of GXM

Glucuronoxylomannan (GXM) was purified from *Cryptococcus neoformans* (Serotype A, Chemotype 5, ATCC 62066) as described before ([4] and this thesis, Chapter 5). Purified GXM was deacetylated in aqueous NaOH (pH 11) at 5 mg/ml for 24 hours. After neutralization with aqueous HCl, the solution was dialyzed against bi-distilled water and lyophilized. Acetyl group determination was performed according to Hestrin[21] using acetylcholine as a standard. The acetyl content of deacetylated GXM is expressed relative to that of unmodified GXM, which contained an average of 2 acetyl groups per 3 mannose residues as determined by ^{1}H NMR spectroscopy at 500 MHz in D_2O. For monosaccharide analysis, samples were subjected to methanolysis (1.0 M methanolic HCl, at 85°C for 24 hrs], followed by trimethylsilylation [5:1:1 pyridine/ chlorotrimethylsilane / hexamethyldisilazane, at room temperature for 30 minutes), and analyzed by Gas chromatography (GC) and combined Gas- Mass spectrometry (GS-MS)[23].
GXM was tested for the presence of LPS using a Limulus Amoebocyte Lysate assay (Coatest Endotoxin Diagnostica, Mölndal, Sweden) with a sensitivity of 25 pg/ml E.coli LPS. The LPS concentration was always less than 0.6 ng/ml.

Animals

Male Wistar rats (280-320 gram, Harlan, Zeist, The Netherlands) were housed according to standard procedures. The experimental procedures were approved by the Ethical committee for the use of experimental animals within the University Medical Centre, Utrecht.

Experimental protocol

Rats were either subjected to coronary artery ligation or sham operation, according to the method of Fishbein *et al*[19] with slight modifications as previously described[36], and this thesis, Chapter 4). Briefly, after anaesthetizing with urethane and initiation of mechanical ventilation, the thorax was opened and a silk (6-0) suture was looped under the left descending coronary artery near the origin of the pulmonary artery. In infarct animals, the coronary artery ligature was closed; in sham animals, the suture was not closed. A bolus of 250 µl normal saline (placebo) or 2.5 mg GXM dissolved in 250 µl normal saline was administered intravenously to rats 15 minutes after coronary occlusion. After a one-hour

period of occlusion, the circulation of the coronary artery was restored by opening the suture. The early influx of neutrophils reaches its peak 2-6 hours after restoration of the blood flow of ischemic myocardium[14,38]. Hence, after a three-hour period of reperfusion, the rats were deeply anesthetized and the hearts were quickly excised and processed for determination of the neutrophil influx in the ischemic area. Blood was collected from the vena cava in plastic tubes without anticoagulant and in tubes containing sodium heparin just before the heart was isolated. The blood was centrifuged immediately in a refrigerated centrifuge at 2100 x *g*. Plasma and serum were collected and stored at -70°C.

Determination of the ischemic area

The coronary arteries of the excised hearts were perfused with cold phosphate buffered saline by infusion of the aorta. The non-perfused area (ischemic region; area at risk) was determined by closing the suture again and perfusion of the isolated heart with trypan blue. As a result, the perfused area was stained blue, whereas the non-perfused area (ischemic area) remained unstained. This area was separated and deeply frozen for later MPO measurements.

Quantification of neutrophil infiltration

Myeloperoxidase (MPO) activity was used as a marker of tissue infiltration by neutrophils[2,38,39,41] and detection was based on its ability to degrade hydrogen peroxide. MPO was extracted from the cardiac tissues according to the method described by Bradley *et al*[2,20]. The dissected ischemic areas were rapidly frozen in liquid nitrogen, pulverized and weighed. The material was homogenized in 0.5% hexadecyltrimethylammonium bromide (HTAB, Sigma) in 50 mM potassium phosphate buffer, pH 6.0 to provide a 10% homogenate (w/v) followed by sonication on ice and three freeze-thaw cycles. After centrifugation at 25.000 x *g*, the resulting supernatants were chromatographed on 1 ml Sephadex G-75 columns (Pharmacia Biotech, Uppsala, Sweden) to separate MPO from tissue myoglobin and vascular hemoglobin that may affect the MPO assay[41]. The second effluent with the size of the void volume was collected and assayed.

The MPO activity was determined by mixing 50 µl sample with 1.45 ml of 50 mM potassium phosphate buffer containing 0.167 mg/ml *o*-dianisidine hydrochloride (Sigma). After the addition of hydrogen peroxide (final concentration of 0.0005%), the change in absorbance at 460 nm was measured spectrophotometrically every 15 seconds for 5 min (Genesys 10 UV Thermo Spectronic, Rochester, NY, USA). The MPO activity was expressed as the change in absorbance per minute per 100 mg of heart tissue.

GXM detection in sera

GXM was determined in serum by the Premier Cryptococcal Antigen ELISA (Meridian Bioscience Europe, Belgium). A standard concentration curve was created by reducing the absorbance data from dilution series of GXM in rat serum by computer software capable of generating a four-parameter logistic curve fit.

Activation of complement in rat plasma

Complement activation by GXM was achieved by mixing 45 µl plasma with 5 µl of GXM diluted in PBS to achieve final GXM concentrations ranging between 10 µg/ml to 1 mg/ml.

The positive control consisted of rat plasma stimulated with 15 mg/ml zymosan at 37°C for one hour, which is a potent inducer of the alternative complement pathway[18].

Detection of C5a in plasma

Undifferentiated U937 cells stably transfected with the human receptor for C5a (U937-C5aR) were a kind gift of E.R. Prossnitz (Department of Pathology, State University of New York, USA[24]). The U937-C5aR cells were loaded with a calcium specific intracellular probe (Fluo-3, acetoxymethyl (AM) ester; 20 minutes at room temperature) in RPMI-1640 containing 0.1% human serum albumin (HSA)[3]. C5a has been shown to induce a rapid and transient increase in free intracellular calcium concentration in the U937-C5aR cells which correlates with an increase in Fluo-3AM fluorescence signal as measured by flowcytometry (at 530nm; FACS®)[3,24]. After washing, the fluorescence of each sample of cells (180 µl cells at 10^6 cells/ml) was first measured to determine the basal calcium level. Then 20 µl of rat plasma was added followed by repetitive measurements during 10 seconds. For each determination, the response was taken as the peak fluorescence occurring within 10 seconds of ligand addition.

Statistical analysis

Data were analyzed by univariate analysis of variance using a two factor ANOVA. To compare multiple treatment groups to each other, the ANOVA was followed by post hoc analysis (Bonferroni's test). P values < 0.05 were considered significant.

	Man	*Xyl*	*GlcA*	*%Ac*
GXM Ac+	3.9	2.2	1.0	100
GXM Ac-	3.7	2.2	1.0	<5

Table 1. Chemical analysis of GXM. The monosaccharide content of GXM was determined by methanolysis, followed by gas chromatography (GC) and GC- mass spectrometry (GC-MS) and expressed as molar ratios. The acetyl content is expressed relative to that of intact GXM derived from ATCC-62066 (serotype A), which contained an average of 2 acetyl groups per 3 mannose residues as determined by ^{1}H NMR spectroscopy. Man = mannose; Xyl = xylose; GlcA = glucuronic acid; Ac = acetyl.

Results

Chemical analysis of GXM preparations revealed that chemically deacetylated GXM contained less than 5% *O*-acetyl when compared to unmodified GXM, while relative mannose, xylose and glucuronic acid contents were comparable (Table 1).

Seven animals were subjected to a sham operation. 26 animals were subjected to coronary artery ligation, of which 16 animals were randomly treated intravenously with either *O*-acetyl deficient or *O*-acetyl-positive GXM, while 10 animals received normal saline (i.e., placebo).

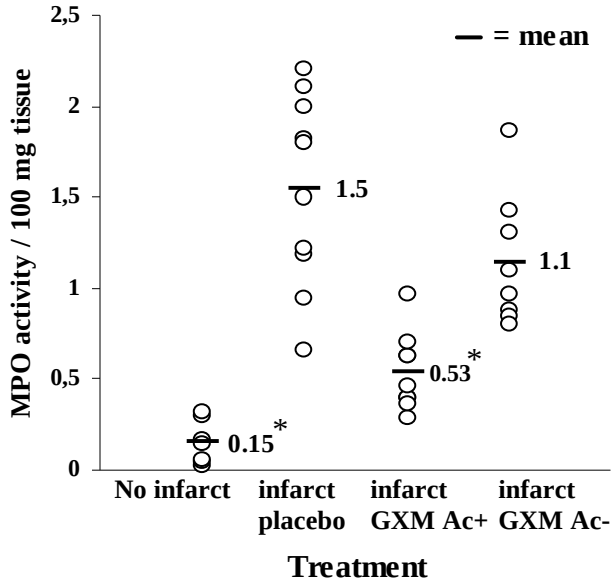

Figure 1. Effect of intravenous GXM on MPO content of ischemic myocardium.
Rats were subjected to coronary artery ligation (infarct animals) or sham operation (no infarct; n=7)) during one hour followed by a three-hour reperfusion period. Infarct animals received either a placebo (250 µl normal saline; n=10) or a 2.5 mg bolus of unmodified GXM (GXM Ac+) or deacetylated GXM (GXM Ac-) intravenously (n=8 in each group). The hearts were removed at the termination of experiments and the non-perfused (ischemic) area was determined, cut out and homogenized. MPO content was determined in the resulting supernatants as a measure of PMN influx. The color development caused by the reaction was detected by spectophotometry. The Y-axis expresses the MPO activity as the absorbance change per minute per 100 mg tissue; the X-axis expresses the different treatment groups. * = $p < 0.05$ when comparing to untreated infarct animals.

The neutrophil accumulation in the dissected ischemic myocardium (i.e., the non-perfused area) was quantified by the detection of tissue MPO. When compared to placebo-treatment, the administration of unmodified (*O*-acetyl-positive) GXM led to a 65% reduction of MPO activity in the ischemic myocardium, while *O*-acetyl-negative GXM was clearly less potent (reduction of MPO not significant, p=0.6; Figure 1).

To exclude that a faster clearance of deacetylated GXM from the circulation caused the reduced effect on neutrophil recruitment in the ischemic myocardium, the serum levels of GXM were compared. Serum levels of *O*-acetyl negative GXM in treated animals were comparable to that of *O*-acetyl-positive GXM (Figure 2A, mean 130 +/-42 µg/ml and 118 +/-30 µg/ml, respectively, p=0.4). For both unmodified GXM and deacetylated GXM treatment a correlation could be demonstrated between the serum concentration of GXM and the degree of MPO reduction (Figure 2B; correlation coefficients -0.45 and -0.68, respectively, two-sided $p<0.05$).

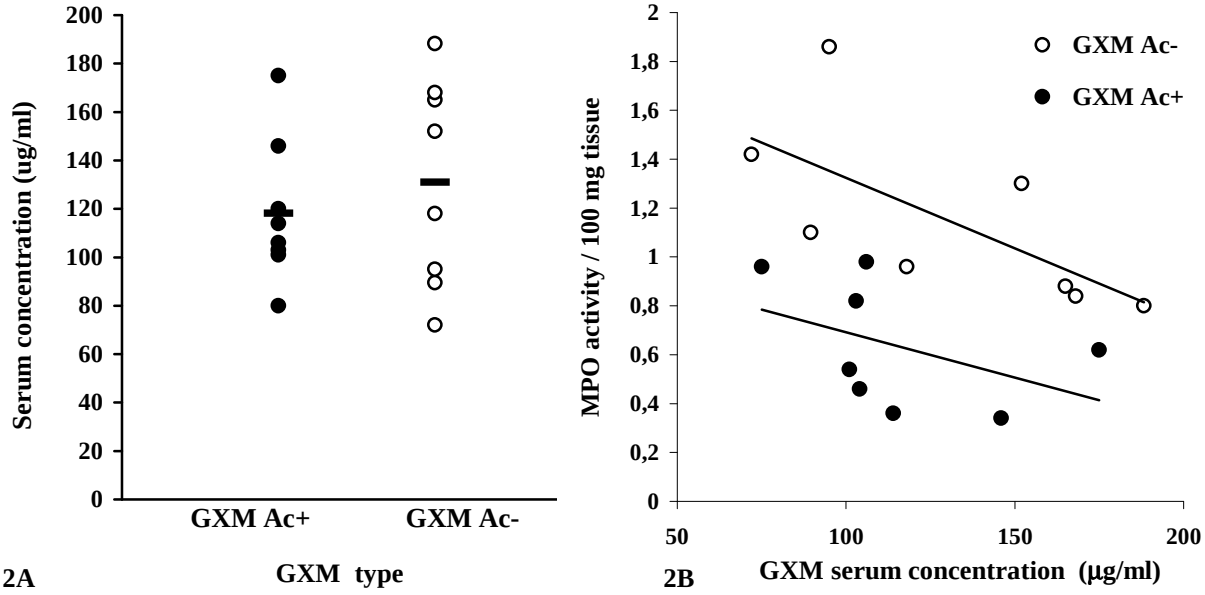

Figure 2. Correlation of theMPO content of ischemic myocardium with GXM serum concentration.
GXM concentrations were measured in serum of rats by a commercial ELISA and calculated using a standard curve of dilution series for both unmodified and of *O*-acetyl-negative GXM. The MPO activity was determined in the ischemic myocardium of corresponding rats. **A**. Comparison between the serum concentrations of GXM from animals that had received either 2.5 mg unmodified GXM (GXM Ac+; n=8) or deacetylated GXM (GXM Ac-; n=8). **B.** Inverse correlation between the serum concentration of GXM and the MPO activity in the ischemic myocardium of corresponding rats. The black dots represent the separate rats treated with *O*-acetyl-positive GXM and the white dots those treated with *O*-acetyl-negative GXM. The black lines represent the trend lines, which were calculated by linear regression (correlation coefficient: –0.45 and -0.68, respectively, n=8 in both groups; two-sided P<0.001).

GXM is known to induce complement activation[27] and the presence of massive amounts of C5a in the bloodstream would theoretically prevent neutrophils from responding properly to other chemoattracting substances present in the inflamed tissues.
Here, no significant amounts of C5a could be detected in plasma samples derived from GXM treated animals (data not shown). However, the inability to detect C5a in these plasma samples might be a consequence of the detection limit of the used assay. We therefore assessed the ability of GXM to generate C5a *in vitro*. Stimulation of plasma from control rats with either unmodified or *O*-acetyl deficient GXM led to a dose-dependent generation of C5a in both cases, when comparing to control plasma kept at 37°C (Figure 3). However, at GXM-concentrations that were comparable to those detected in animals treated with GXM (80-188 µg/ml), *in vitro* no or little C5a was generated, especially when compared to zymosan-induced C5a (Figure 3).
We therefore concluded that complement activation by GXM plays little or - presumably - no role in the observed interference with neutrophil migration.

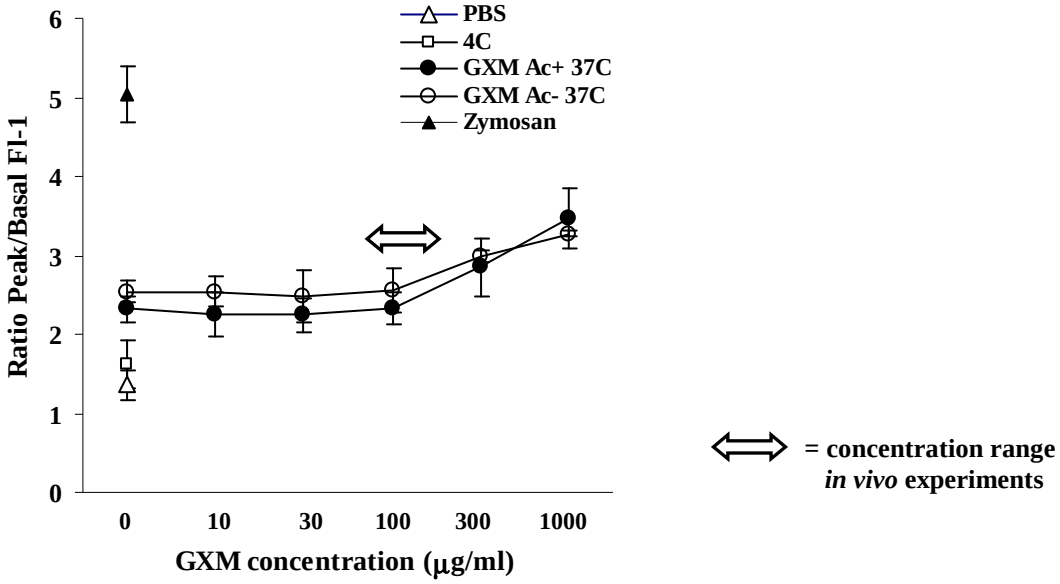

Figure 3. Complement activation by GXM. Plasma of control rats (n=4) was stimulated *in vitro* at 37°C Zymosan (positive control) or increasing concentrations of acetyl positive or acetyl-negative GXM. These were compared to the C5a activity in normal saline (PBS), plasma kept at 4°C (4C), plasma stimulated with PBS at 37°C (labeled as "0 mg GXM") and zymosan-stimulated rat plasma (positive control). The presence of C5a in samples was detected by the ability of C5a to induce a rapid mobilization of calcium in U937-C5aR transfectants loaded with a calcium specific intracellular probe. The resulting increase in the fluorescent signal was detected by FACS® by repetitive measurements during 10 seconds. The data are expressed as relative values comparing the peak fluorescence to the basal fluorescence value before stimulation (increase in fluorescence = peak value : basal value). The results are the averages of separate stimulations of plasma from four different control rats. Error bars represent the standard deviations.

Discussion

The cellular mechanisms by which GXM accomplishes its effects on neutrophils are largely unknown. GXM binds to and is subsequently internalized by leukocytes and potential cellular surface receptors have been described (TLR4, CD14, CD18)[13,32,37]. However, the intracellular sequences following receptor binding and internalization remain to be clarified. Structure-function studies are an additional approach to the clarification of cellular mechanisms. For example, based on functional studies of naturally occurring variants, chemical modified GXM or *O*-acetyl deficient GXM derived from cas1Δ mutant strains, it has been established that *O*-acetyl is a major determinant of the antigenic activity of GXM and *O*-acetyl and the most important epitope for antibody binding[1,5,25,26]. Additionally, O-acetyl appears to be involved in the clearance of GXM from the blood[26]. We recently showed that chemically deacetylated GXM and *O*-acetyl deficient GXM derived from mutant strains were considerably less potent than *O*-acetyl-positive GXM in the inhibition of chemokinesis and endothelial adhesion of PMN (This thesis, Chapter 5; submitted). Thus, 6-*O*-acetyl-mannose appears an important epitope for these functions of GXM and might mediate binding of GXM to its cellular receptors.

In the present study, the importance of *O*-acetylation of GXM for its effects on neutrophil migration was affirmed *in vivo*. Chemical de-*O*-acetylation greatly reduced the capacity of GXM to diminish neutrophil influx in the injured myocardium in a model of ischemia/reperfusion.
We established that a faster clearance of GXM from the blood did not cause the observed difference, since serum concentrations of GXM and deacetylated GXM were comparable. Although the commercial ELISA had a lower detection limit for GXM than for deacetylated GXM (1 ng/ml versus 100 ng/ml, respectively), serum levels of both could be deduced from the separate standard curves that were created for each type of GXM. Since *O*-acetyl is a major binding epitope for polyclonal and many monoclonal antibodies[1,5,15,25], the higher detection limit in the ELISA can be well explained by its near absence in deacetylated GXM, and several studies have described this lower reactivity of *O*-acetyl-negative GXM with a series of monoclonal and polyclonal antibodies[1,26].

In vivo, the generation of C5a due to complement activation by GXM could influence the effects on neutrophil migration. Recently, however, we obtained evidence that the observed interference with neutrophil migration toward inflammatory sites is not caused by GXM-related complement activation and the subsequent generation of chemotactic C5a (Chapter 5; submitted). In that study, no significant amounts of C5a could be detected in plasma of rats treated with (unmodified) GXM. Additionally, *in vitro* stimulation of plasma with GXM at concentrations comparable to those detected in treated animals generated no or little amounts of chemotactic C5a. We now show that - although different in their effect on neutrophil recruitment - *O*-acetyl-positive and *O*-acetyl-negative GXM induce low levels of C5a to a similar degree. Although pitfalls exist in the detection of C5a - since C5a breaks down rapidly - this observation further supports the hypothesis that the effects of GXM on chemokinesis is complement-independent and relies on direct effects of GXM on PMN.

In summary, this paper underscores that 6-*O*-acetylation of the mannose backbone of GXM is crucial for the interference with neutrophil migration. Future research must focus on the role of *O*-acetyl in GXM binding to potential cellular receptors and in the subsequent internalization and intracellular sequences. Additionally, although xylose appeared to play a minor role in the inhibition of neutrophil chemokinesis and adhesion *in vitro*, we may need to verify this *in vivo* as well.

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CHAPTER 7

Summary and discussion

Summary and discussion

Cryptococcus neoformans is an important pathogen causing severe mycological infection of the central nervous system, especially in immunocompromised patients. Cryptococci possess several virulence factors that enhance their infectivity and provide tools to escape the immune response of the infected host. The thick polysaccharide capsule has been recognized as a major virulence factor that physically impedes recognition and phagocytosis of the fungus and affects the host response by shedding its immunomodulating polysaccharide components into the bloodstream of the host during disseminated cryptococcosis.
It has been shown, among others by our group, that impairment of neutrophil migration is an important effect mediated predominantly by the major capsular polysaccharide GXM[15,47,48,]. A major focus of research of our group in recent years has been the identification of the mechanism underlying inhibition of migration by GXM.

Prerequisites for the recruitment of leukocytes into inflammatory sites are the formation of chemotactic gradients that enable attraction of leukocytes and the process of their migration through the blood-tissue barrier. The best documented mechanism by which GXM impedes leukocyte migration is interference with chemotaxis[10,14,15,46] The intrinsic chemoattracting properties of circulating GXM[13,14] and down–regulation of chemokine receptors[55] are recognized mechanisms that prevent leukocytes from reacting to chemoattractants. In **Chapters 5 and 6**, we show that GXM–related generation of C5a plays no role in preventing PMN from responding to other chemoattractants present at inflammatory sites. Interference with the process of leukocyte transmigration through the endothelium is a second major mechanism, which might add to reduced leukocyte migration. Although GXM has been shown to modulate certain adhesion molecules at the surface of leukocytes[10,16,17], the actual impact on leukocyte adhesion to the endothelium had not been described previously.

This thesis has focused on several aspects of GXM-related interference with neutrophil migration into inflammatory sites. First, the effect of GXM on neutrophil adhesion (rolling and firm adhesion) to the endothelium was investigated and potential mechanisms were explored. Second, we investigated the outcome of GXM treatment on neutrophil recruitment in a model of myocardial ischemia. Finally, we examined the role of certain structural elements of GXM in the effects on neutrophil migration.

GXM inhibits neutrophil adhesion to the endothelium

(1) Our observations
In **Chapter 2 and 3**, we established that GXM treatment of PMN interferes with both rolling and firm adhesion to the endothelium (40% and 44% inhibition, respectively). In addition, GXM-treatment of endothelial cells inhibited firm adhesion of PMN by 27%, but did not affect PMN rolling. Although these effects were partial, they will add to other migration-reducing mechanisms, such as interference with chemokinesis. We showed that GXM exerts its effect on PMN by interfering with E-selectin mediated binding, which was confirmed in a more selective rolling model using monolayers of E-selectin transfectants. The effect of GXM on endothelial cells presumably involves ICAM-1 and E-selectin. Although GXM has been reported to shed L-selectin and bind to CD18, these effects have only been

demonstrated at higher GXM concentrations (500 μg to 1,000 μg/ml) than used here and we were unable to link these findings to effects on adhesion.

(2) Explaining some controversies
Treatment of both PMN and endothelium did not lead to a cumulative inhibition of adhesion. This might be explained by interference with the same binding pathway (i.e., E-selectin). Alternatively, the role of other available adhesion pathways might increase when certain pathways are blocked (i.e., redundancy). For instance, it has been described that CD11b/CD18 will bind to ICAM-2 when ICAM-1 is unavailable[24].

Importantly, we were the first to report effects at GXM levels ranging between 10 ng/ml and 10 μg/ml), whereas the majority of previously reported effects occurred at GXM levels above 100 μg/ml. For instance, L-selectin shedding, upregulation of CD11b/CD18 and shedding of the TNF receptor (i.e., activation of PMN) were observed at GXM concentrations ranging between 250 and 1,000 μg/ml[10,16,19]. Other effects of GXM, such as binding of CD18, interference with chemokinesis and cytokine induction were demonstrated at GXM concentrations between 50 and 1,000 μg/ml[10,17,46,79]. The clear dose-dependency of GXM-related effects may explain our observation that effects on L-selectin or CD18 did not play a role in the GXM-induced inhibition of PMN adhesion at the lower concentration range used. During cryptococcosis, GXM levels above 100 μg/ml have been detected in serum of AIDS patients, however, in most cryptococcosis patients they are usually a hundred-fold lower[12,20,53,81]. Although the *in vitro* effects of GXM at certain concentrations should not be indiscriminately extrapolated to *in vivo* levels of GXM in blood, it may be expected that the nature of GXM-related effects will depend on its serum concentration. Consequently, effects will differ between individual cases of cryptococcosis. Currently, no *in vivo* data are available regarding the variability of GXM-related effects in relation to serum levels.

In fact, in our experiments the magnitude of interference with PMN rolling and firm adhesion diminished at GXM concentrations above 100 μg/ml. Similarly, other researchers observed that GXM binding to CD18 diminished above 250 μg/ml. A first plausible mechanism is the tendency of the large GXM molecules to aggregate at higher concentrations (**Chapter 2),** which could lower the actual numbers of free reactive molecules. A second explanation might be GXM-related activation of PMN at GXM concentrations above 250 μg/ml[16], leading to upregulation of CD11b/CD18[10,16] and increased adhesiveness of PMN to endothelium. We proved the latter mechanism to be unlikely, since (1) the curve of inhibition did not change when CD18 was blocked by antibodies, and (2) the ratio of firmly adhered cells to rolling cells did not increase when higher GXM concentrations were used in the flow-experiments (**Chapters 2 and 3**).
When GXM-treated PMN were flown over E-selectin transfectants (**Chapter 3**), optimal inhibition was reached at somewhat higher GXM concentrations when compared to PMN rolling on endothelium. Additionally, whereas on endothelium the inhibition of rolling decreased above 10 μg/ml, on E-selectin monolayers the magnitude of inhibition did not decrease, but stabilized. These observations concur less with the theory of GXM aggregation. However, the curves of inhibition on endothelium and E-selectin cannot be compared easily, since the nature and distribution of ligands for PMN adhesion differ

considerably when comparing these two cell types. HUVEC (endothelial) cells express a variety of ligands for PMN rolling and firm adhesion, whereas the E-selectin transfectants exclusively and more abundantly express E-selectin. Since GXM appears to inhibit rolling primarily through inhibition of E-selectin mediated pathways, it seems plausible that a higher concentration would be required to reach optimal inhibition on the E-selectin transfectants.

(3) Some clues leading to the mechanism

Simple mechanical hindering by the large and sticky polysaccharide molecules is an unlikely mechanism, since washing of GXM-treated PMN or endothelium did not decrease the interference with adhesion. In our experiments, GXM appeared to decrease rolling and firm adhesion of PMN by specifically interfering with binding of PMN to E-selectin. However, we could not identify an effect on known E-selectin ligands (i.e., L-selectin, PSGL-1) or associated sugar-moieties (i.e., sLewis^x) on the surface of PMN. Then again, the selectins have been found to bind to a variety of carbohydrate ligands, and most likely, not all have been identified to date. In addition, interference with the glycosylation of selectin ligands[51] seems unlikely, since GXM did not change the surface expression of sLewis^x, (i.e., CD15), which is one of the carbohydrate structures expressed on the ligands of selectins.

With regard to the effect on endothelial cells, GXM did not affect the cellular synthesis or the surface expression of E-selectin or ICAM-1 on the endothelium (**Chapter 2**), nor did GXM selectively bind to E-selectin transfected CHO-cells (**Chapter 3**). However, since GXM was shown to bind to both PMN[17,56] and endothelial cells (**Chapter 2**), the mechanism of interference may need to be explored at a different level.

Exploring cellular receptors for GXM is another approach to clarify the mechanism by which GXM exerts its effects on adhesion. TLR4, CD14 and CD18 have been described as potential cellular surface receptors that bind GXM[17,56,69].

CD11b/CD18 (CR3) regulates important cell functions in inflammation, including adhesion, oxidative burst, and phagocytosis of a range of microorganisms[18,42]. TLR4 and CD14 are recognition and signaling receptors for LPS, and in cooperation with CD11b/18 elicit full signaling responses in reaction to LPS binding[62,75]. LPS binding to these receptors on leukocytes results in cell activation, i.e., the release of cytokines[6,28], L-selectin shedding[68], an increased adhesive capacity of the integrins CD11b/CD18[82] and priming of neutrophils for enhanced production of oxygen radicals[66].

PMN have been shown to phagocytose whole cryptococci with the aid of CD11b/CD18 (CR3)[11,73]. Moreover, macrophages and PMN internalize GXM[26,56], the latter presumably with the help of CD14 and CD18[56]. In our rolling experiments, CD14 and TLR4, but not CD18, appeared to mediate the interference with PMN rolling (**Chapter3)**.

The responses of PMN to GXM show some similarities to LPS binding to TLR4 and CD14 and subsequent cell activation (i.e., L-selectin shedding, upregulation of CD11b/CD18 and cytokine production), yet PMN have been shown not to respond with a respiratory burst[56]. Binding of GXM to CD14 and TLR4 induced intracellular signaling in TLR4 and CD14 transfected CHO cells[69], which did not lead to cytokine production in these particular transfectants. Notably, most of these effects have only been described at GXM concentrations above 500 μg/ml and not at concentrations where we observed inhibition of cell adhesion. Therefore, it is unlikely that binding of GXM to CD14, TLR4, and CD18 will

affect adhesion by activating signaling pathways that lead to activation of PMN and premature L-selectin shedding.

It is unusual for a polysaccharide (i.e., non-enzymatic) to exert such a broad range of effects, and the range of reported immunological effects of GXM are likely to share a common mechanism. Therefore, the receptors for GXM might merely mediate the phagocytosis of GXM by PMN, which may temporarily deviate the cell metabolism toward processing of intracellular GXM. For example, it has already been demonstrated that internalization of GXM reduces the fungicidal capacity of PMN[56]. Phagocytosis of GXM might also cause a temporary morphological change of the cellular surface. Changes in the cellular morphology (e.g., by hypo-osmotic swelling) alter the distribution of adhesion receptors on the microvilli of neutrophils, which is crucial for the initial contact between leukocytes and endothelial cells[2,70], thereby reducing tethering and rolling[22]. However, in that case GXM would indiscriminately affect several adhesion molecules and not E-selectin binding only. These remain important hypotheses to be addressed in future research.

The capacity of GXM to interfere with leukocyte mobilization *in vivo*

The study described in **Chapter 4** endorses the few reports that demonstrated the capacity of purified GXM to diminish neutrophil recruitment in models of inflammation[15,47]. Here, we showed that GXM potently reduced neutrophil recruitment in a rat model of post-ischemic myocardial reperfusion injury (65% reduction).
Optimal inhibition of neutrophil influx was reached at a GXM serum concentration of 100 μg/ml, whereas *in vitro* optimal effects on chemokinesis and adhesion were reached at GXM concentrations of 100 μg/ml and 100 ng-10 μg/ml, respectively (**Chapters 2, 3** and **5**). These data suggest that, in this model, the emphasis might be on interference with chemotaxis rather than with transmigration of the endothelium. However, the effects of *in vitro* and *in vivo* GXM concentrations might not be compared that simply, since blood contains many different components that might functionally affect GXM, such as protein binding. We provided new data regarding the mechanism of interference with chemokinesis; since we showed that, the reduced leukocyte influx is not associated with, and thus not caused by, massive complement activation and C5a generation by GXM.
Although treatment with GXM or a mixture of polysaccharides (CneF) in other models of inflammation or infection reduced tissue damage, in our model the substantial reduction of neutrophil infiltration was not accompanied by a significant reduction in infarct-size. However, the used experimental set-up has several drawbacks. First, the treatment groups were small and considering the used method of macroscopic determination of infarct size in small animals the numbers would have to be considerably enlarged to prove modest effects. Second, although PMN infiltration has been related to aggravation of ischemic injury, and experimental PMN depletion has been shown to decrease infarct size[30,36,37,67], not all studies were able to demonstrate a significant effect[4,5,61,74]. The discrepancies in the results can partly be explained by the nature of anti-neutrophil therapy and differences in the experimental procedures, e.g., the animal species, the duration of the ischemic and reperfusion periods, the size of the non-perfused area, or the method of measuring infarct size. Thus, future experiments that investigate the effect of GXM on ischemic injury might include (1) variation in the duration of the ischemic period (2) the use of larger animals or a

different method of infarct size determination (3) exploration of the potential benefit on the later stages of infarct healing and remodeling and (4) assessment of the effects on cardiac function and arrhythmias.

Structure-function studies

The variability of *O*-acetylation and the degree of side-chain substitution of the mannose backbone have been associated with functional aspects of GXM (for structure of GXM see **Chapter 1**, **Figure 1**). For instance, *O*-acetyl has been recognized as a major antigenic factor and epitope for antibody binding that determines antibody responses and additionally is involved in the clearance of GXM from the circulation[40]. Xylose substitution is important for accumulation of complement factor C3 on the surface of cryptococci, accumulation of GXM in the spleen, as well as for antigenic activity[40]. Glucuronyl side-chains provide a negative cellular charge to GXM, and thus far, modification by decarboxylation has not revealed other functions[38,39,41].

In **Chapter 5** we show that 6-*O*-acetylation of mannose in GXM is crucial for the interference with several phases of PMN migration, whereas xylose substitution played no convincing role. We demonstrated that chemical and genetic removal of acetyl dramatically reduced the ability of GXM to interfere with the chemokinesis and endothelial adhesion of PMN. The importance of *O*-acetylation for interference of GXM with PMN migration was endorsed *in vivo* in the model of myocardial ischemia/reperfusion (**Chapter 6)**. There, chemical de-*O*-acetylation markedly reduced the capacity of GXM to diminish neutrophil influx in the injured myocardium. The effect of deacetylation appears specific since physical properties of GXM, such as viscosity and molecular size did not change.

Thus, 6-*O*-acetyl mannose might be the principal binding site of GXM to surface receptors on leukocytes and/or mediate the subsequent internalization of GXM leading to interference with migration.

The impact of GXM-related interference with neutrophil migration during cryptococcosis

Chemokine-induced leukocyte recruitment to the primary site of infection (i.e., the lungs) by *Cryptococcus neoformans* is critical for clearance of the fungi and prevention of their dissemination to the intravascular compartment and the brain, and to control the infection once disseminated[32,33,34]. The data available thus far indicate that GXM reduces neutrophil influx by interference with chemokinesis and endothelial adhesion, which depends on GXM concentration.

The actual impact of the inhibition of neutrophil influx into sites of infection during cryptococcosis will depend on **(a)** the importance of neutrophil involvement for clearance of cryptococci, **(b)** the time span of neutrophil participation in the inflammatory response against cryptococci, **(c)** the serum levels of GXM during that period, and **(d)** the additional effects of GXM and other secreted cryptococcal products on the host-response. Furthermore, since leukocytes share many molecular mechanisms involved in their chemokinesis and adhesion, GXM might also affect migration of other inflammatory cells, such as lymphocytes and mononuclear cells, which remains to be investigated.

Ad (a). The importance of neutrophils for the clearing of cryptococci during infection is unclear. Even though neutropenia is not a proven risk factor for cryptococcosis, PMN are often found in inflamed tissues early in infection in close association with cryptococci[21,63] and infection with a highly virulent strain has been associated with high neutrophil counts1. *In vitro*, neutrophils effectively phagocytose and kill cryptococci[52,54], although susceptibility to killing differs considerably between cryptococcal strains. Even though PMN are more potent than macrophages in killing cryptococci *in vitro*[52,54], macrophages appear to be the predominant phagocytic cells of cryptococci *in vivo*[25,57].

Ad (b). Neutrophils will primarily participate in host response against cryptococci during early cryptococcosis, whereas lymphocytes and mononuclear cells are involved during the entire period of infection[7,25,32,33,49]. Additionally, PMN participate in a later stage of infection in the early phase of granuloma formation[3,35].

Ad (c). GXM can be detected early in the lung tissues during the 'primary' pulmonary stage of cryptococcal infection[25]. However, the effects on neutrophil migration are expected to become apparent when GXM is shed into the blood. Few data are available on the serum levels of GXM during early disseminated infection, and are all derived from experimentally induced cryptococcosis in animals. These studies show that the course of GXM levels in time depend highly on the inoculum, growth rate and dissemination status of the cryptococci[23,50,58,59]. GXM levels are barely detectable during primary pulmonary cryptococcosis in animals; however, during disseminated cryptococcosis levels can rapidly increase[23,25,50,59]. In humans, the early kinetics of GXM shedding are unknown, since the disease has usually evolved to full-blown disseminated or extended pulmonary infection at the time of diagnosis. Moreover, at that time, GXM titers vary considerably between subjects[12,20,53,81].

Ad (d). Besides interference with neutrophil migration, other reported effects of GXM include inhibition of phagocytosis, activation of leukocytes, complement activation, and depression of T-cell responses[8]. All cryptococcal polysaccharides induce pro-inflammatory as well as anti-inflammatory cytokine responses[77,78,79]. Several mannoproteins have been shown to stimulate T-cell responses[31,44,65] and MP4 interferes with chemokinesis of neutrophils by cross-desensitization of chemokine receptors[10]. Other virulence factors of cryptococci that may affect the host response include mannitol, the anti-oxidant melanin, prostaglandins, and extra cellular phospholipase activity[8,9,60]. The net outcome of all these effects on the host-response is unknown.

Taking all these considerations into account, the isolated impact of GXM-related interference with neutrophil migration will be difficult to evaluate during cryptococcal infection.
Since GXM serum levels are presumably low or absent during the first days of the initial pulmonary infection, an effect on neutrophil influx is not expected during this stage. Rather, the phagocytosis-inhibiting capacities of the capsule will be more important during the primary phase of infection. When the disease progresses to disseminated disease, GXM and mannoproteins will be shed into the blood and might add to progression of the disease by interference with neutrophil migration, thereby favoring further multiplication and

dissemination of cryptococci. However, we expect the nature of GXM-related effects to depend on the serum levels. Based on our observations, inhibiting effects on neutrophil migration can be expected at GXM levels below 100 μg/ml, whereas GXM levels above 100 μg/ml will cause leukocyte activation (i.e., upregulation of integrins, cytokine induction, shedding of TNFα receptors) which might nullify the inhibition of migration.

Future directions for clarification of the mechanism.

Combining all the data on the working mechanisms by which GXM interferes with neutrophil movement, a complex picture emerges with multiple possible receptors to interact with and many pathways to interfere with. Furthermore, at least some of the effects ascribed to GXM are due to GXM aggregation occurring at higher concentrations or might be influenced by impurities or badly characterized polysaccharide preparations. Starting points to continue the clarification of molecular mechanisms are (a) the fact that GXM binds to and is subsequently phagocytosed by neutrophils (and possibly other cell types), (b) the potential receptors that have been recognized (i.e., CD18, CD14 and TLR4), and (c) the recognition of 6-*O*-acetyl-mannose as a crucial structural motive for the effects of GXM on neutrophil migration. To our opinion, the elucidation of working-mechanism of GXM can be approached by investigating the following aspects:

(1) Functionally important carbohydrate moieties of GXM.
Quite a few carbohydrate structures have been shown to interfere with adhesion by an effect on leukocytes, such as heparin, heparan sulfate[43,83], fucoidin (a sulfated fucan from algae)[45,71], and mannose-6-phosphate[72]. In most examples, these saccharides share no common structural elements with GXM and sulfation or phosphorylation appears essential for their effects. However, smaller non-sulfated or phosphorylated sugars, such as L-fucose, D-mannose and disaccharides derived from heparin also affect leukocyte adhesion[29,72].
Our observation that the 6-*O*-acetyl-mannose epitope of GXM is involved in the interference with PMN migration (**Chapters 5 and 6**) might form the basis for future projects. Isolation of simplified polysaccharides, such as 6-*O*-acetylated (1→3)-α-D mannan from *Dictyophora indusiata*[27,76] and the development of synthetic polymers with chemically added side-groups of xylose, glucuronic acid or acetyl, will make it possible to relate certain groups to a specific function in a more straightforward way. Additionally, these modified carbohydrates will help to identify cellular receptors for GXM, in particular receptors that bind 6-*O*-acetylated-α-D mannose.

(2) Targets for GXM with respect to neutrophil migration on both endothelium and PMN
Although heparin has been shown to bind CD11b/CD18 and P-selectin[64,80], no specific receptors on leukocytes have been identified for other adhesion-inhibiting carbohydrates. Studies using isolated adhesion molecules such as selectins or sLe^x-related structures might reveal structures that are directly bound by GXM.
The knowledge of functional important epitopes of GXM can be used to demonstrate the involvement of previous recognized cellular receptors for GXM (i.e., CD11b/CD18, CD14 and TLR4). Comparing *O*-acetyl-positive and *O*-acetyl-deficient GXM in their ability to bind these receptors might verify their participation in GXM-related effects. Direct binding assays also enable the investigation of binding specificity by using inhibiting

monosaccharides (GlcA, Xyl and Man). The search for other cellular receptors for GXM might be approached by binding studies of GXM to isolated cellular surface receptors on PMN and endothelium or alternatively by capturing of all proteins binding to GXM in extracts of cell membranes.

(3) Intracellular and morphological sequences following binding and uptake of GXM
The identification of cellular receptors for GXM will add to the elucidation of subsequent intracellular signaling pathways. Effects of phagocytosis and intracellular processing of GXM on the cell metabolism of PMN might be studied by measuring markers of energy metabolism and the anti-oxidant system, as well as functional aspects, such as degranulation and oxidative burst. (Transient) changes in cell morphology might accompany the phagocytosis of GXM, which can affect the numbers of microvilli and distribution of adhesion receptors, such as the selectins, on the surface neutrophils. These matters can be studied using electron microscopy or high-resolution field emission.

Finally, unraveling the mechanism by which GXM interferes with neutrophil movement may yield points of departure to ameliorate the treatment of cryptococcosis and create tools to therapeutic tool to reduce unwanted (excess) PMN infiltration in other types of disease, such as autoimmune disorders or ischemia.

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Nederlandse samenvatting

Introductie

Wat is een cryptococ?

Cryptococcus neoformans is een schimmel die wereldwijd voorkomt in de natuurlijke omgeving. We ademen waarschijnlijk frequent cryptococcen in, maar deze worden meestal direct en onopgemerkt opgeruimd door de afweer in de longen. De cryptococ veroorzaakt dus zelden een infectie bij gezonde mensen, maar wel bij mensen met een gestoorde afweer, zoals AIDS patienten. Als cryptococcen ontsnappen aan het immuunsysteem (de afweer) van de gastheer, kunnen zij zich verder vermeerderen en zich vanuit de longen via de bloedbaan verspreiden naar de hersenen, waar ze een ernstige vorm van hersenvliesontsteking veroorzaken. Een dergelijke infectie wordt behandeld met antibiotica gericht tegen schimmels, maar de infectie kan bij AIDS patienten vaak erg hardnekkig zijn en recidiveren. Met name in derde-wereld landen is cryptococcosis een groot probleem vanwege de enorme aantallen AIDS patienten en het niet voorhanden zijn van de juiste medicijnen. Wereldwijd overlijden dan ook honderdduizenden mensen per jaar aan cryptococcose.

Hoe weert een cryptococ zich tegen het immuun systeem van de gastheer?

Ons immuun systeem is er op gericht zo snel mogelijk binnengedrongen microben op te sporen en te verwijderen. Het dikke polysaccharide kapsel van de cryptococ is één van zijn voornaamste wapens om zich te weren tegen het immuunsysteem van de gastheer. Tijdens een infectie schermt het kapsel de cryptococ af van de onstekingscellen van de gastheer. Tevens scheidt de cryptococ de kapselpolysacchariden uit in de bloedbaan van de patient, van waaruit deze het immuunsysteem negatief kunnen beinvloeden. Waarschijnlijk hebben cryptococcen hierdoor minder last van de afweer zodat zij zich verder kunnen vermeerderen en verspreiden. Glucuronoxylomannan (GXM) is één van de belangrijkste kapsel-polysacchariden van de cryptococ en de mechanismen waarmee GXM het immunsysteem van de gastheer negatief beïnvloed zijn al jaren het onderwerp van onderzoek.

De effecten van het kapsel polysaccharide GXM op de migratie van ontstekingscellen

Onstekingscellen ruimen lichaamsvreemde eiwitten en microben op die ons lichaam binnendringen. Om ontstekingscellen (witte bloedcellen) uit de bloedbaan te mobiliseren naar de lokatie waar de microben zich bevinden (infectiehaard) moeten ze (1) aangetrokken worden door signaal moleculen (chemokines) die afgegeven worden door reeds aanwezige ontstekingscellen (macrophagen) in de infectiehaard en (2) ter plaatse de vaatwand passeren door er over heen te rollen, vast te hechten en tussen de vaatwand cellen (endotheel) door te passeren.

Er zijn een aantal aanwijzingen dat GXM de migratie van ontstekingscellen (met name neutrofiele granulocyten, PMN) vanuit de bloedbaan naar de haard van infectie remt. Het viel op dat er relatief weinig witte bloedcellen aanwezig waren in de geïnfecteerde weefsels van patienten met cryptococcose. Om te testen of GXM daarvoor verantwoordelijk was, hebben onderzoekers gezuiverd GXM toegediend in de bloedbaan van proefdieren met een bacteriële hersenvliesontsteking. Tengevolge van deze behandeling bleken er daadwekelijk minder PMN naar de ontstekingshaard toe te gaan. Verder is aangetoond dat GXM verhindert dat PMN de lokkende signalen herkennen die vanuit de infectiehaard worden afgegeven. Als men in een bakje met onder-en bovencompartiment, gescheiden door een

filter, in het benedencompartiment signaal-moleculen (chemokines) en in het bovencompartiment PMN aanbrengt, lopen de cellen normaal gesproken door het filter naar de lokkende chemokines in het ondercompartiment toe. Wanneer men echter gezuiverd GXM aan de PMN in het bovencompartiment toevoegde, migreerde een aanzienlijk minder PMN naar beneden toe. Waarschijnlijk vermindert GXM de werking van de receptoren waarmee PMN deze signaal-moleculen herkennen en binden.

Dit proefschrift:

In dit proefschrift hebben wij onderzocht of

(1) er een tweede potentieel mechanisme is waarmee GXM de migratie van PMN remt, namelijk remming van de hechting aan en de passage van de vaatwand (= endotheel)
(2) GXM *in vivo* de migratie van PMN remt in een 'niet-infectieus' model (een hartinfarct)
(3) een specifieke bouwsteen van GXM verantwoordelijk is voor de effecten op PMN migratie

(1) GXM remt de adhesie van neutrofiele granulocyten (PMN) aan endotheel

In **hoofdstuk 2 en 3** laten we zien dat GXM behandeling van PMN *in vitro* zowel het rollen als de hieropvolgende vaste hechting van PMN aan endotheel cellen remt 40-44% met door aan te grijpen op de binding van PMN aan het adhesiemolecuul E-selectine op het endotheel. Wat GXM precies verandert aan de PMN is nog niet duidelijk. Na behandeling met GXM lijken alle adhesie moleculen die binden aan E-selectine nog in voldoende mate aanwezig op het oppervlakte van de PMN. Voorts vonden we aanwijzingen dat (potentiële) specifieke receptoren voor GXM op het oppervlak van PMN betrokken zijn bij de remming van PMN adhesie. Als we die specifieke receptoren (TLR4 en CD14) namelijk remden, had GXM geen effect meer. CR3 (CD11b/CD18), een andere beschreven receptor voor GXM, blijkt niet betrokken te zijn.

(2) GXM is in staat de toestroom van PMN in ischemisch hartweefsel te remmen

Er zijn maar een beperkt aantal studies die hebben aangetoond dat als je GXM 'buiten' cryptococcen infecties toedient het ook de instroom van PMN in de weefsels beperkt.

In **hoofdstuk 4** hebben we onderzocht of GXM *in vivo* ook de toestroom van PMN en weefselschade kan remmen in een hartinfarct model in ratten. Als de bloedtoevoer naar de hartspier onderbroken wordt, raakt de hartspier verstoken van nutriënten en zuurstof (ischemie) en lopen de hartcellen schade op (hartinfarct). Als de bloedstroom weer hersteld wordt, snellen er ontstekingscellen toe (met name PMN) om de beschadigde hartcellen op te ruimen. In zo'n geval kan een overmaat aan PMN echter ook schadelijk zijn, omdat PMN tevens intacte hartcellen beschadigen. Een aantal studies heeft laten zien dat het beperken van de toevoer van PMN de schade aan de hartspier (het hartinfarct) kan beperken.

We dienden een hartinfarct toe aan ratten, door onder narcose tijdelijk de grote kransslagader van het hart af te binden en na een uur de bloedstroom weer te herstellen, waardoor ontstekingscellen weer konden toestromen. De hoeveelheid PMN in het hartweefsel werd gemeten door in een homogenisaat (een 'soepje') van het vermalen hartweefsel het enzym myeloperoxidase (MPO) te bepalen, dat vrijwel uitsluitend in PMN voorkomt. Ratten die een

GXM injectie kregen hadden gemiddeld 65% minder MPO in het beschadigde hartweefsel, hetgeen bevestigt dat GXM inderdaad de migratie van PMN kan remmen.
Door de toestroom van PMN te remmen verminderde GXM niet duidelijk de groottte van het hartinfarct (i.e., de weefselschade). Dat kan aan een aantal dingen liggen. Ten eerste was het aantal ratten per behandelingsgroep klein en om kleine effecten aan te tonen moeten de groepen sterk uitgebreid worden. Ten tweede is het resultaat afhankelijk van de gekozen methodiek (i.e., duur van de kransslagader afsluiting en methode van bepaling van de grootte van het hartinfarct) en zouden veranderingen hierin de resultaten nog kunnen beïnvloeden. Ten derde is nu slechts naar de grootte van het hartinfarct gekeken als directe maat van weefselschade. Het zou ook interessant zijn om het effect van GXM op de hartfunctie en ritmestoornissen na een hartinfarct te bekijken.

(3) Welke bouwstenen van GXM zijn belangrijk voor de effecten op PMN migratie?
GXM is een polysaccharide opgebouwd uit verschillende enkelvoudige suikers (monosacchariden) en een molecuul bestaat uit geschakelde mannose moleculen met zijgroepen van glucuronzuur, xylose en acetyl (zie **Hoofdstuk 1**, **Figuur 1**). Tussen verschillende cryptococcen stammen wisselt de samenstelling van GXM in de hoeveelheid en de locatie van de zijgroepen.
In **hoofdstuk 5** hebben we *in vitro* onderzocht of xylose en *O*-acetylering belangrijk zijn voor de remmende functie van GXM op de migratie van PMN. Daartoe hebben we verschillende soorten GXM getest die wel of niet acetyl of xylose bevatten. De acetyl werd chemisch van GXM verwijderd door het verhogen van de zuurgraad, waardoor de acetyl groepen vanzelf los laten. Ook werd GXM gezuiverd van speciale cryptococcen stammen die de genen misten die coderen voor de aanmaak van xylose of acetyl, waardoor het GXM van deze stammen (mutanten) geen xylose of geen acetyl bevatten. Integenstelling tot intact GXM was chemisch of genetisch gemanipuleerd GXM zonder acetyl nauwelijks meer in staat de verschillende migratie fasen van PMN te remmen (i.e., chemokinesis en de adhesie van PMN aan endotheel). GXM zonder xylose remde echter in de meeste testen vergelijkbaar als intact GXM. Acetylering van de mannose keten lijkt dus onmisbaar voor deze functie van GXM en xylose speelt nauwelijks een rol. In **hoofdstuk 6** wordt dit bevestigd in een *in vivo* studie: de toediening van intact GXM aan ratten verminderde de toestroom van PMN in ischemisch hartweefsel met 65%, terwijl acetyl-vrij GXM geen effect had. Concluderend is *O*-acetylering van GXM moleculen belangrijk voor de effecten die GXM heeft op PMN migratie. Het is zeer wel mogelijk dat GXM via de *O*-acetyl groepen bindt aan receptoren op het oppervlak van PMN, hetgeen dan leidt tot remming van bepaalde functies.

De impact van verminderde PMN migratie op het beloop van cryptococcosis
Theoretisch zou tijdens een cryptococcen infectie het weghouden van PMN (en e.v.t. ook andere ontstekingscellen) bijdragen aan het onstnappen van cryptococcen aan de afweer en dus aan verdere vermeerdering en verspreiding. Echter zo simpel valt dat niet te voorspellen. Ten eerste beïnvloed GXM meer processen dan alleen PMN migratie, zoals de inductie van cytokinen, activatie van PMN en het onderdrukken van T-cellen. Ten tweede spelen PMN voornamelijk een rol in de beginfase van een cryptococcen infectie en is het niet duidelijk hoe snel en in welke mate GXM wordt afgescheiden in de bloedbaan tijdens deze fase.

Bovendien hangen de effecten van GXM op PMN migratie sterk af van de concentratie GXM in de bloedbaan en verschilt deze per patient tijdens cryptococcose. Ten derde is het de vraag of het weghouden van PMN in de onstekingshaard het beloop van een cryptococcen infectie beïnvloedt, aangezien er ook andere witte bloedcellen zoals T-cellen en macrophagen op de ontstekingshaard afkomen die al het opruimwerk net zo goed kunnen verrichten. Ten vierde beschikken cryptococcen over meerdere virulentie factoren (zowel suikers als eiwitten) die allerlei effecten hebben op de immuunrespons van de gastheer. Concluderend speelt remming van PMN migratie door GXM een rol tijdens de initiële fase van een cryptococcen infectie, maar dat is moeilijk experimenteel te onderzoeken.

Toekomstig onderzoek

Toekomstig onderzoek zal gericht zijn op het verder ontrafelen van het mechanisme waarmee GXM de migratie van PMN remt. Hiervoor is het belangrijk te weten hoe GXM deze cellen beïnvloedt. Er is aangetoond dat GXM aan het oppervlak van cellen kan binden gevolgd door opname in de cel. Waarschijnlijk bindt GXM aan specifieke receptoren op het cel oppervlak met een bepaald gedeelte van de GXM molecuul en wordt vervolgens via deze receptoren in de cel opgenomen. De binding van GXM aan deze receptoren en de opname van GXM in de cel zal dan waarschijnlijk leiden tot veranderingen in bepaalde functies van de cel.

Aangezien uit ons onderzoek is gebleken dat de *O*-acetyl groepen op de GXM moleculen betrokken zijn in de remmende effecten op migratie, kan dit gegeven gebruikt worden om de receptoren voor GXM op het celoppervlak te identificeren via welke deze remming wordt bereikt. Verder dient opgehelderd te worden wat er precies aan het metabolisme van de PMN verandert als GXM er aan bindt en door wordt opgenomen.

Tenslotte: opheldering van het mechanisme waarmee een microbe de weerstand van de mens beïnvloedt kan aanknopingspunten opleveren voor de ontwikkeling van nieuwe behandelingsmethoden van cryptococcosis of andere ziektebeelden.

Afkortingen en definities:

AIDS	Acquired immuno deficiency syndrome. Een ziekte veroorzaakt door infectie met het HIV-virus, waarbij de specifieke afweercellen die door HIV geinfecteerd zijn ten gronde gaan. Dit leidt tot een verminderde weerstand en verhoogde vatbaarheid voor bepaalde infecties.
Fagocytose	het proces waarbij een ontstekingscel een microbe of substantie in zich opneemt (‘opeet’) teneinde het te verwijderen.
Gastheer	het dier of mens waarin een micro-organisme binnendringt.
GXM	glucuronoxylomannan, de belangrijkste polysaccharide (suiker) in het kapsel dat cryptococcen omringt.

Immuunsysteem	Het georganiseerde systeem van afweer tegen lichaamsvreemde eiwitten of microben (bacteriën, schimmels, virussen, etc.). De afweer bestaat uit diverse typen onstekingscellen, bloedeiwitten (antistoffen, complement) en activatie- en signaaleiwitten (geproduceerd door ontstekingscellen) en is erop gericht om lichaamsvreemde substanties en beschadigd weefsel zo spoedig mogelijk op te sporen en te verwijderen. Het beenmerg, lymfeklieren, bloed en lymfebanen zijn de aanmaakplaatsen en transportbanen van deze componenten.
Infectie	Onsteking veroorzaakt door een microbe (bacterie, virus, schimmel, parasiet, etc.)
Ontsteking	Wanneer het immuunsysteem in werking treedt als reactie op het binnendringen van een microbe of op weefselschade door andere oorzaken (bijv., door trauma of verbranding) heet dit proces “ontsteking” of “inflammatie”. De symptomen van een ontsteking worden veroorzaakt door de effecten van ontstekingsmediatoren, het instromen van ontstekingscellen op de plek des onheils en weefselschade. Een uitwendig gelocaliseerde ontsteking kenmerkt zich door de combinatie van roodheid, zwelling van de weefsels, pijn, en verlies van functie en gaat soms gepaard met koorts. Bij een inwendige ontsteking staan algemene symptomen als koorts en algeheel onwelzijn op de voorgond, naast symptomen van de aangedane locatie (bijv. hoesten bij longontsteking, pijn bij onsteking in de buik).
PMN	letterlijk: polymorpho nuclear cell, ofwel neutrofiele granulocyt; type onstekingscel of witte bloedcel die in onze bloedbaan circuleert en helpt lichaamsvreemde eiwitten of microben te fagocyteren.
Polysaccharide	meervoudige suiker, samengesteld uit enkelvoudige suikers.
MPO	myeloperoxidase, een enzym, aanwezig in PMN.

Reflecties en dankwoord

Tegen het einde van de opleiding tot Internist stortte ik mij in 2000 in de basale wetenschap met sterk verjaarde lab-ervaring. De gehele periode van onderzoek kan gemakkelijk als volgt worden samengevat: vallen en opstaan. Ik was als arts gewend geraakt om op efficiente wijze relatief snel doelen te behalen, garant staande voor een dagelijkse portie voldoening. En dat viel tegen…. de resultaten lieten nog wel eens op zich wachten. Het duurde even voor ik kon omgaan met de teleurstelling van herhaaldelijk mislukte experimenten. Ook moest ik een stap terug nemen in het 'race' tempo dat ik mij in de kliniek had aangemeten (letterlijk: vele EWI collega's herkennen mijn hooggehakte marcheren door de gangen). Immers: beter 1 goed doordachte proef dan 3 gehaaste (mislukte) proeven per dag. Zitvlees om te filosoferen en resultaten te evalueren moest ik cultiveren. De voldoening kwam geleidelijk aan toen ik de 'schwung' te pakken kreeg van het opzetten van technieken, hypotheses stellen en toetsen. Het opschrijven was de 'icing' op de cake.

Professor Andy Hoepelman heeft mij de unieke kans gegeven om op fulltime basis onderzoek te doen. Andy, hier wat veren. Ten eerste stond je altijd pal achter me om me te stimuleren en straalde je altijd vertrouwen uit, ook als het onderzoek wat minder vlotte. Mijn vertrouwen had je al snel gewonnen door je loyaliteit, eerlijkheid, openheid voor nieuwe ideeën, betrokkenheid bij al je medewerkers, en het altijd nakomen van afspraken. Je mild plagerige aanpak van mijn eigenwijsheid werkte goed en er was altijd ruimte voor een grapje. Je hebt een talent voor management! Zo, nu kan ie wel weer.

Professor Willem Erkelens was mij zeer dierbaar. Als inspirerend opleider in de Interne geneeskunde, gaf hij mij blindelings zijn vertrouwen en een duwtje in de goede richting, toen ik halverwege de opleiding tot Internist weifelde over mijn toekomst.

Frank Coenjaerts, mijn co-promotor, heeft mij op de werkvloer begeleid met de praktische struggelingen en nieuwe ideeën om verder te gaan. Frank, ook al was de immunologie voor jou een relatief nieuw terrein, je stak er altijd energie in om samen met mij hindernissen af te breken en oplossingen te zoeken als ik er niet uit kwam. Ik heb je af en toe overvallen met mijn Amsterdamse directheid, jij bent nu eenmaal als Limburger wat subtieler; je remde me af waar ik stappen over dreigde te slaan, ik heb je geduld en je zachtheid altijd erg gewaardeerd.

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Ik heb genoten van de warme en zeer collegiale deken van het EWI en ik wil daar iedereen voor bedanken. Speciaal noem ik even een paar mensen: de deur van Jos van Strijp en Kok van Kessel stond altijd open om van gedachten te wisselen over immunologische problemen en me met een waardevolle tip verder te helpen. In Piet, de zeer betrokken en ervaren 'opper-analist' van '602' vond ik iemand die bij 'acute' problemen (die %&$@# machine doet het niet!) alles liet vallen en me te hulp schoot. Even belangrijk: iemand die mijn schoonmaak-neurose deelde (sterkte verder met al die viezeriken op het lab). Systeem beheerder Henk Versteeg en de ervaren '406' analisten Erik, Maartje, Bertie, Mirjam, Loek, Hennie en Machiel stonden zonder nadenken klaar als ik een praktische vraag had. De mannen van de medium-room waren nooit te beroerd even tussendoor iets te steriliseren en Jos hielp me enthousiast met het traceren en bespoedigen van bestelde chemicalien.

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Mijn naaste familie heeft altijd met veel interesse mijn vorderingen gevolgd. Van mijn vader en moeder leer ik nog elke dag, zij zijn mijn spiegels. Ik wordt nog steeds verrast door het vertrouwen, de warme betrokkenheid en onbaatzuchtige ondersteuning die zij - volstrekt ongehinderd door vooroordelen – aan iedereen geven.

Edje, als 'natural work-aholic' accepteerde je het als vanzelfsprekend dat ik soms als een onbereikbaar spook achter mijn computer zat. Indien ik bij tegenslagen neigde tot filosoferend zelfbeklag, wist je dat consequent om te buigen naar de praktische probleem-oplossende sfeer, zodat ik er snel weer een uitdaging in zag. Je onuitputtelijke energie en nieuwsgierige speelsheid (alles uitproberen!) leveren dagelijks gekke plannetjes op die me zonodig uit de research-dalletjes omhoog stuwden. Wat een mazzelaar ben ik!

Curriculum vitae en publicaties

Curriculum vitae.

De auteur van dit proefschrift werd op 2 juni 1967 geboren te Amsterdam. In 1985 werd het VWO diploma behaald op het Visser ’t Hooft Lyceum te Leiden.
Van 1985 tot 1986 volgde zij Frans en literatuur aan de Universiteit van Dijon in Frankrijk. Niet minder belangrijk werd ook een diploma in wijnproeven behaald.
Vanaf 1986 studeerde zij Geneeskunde aan de Universiteit van Amsterdam en in 1990 werd het doctoraal diploma behaald. Hierna verrichte zij een jaar wetenschappelijk onderzoek op het Centraal Laboratorium voor Bloedtransfusie (C.L.B.) in Amsterdam naar de rol van antistoffen tegen neutrofiele granulocyten (ANCA) bij het ontstaan van chronische darmontsteking (chronic inflammatory bowel disease; IBD). In 1991 werd met de co-schappen aangevangen, tijdens vakanties werkte zij als invalkracht op het C.L.B. bij de diagnostiek naar trombocyten en leukocyten antistoffen.
Na het behalen van het artsdiploma in 1994 werkte zij als basisarts een jaar op de afdeling Interne Geneeskunde van het Streekziekenhuis Hilversum alvorens in 1995 met de specialisatie tot Internist aan te vangen in het Universitair Medisch Centrum te Utrecht (U.M.C.U.). De specialisatie tot Internist werd alternerend in het Streekziekenhuis Hilversum en het U.M.C.U. doorlopen en in 2001 vond de registratie als Internist plaats.
Mei 2000 werd aangevangen met promotie onderzoek binnen de afdeling Interne Geneeskunde, onderafdeling Acute geneeskunde en Infectieziekten in het U.M.C.U. onder leiding van Prof. I.M. Hoepelman, hetgeen heeft geleid tot het huidige proefschrift. Het onderzoek werd hoofdzakelijk uitgevoerd in het laboratorium van het Eijkman Winkler Instituut voor Microbiologie en deels op de afdeling Farmacologie van de Erasmus universiteit in Rotterdam. Daarnaast verzorgde zij op beperkte schaal poliklinische zorg van HIV besmette patienten. Momenteel werkt zij als fellow op de afdeling Infectieziekten in het U.M.C.U.

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