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Binding of Glucuronoxylomannan to the CD14 Receptor in Human A549 Alveolar Cells Induces Interleukin-8 Production[▽]

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Glucuronoxylomannan (GXM) is the major capsular polysaccharide of *Cryptococcus neoformans*. GXM receptors have been characterized in phagocytes and endothelial cells, but epithelial molecules recognizing the polysaccharide remain unknown. In the current study, we demonstrate that GXM binds to the CD14 receptor in human type II alveolar epithelial cells, resulting in the production of the proinflammatory chemokine interleukin-8.

Cryptococcus neoformans is an encapsulated fungal pathogen infecting mainly immunosuppressed patients. Infections are acquired by inhalation of desiccated cells, which are available in the environment as basidiospores or poorly encapsulated yeasts. Inhaled cells are deposited in the alveolar space, where they are ingested by macrophages (24) and interact with epithelial cells (2, 7, 8). An effective interaction of *C. neoformans* with epithelial alveolar cells is probably essential for the establishment of pulmonary infection. Microscopic studies of pulmonary cryptococcal infection reveal that yeast cells are in close apposition to lung epithelial cells (7). Once *C. neoformans* becomes established in the lung, it proliferates locally and causes a primary lesion that is usually contained by granuloma formation (10, 24). Two components of *C. neoformans* that are important for adhesion to human type II alveolar epithelial cells are phospholipase B (8) and glucuronoxylomannan (GXM) (2).

GXM is a virulence factor (4) that represents a potential vaccine component and is the target of therapeutic antibodies (3, 11, 17, 19). As the major constituent of the capsule, it is the primary component of a structure that is antiphagocytic and thus protects the fungal cell from immune cells. However, cryptococcal infections are also associated with the release of large amounts of GXM into host tissues, where they have many deleterious effects on the host immune response through multiple mechanisms (15). GXM has been reported to interact with numerous cellular receptors. Receptors for GXM in macrophages, neutrophils, and endothelial cells include Toll-like receptor 2 (TLR2), TLR4, CD14, and CD18 (12, 13, 16, 25). The receptors for GXM in epithelial cells, however, remain to be characterized.

GXM was purified from strain T_1-444 of *C. neoformans* (serotype A) by following standard methods (5) and incubated with A549 cells (100 μg/ml) for 1 h at 37°C (2). Binding of GXM to host cells was confirmed by immunofluorescence with the GXM-binding monoclonal antibody (MAb) 18B7 (3) (Fig. 1, inset). Lysates were obtained as described previously (21) and immunoprecipitated by sequential incubation with MAb 18B7 (3) and Sepharose-bound protein G. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the immunoprecipitated material indicated the presence of a major band with a molecular mass corresponding to 55 kDa (Fig. 1A). The same mixture analyzed by SDS-PAGE was boiled for 5 min to disintegrate protein-polysaccharide conjugates. GXM was then removed from this preparation by ultrafiltration (cutoff, 100 kDa), and the filtrate was incubated with Sepharose-bound protein G for antibody depletion. The purified fraction containing the GXM-binding protein, but no polysaccharide or antibodies, was concentrated to dryness and used to coat polystyrene 96-well plates (5 μg/ml, 100 μl/well). After the addition of GXM in serial dilutions, the plates were sequentially incubated for 1 h with MAb 12A1, a mouse immunoglobulin M (IgM) MAb with specificity for GXM (6), and a phosphatase-labeled goat anti-mouse antibody with specificity for IgM. Reactions were developed after the addition of *p*-nitrophenyl phosphate, followed by a reading at 405 nm. As demonstrated in Fig. 1B, the purified fraction binds GXM in a dose-dependent fashion.

CD14 is a 55-kDa glycosylphosphatidylinositol-anchored membrane protein found mainly on cells derived from the monocyte/macrophage lineage, as well as neutrophils and B lymphocytes (8, 18). This molecule, which is a member of the heteromeric lipopolysaccharide (LPS) receptor complex that also contains TLR4 and MD2 (17), is a GXM receptor in endocytic cells (12, 16, 25). This information and the molecular mass of the purified GXM-binding protein presented in Fig. 1A led us to investigate whether CD14 could be the GXM receptor expressed by A549 cells. Human cells were detached from culture plates using 1 mM EDTA and fixed in 4% para-

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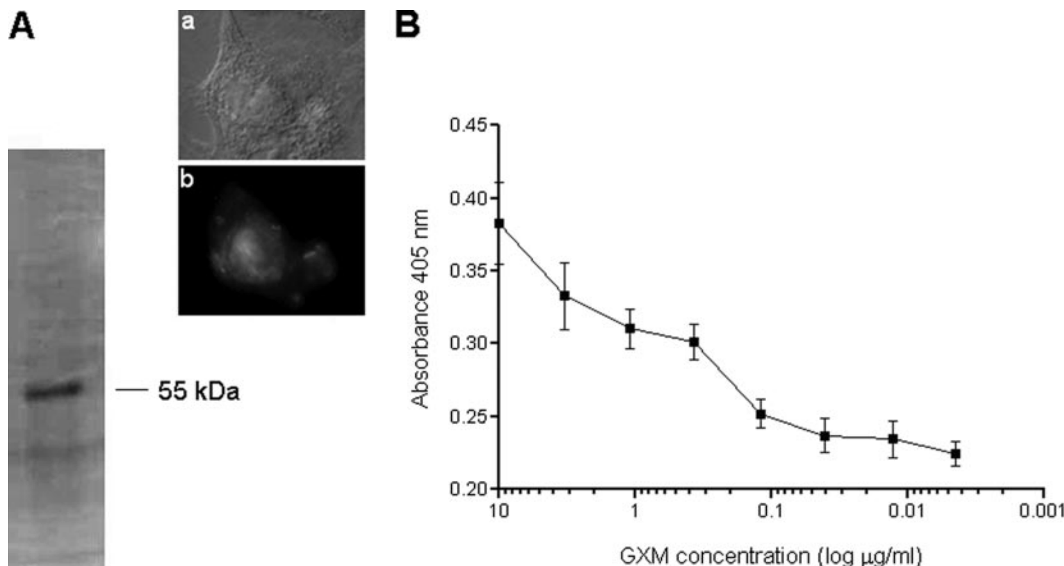

FIG. 1. Purification of a GXM-binding molecule from A549 cells by immunoprecipitation. (A) Protein extracts from GXM-treated human cells were sequentially incubated with MAb 18B7 and Sepharose-bound protein G for further analysis by SDS-PAGE. GXM binding to epithelial cells was confirmed by immunofluorescence with MAb 18B7 (inset). An A59 cell observed under differential interferential contrast (a) and fluorescence (b) modes is shown. A single major protein with a molecular mass corresponding to 55 kDa was detected. The purified fraction was used in GXM-binding assays in 96-well plates. (B) Dose-dependent binding of the 55-kDa protein to GXM detected by an IgM MAb to GXM.

formaldehyde. After blocking with Tris-buffered saline containing bovine serum albumin, A549 cells were incubated with a phycoerythrin (PE)-labeled mouse MAb to CD14 (BD Biosciences, San Jose, CA). Analysis in a FACSCalibur (BD Biosciences, San Jose, CA) flow cytometer revealed that A549 cells express CD14 (Fig. 2A), confirming previous reports (9, 20, 23). Control cells, incubated with an irrelevant PE-labeled antibody, presented low levels of fluorescence.

Given our recent observations that *C. neoformans* efficiently infects A549 cells (2), we evaluated whether a CD14-binding molecule would interfere with this process. Untreated human cells or an A549 population that was pretreated with LPS (10 μg/ml) for 1 h was incubated with *C. neoformans* under previously established conditions (2). The index of association between cryptococci and alveolar cells was measured as the reactivity of A549 cells with MAb 18B7 in flow cytometry assays (2), which showed that the efficacy of the interaction of cryptococci with untreated cells was higher than that observed with LPS-treated epithelia (Fig. 2B). Controls consisted of similar preparations that were not infected with *C. neoformans*. Pretreatment of A549 cells with anti-CD14 antibodies also resulted in a lower association between *C. neoformans* and host cells (data not shown).

To confirm that CD14 is indeed a GXM-binding molecule in human alveolar cells, a modification of classical immunoprecipitation methods was used. Lysates of GXM-treated cells, presumably containing polysaccharide-CD14 complexes, were added to the wells of a 96-well plate previously coated with the

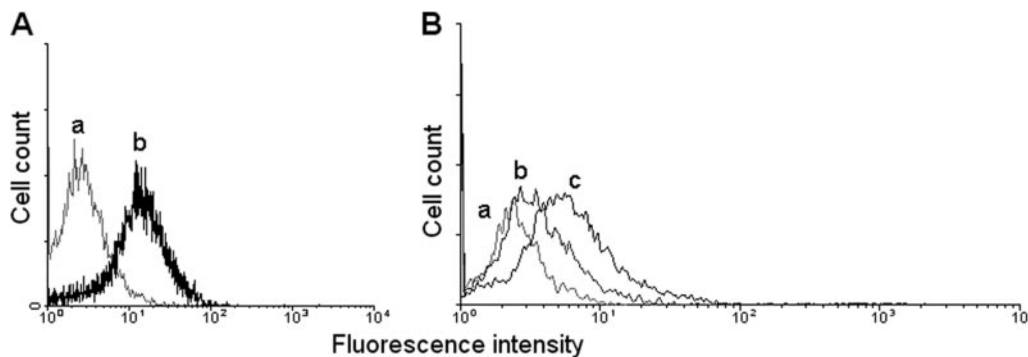

FIG. 2. Expression of CD14 by A549 cells. (A) Flow cytometric analysis of human cells incubated with a PE-labeled MAb to CD14 demonstrating that the receptor is expressed on the surface of A549 cells. Unstained (a) and antibody-treated (b) cells are shown. (B) Pretreatment of human cells with LPS, a CD14-binding compound, decreases their association with cryptococci. The increase in fluorescence after incubation of infected cells with MAb 18B7 was used to identify A549 cells in association with cryptococci. Infected cells that were pretreated with LPS (b) presented a reduced reaction with MAb 18B7, in comparison with untreated cells (c). The fluorescence levels of uninfected cells is shown in panel a.

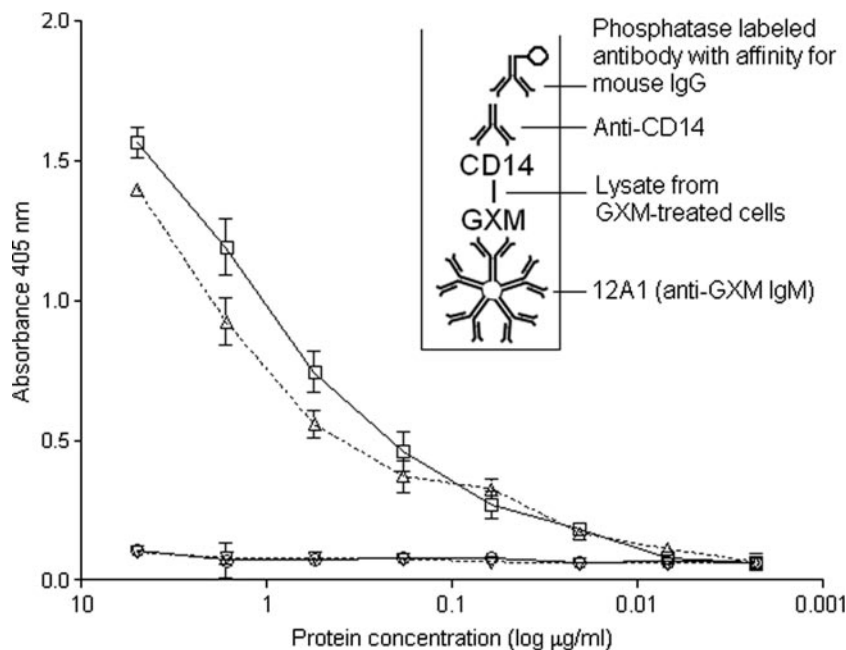

FIG. 3. Identification of CD14 as the epithelial receptor for GXM in A549 cells. CD14-GXM complexes (triangles) from polysaccharide-treated cells were captured by IgM antibodies in ELISA. An anti-CD14 was used as the detection probe, as demonstrated in the schematic presentation of the capture ELISA (inset). No significant reactions with the anti-CD14 antibody were observed when protein extracts from untreated cells (inverted triangles) were used. Additional controls included an ELISA in which A549 lysates (positive control; squares) and protein extracts from Chinese hamster ovary cells (negative control; circles) were used to coat the plates, followed by sequential incubation with the anti-CD14 MAb and phosphatase-labeled secondary antibodies.

anti-GXM IgM 12A1. After successive blocking and washing, the plate was incubated with a mouse MAb (IgG) to CD14 (BD Biosciences, San Jose, CA) and then with a phosphatase-labeled goat antibody with specificity for mouse IgG. CD14 was coprecipitated with GXM, as suggested by the dose-dependent recognition of complexes by the anti-CD14 antibody (Fig. 3). Controls consisted of similar assays in which lysates from GXM-treated cells were replaced by extracts from untreated alveolar cells. Additional controls included enzyme-linked immunosorbent assays (ELISAs) in which A549 lysates (positive control) and protein extracts from Chinese hamster ovary cells (negative control) were used to coat the plates, followed by blocking and sequential incubation with the anti-CD14 MAb and phosphatase-labeled secondary antibodies.

The association of microbial compounds with CD14 in alveolar cells can elicit the secretion of proinflammatory cytokines (20). We therefore speculated whether A549 cells could produce a cytokine response activated by GXM. For cytokine determinations, the culture medium was replaced by fresh medium containing no serum but supplemented with 10 μg/ml GXM. After 4 h at 37°C in a 5% CO_2 atmosphere, culture supernatants were collected and assayed for cytokines by using a RayBio human cytokine antibody array (RayBiotech, Inc.). Procedures followed the manufacturer's protocol. Cytokine production was quantified by using Scion Image 2000 software (Scion Corporation, NIH). Twenty cytokines were assayed, and under our experimental conditions, the production of interleukin-8 (IL-8) by GXM-treated cells was considered to be significantly different from that detected in nonstimulated supernatants (Fig. 4). GXM induced the secretion of growth-related oncogene α and IL-3 by human cells (data not shown), although the results obtained with the control and GXM-treated cells were not significantly different.

Since the CD14-LPS association resulted in IL-8 production by epithelial cells in previous studies (20), we investigated whether binding of GXM to CD14 molecules would have the same effect. Supernatants of nonstimulated A549 cells or of human cells after treatment for 1 h with LPS (positive control, 10 μg/ml), *C. neoformans* (2), or GXM (100 μg/ml) were col-

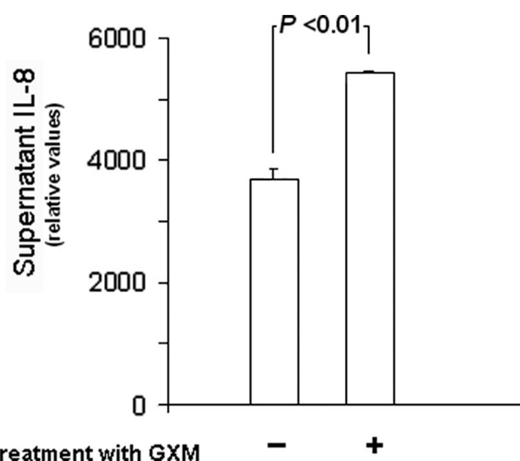

FIG. 4. Production of IL-8 by GXM-treated cells. Supernatants of untreated (−) or polysaccharide-treated (+) cells were collected for cytokine detection. An increased production of IL-8 was observed in supernatants of GXM-treated cells.

TABLE 1. Stimulation of A549 cells with GXM results in CD14-mediated production of IL-8[a]

Treatment of A549 cells[b]	Supernatant IL-8 (ng/ml) (mean ± SD)	P^c	Statistical significance
None	0.82 ± 0.06		
LPS (10 μg/ml, 1 h)	1.33 ± 0.01	0.007	Yes
C. neoformans (10 yeast cells/host cell)	1.06 ± 0.01	0.030	Yes
GXM (100 μg/ml, 1 h)[d]	1.18 ± 0.03	0.017	Yes
Anti-CD14 (10 μg/ml), then GXM (100 μg/ml)	0.54 ± 0.12	0.085	No

[a] Results are representative of three different assays producing similar results. The error bars were generated from multiple trial wells ($n = 3$) of each single sample.

[b] A549 cells were treated with LPS, fungal cells, GXM, or anti-CD14 antibodies for 1 h at 37°C.

[c] P values were obtained by the use of Student's t test, after comparison with results of control systems (no stimulation). Differences between results with stimulated systems and control cells were considered statistically significant when P values were smaller than 0.05.

[d] Results obtained for A549 cells treated with GXM alone were statistically compared to the data obtained for alveolar cells sequentially treated with the antibody to CD14 and GXM. IL-8 levels detected in cells after treatment with GXM alone were significantly higher ($P < 0.01$) than those obtained in A549 cells that were first treated with the antibody to CD14.

lected, and the presence of IL-8 was assayed by ELISA-based techniques (human CXCL8/IL-8 detection kit; R&D Systems, Minneapolis, MN). Under any of the conditions used for chemokine determination, the viability of the A549 population was significantly affected (data not shown). A significant increase in IL-8 production was observed when fungal cells, GXM, or LPS was incubated with A549 cells (Table 1). The levels of IL-8 produced under our experimental conditions were very similar to those observed in a previous study using LPS-treated A549 cells (14). Supplementation of the polysaccharide solution with polymyxin B produced similar ($P = 0.18$) levels of IL-8 (data not shown), indicating that the results obtained were not due to LPS contamination. When human cells had been previously incubated with antibodies to CD14, however, GXM-treated and nonstimulated cells expressed similar levels of IL-8 (Table 1).

In microbial infections, a primary function of the airway epithelium is to act as a physical barrier for the exclusion of inhaled infectious propagules. However, there is increasing evidence that lung cells can induce a localized immune response through the production of a variety of mediators, including proinflammatory cytokines and chemokines (1). These mediators could act, for example, to recruit polymorphonuclear leukocytes from the pulmonary vasculature into the alveolar space. With fungi, it has been demonstrated that *Aspergillus fumigatus* proteases elicit IL-6 and IL-8 production in lung epithelial cells in vivo and in vitro (26). In cases of cryptococcosis, the production of these proinflammatory mediators is associated with the survival of human patients (22). The production of such molecules by the airway epithelium could therefore represent an effective mechanism for the establishment of a local immune response controlling microbial lung infections.

GXM receptors have been characterized in a number of cells (12, 13, 16, 25). This polysaccharide mediates the interaction of *C. neoformans* with different host cells and induces a deleterious effect on the immune system (15). Interactions of cryptococci with epithelial cells are influenced by GXM (2), but the host receptors involved in this process are unknown. The identification of CD14 as an epithelial molecule interacting with GXM with the consequent production of IL-8 brings to light the potential role of epithelial respiratory cells in immunity against *C. neoformans*.

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