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Binding and Internalization of Glucuronoxylomannan, the Major Capsular Polysaccharide of *Cryptococcus neoformans*, by Murine Peritoneal Macrophages

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Glucuronoxylomannan (GXM), the major component of the capsular polysaccharide of *Cryptococcus neoformans*, is essential to virulence of the yeast. Previous studies found that the interaction between GXM and phagocytic cells has biological consequences that may contribute to the pathogenesis of cryptococcosis. We found that GXM binds to and is taken up by murine peritoneal macrophages. Uptake is dose and time dependent. Examination of the sites of GXM accumulation by immunofluorescence microscopy showed that the pattern was discontinuous and punctate both on the surfaces of macrophages and at intracellular depots. Although resident macrophages showed appreciable accumulation of GXM, uptake was greatest with thioglycolate-elicited macrophages. A modest stimulation of GXM binding followed treatment of resident macrophages with phorbol 12-myristate 13-acetate, but treatment with lipopolysaccharide or gamma interferon alone or in combination had no effect. Accumulation of GXM was critically dependent on cytoskeleton function; a near complete blockade of accumulation followed treatment with inhibitors of actin. GXM accumulation by elicited macrophages was blocked by treatment with inhibitors of tyrosine kinase, protein kinase C, and phospholipase C, but not by inhibitors of phosphatidylinositol 3-kinase, suggesting a critical role for one or more signaling pathways in the macrophage response to GXM. Taken together, the results demonstrate that it is possible to experimentally enhance or suppress binding of GXM to macrophages, raising the possibility for regulation of the interaction between this essential virulence factor and binding sites on cells that are central to host resistance.

Cryptococcus neoformans is the etiological agent of cryptococcosis, an opportunistic infection that may produce a life-threatening meningoencephalitis in immunocompromised patients. The yeast is surrounded by a polysaccharide capsule that is essential to virulence (3, 11). The primary constituent of the capsule is glucuronoxylomannan (GXM), a high-molecular-weight polysaccharide that can be isolated from supernatant fluids of yeast cultures and may be found in high concentrations in serum and cerebrospinal fluid samples from patients with cryptococcosis. GXM has an α-1,3-linked mannose backbone that is O acetylated and substituted with single side chains of xylose and glucuronic acid (2, 5).

There are several direct and indirect lines of evidence for binding and/or uptake of GXM by phagocytic cells. First, large amounts of GXM are shed in vivo, and immunohistochemistry studies have found that GXM accumulates and is stored in tissue macrophages (12, 14, 16, 22). Second, soluble GXM blocks binding of CD18 antibodies to human neutrophils, suggesting an interaction between GXM and neutrophil CD18 (9). Third, Shoham et al. found that GXM bound to Chinese hamster ovary (CHO) cells that were transfected with Toll-like receptors 2 and 4 and/or CD14 (27). Fourth, GXM on the yeast surface interacts directly with phagocyte receptors for GXM to mediate phagocytosis of the yeast (23, 28). Finally, Monari et al. directly demonstrated binding and uptake of GXM by human neutrophils and monocytes (21).

Binding and/or ingestion of GXM by macrophages has biological consequences that may contribute to the pathogenesis of cryptococcosis. Potential biological consequences of GXM-phagocyte interaction include inhibition of neutrophil influx into sites of inflammation (8), induction of shedding of L-selectin from neutrophils (7), blockade of phagocyte CD18 (9), alterations in cytokine secretion by leukocytes (6, 24, 29, 30), blockade of interaction between yeast-bound GXM and phagocyte receptors for GXM (23, 28), and reduced killing of encapsulated and acapsular cryptococci (21).

Despite abundant evidence for GXM-phagocyte interaction, little is known of the cellular events that occur on binding or uptake of GXM by phagocytes. The objective of our study was to further characterize the factors that influence attachment and ingestion of GXM by macrophages. The parameters that were examined included (i) kinetics for binding and uptake of GXM, (ii) the cellular pattern of GXM binding, (iii) requirements for cytoskeleton, (iv) requirements for macrophage activation, and (v) involvement of signal transduction pathways.

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MATERIALS AND METHODS

GXM. *C. neoformans* serotype A strain CN6, used as the source of GXM, was provided by R. Cherniak (Georgia State University, Atlanta, GA). Yeast cells

were incubated for 4 days at 30°C in a synthetic medium (4) and killed by treatment overnight with formaldehyde. GXM was isolated from culture supernatant fluid by differential precipitation with ethanol and cetyltrimethylammonium bromide as described previously (4). A stock solution of GXM in phosphate-buffered saline (PBS) at a concentration of 8 mg/ml was prepared, sterilized by filtration, and kept at 4°C. A 1 mg/ml solution of GXM produced a negative result when assayed by the *Limulus* amebocyte lysate test (QCL-1000; Cambrex Bio Science, Walkersville, Md.).

MAbs. GXM monoclonal antibody (MAb) 3C2 is a murine antibody of the immunoglobulin G1 (IgG1) isotype that is reactive with GXM of *C. neoformans* serotypes A, B, C, and D (10, 26). MAb 3C2 was produced in an in vitro culture system and was isolated from the culture medium by affinity chromatography on protein A. MAb 3C2 was coupled to horseradish peroxidase (HRPO) with a peroxidase labeling kit (EZ-Link activated peroxidase kit; Pierce, Rockford, IL) according to the manufacturer's directions. MAb 3C2 was coupled to the fluorescent dyes Alexa Fluor 555 and Alexa Fluor 488 (Molecular Probes, Eugene, OR) using labeling kits and directions provided by the manufacturer.

Murine peritoneal macrophages. Female Swiss Webster mice were obtained from the Animal Production Program, Frederick Cancer Research & Development Center (Frederick, MD) and were used at 8 to 10 weeks of age. To collect resident peritoneal macrophages, mice were euthanized, the peritoneal cavity was injected with 5 ml of ice-cold DPBS (Dulbecco's phosphate-buffered saline; Cellgro, Mediatech, Inc., Herndon, VA), and the peritoneal cells were harvested. Elicited macrophages were harvested 3 days after mice were given an intraperitoneal injection of 1 ml of sterile 10% thioglycolate medium (Sigma-Aldrich, St. Louis, MO). The cells were washed two times with DPBS and resuspended in Iscove's modified Dulbecco's medium (Cellgro, Mediatech, Inc.).

Analysis of GXM binding to macrophages by an enzyme-linked immunosorbent assay (ELISA). Tissue culture plates (96-well plates; Falcon 35307, Becton Dickinson Labware, Franklin Lakes, NJ) were seeded with peritoneal cells (1.25×10^5/well) in a culture medium of Iscove's modified Dulbecco's medium containing 10% fetal bovine serum (Cellgro, Mediatech, Inc.) and 100 μg/ml kanamycin (Sigma-Aldrich) and incubated at 37°C in a humidified atmosphere with 5% CO_2. After 1 day of incubation, the nonadherent cells were removed by gentle washing, and the macrophage monolayers were overlaid with fresh culture medium and incubated for an additional 24 h.

Three protocols were used to assess GXM binding: (i) total GXM bound to macrophages, (ii) GXM bound to the cell exterior, or (iii) GXM in the cell interior. In all cases, macrophage monolayers were incubated with various concentrations of GXM for the times indicated for each experiment. To assess total bound GXM, the cells were washed three times with PBS, fixed in 4% paraformaldehyde for 10 min on ice, and washed four times with PBS. The cells were permeabilized by treatment with 0.1% saponin for 30 min at room temperature and washed an additional four times with PBS. Potential nonspecific binding sites were blocked by incubation of monolayers for 30 min at room temperature with 2.5% bovine serum in PBS (blocking buffer). Bound GXM was identified by incubation of macrophages for 30 min at room temperature with a working dilution (1 μg/ml) of horseradish peroxidase-labeled MAb 3C2 (HRPO-MAb 3C2) in blocking buffer. Preliminary experiments established that the HRPO-labeled antibody was not limiting at the working dilution. After incubation with HRPO-MAb 3C2, the wells were washed four times with PBS containing 0.05% Tween 20 and incubated for 30 min at room temperature with 100 μl of 3,3′,5,5′-tetramethylbenzidine peroxidase substrate solution (Kirkegaard & Perry Laboratories; Gaithersburg, MD). The reaction was stopped by addition of 100 μl of 1 M H_3PO_4, and the absorbance at 450 nm was read with an ELISA plate reader (Versamax; Molecular Devices, Sunnyvale, CA). Results are reported as the optical density at 450 nm (OD_{450}) after correction for background. Background levels were characteristically ≤0.07. Different experiments often utilized different lots of HRPO-labeled antibody. Since the labeling efficiency varied somewhat from one labeling to another, the OD_{450} observed for one experiment often cannot be directly compared to optical densities observed in other experiments. However, when data from replicate experiments were pooled, the data represented experiments done with the same lot of labeled MAb.

Binding of GXM to the exterior of the macrophages was done as described above for determination of total GXM binding with the exception that permeabilization with saponin was omitted. To assess the amount of GXM inside macrophages, macrophage monolayers were treated with GXM as described above. The macrophages were fixed with paraformaldehyde, and nonspecific sites were blocked by treatment for 30 min at room temperature with blocking buffer. GXM bound to the macrophage surface was blocked by incubation with unlabeled MAb 3C2 (10 μg/ml) for 30 min at room temperature. Preliminary experiments demonstrated that such treatment reduced binding of HRPO-MAb 3C2 to nonpermeabilized GXM-treated macrophages by >95%. The cells were then permeabilized with saponin and incubated for 30 min with blocking buffer, and the amount of intracellular GXM was determined by use of HRPO-MAb 3C2 as described above.

Analysis of GXM binding by confocal microscopy. Peritoneal exudate cells (2.5×10^4), prepared as described above, were added to each chamber of an eight-well glass chamber slide (Nalge Nunc International Corp., Naperville, IL). After incubation for 24 h, the chambers were washed three times with culture medium to remove nonadherent cells. Next, the medium was replaced, and the monolayers were incubated for an additional 24 h. Macrophage monolayers were incubated with GXM (200 μl, 80 μg/ml) for 4, 16, 64, or 256 min. Subsequently, the medium was removed and the cells were washed three times with PBS. The macrophages were fixed with paraformaldehyde as described above, and nonspecific sites for protein binding were blocked by incubation for 30 min with blocking buffer. GXM bound to the surfaces of the cells was stained by incubation for 30 min at room temperature with Alexa Fluor 555-labeled MAb 3C2 (5 μg/ml). The macrophages were then permeabilized by treatment with saponin as described above, and nonspecific sites were blocked by incubation with blocking buffer for 30 min. GXM located in the interior of the macrophages was stained by incubation with Alexa Fluor 488-MAb 3C2 (5 μg/ml) for 30 min at room temperature. Preliminary experiments established that preincubation of paraformaldehyde-fixed cells with unlabeled MAb 3C2 prevented subsequent binding of Alexa 555-MAb 3C2 to the cells. After the cells were stained, the slides were overlaid with Vectashield (Vector Laboratories, Inc., Burlingame, CA) and covered with a coverslip. Binding of various fluorescently labeled antibodies was determined with a Nikon confocal microscope C1 unit that was fitted to a Nikon Eclipse E800 microscope equipped with differential interference contrast optics. Confocal images were acquired with Nikon EZ-C1 software, version 1.70. Merging and cropping of images were done with SimplePCI (Compix, Inc., Cranberry Township, PA).

Effects of activators and inhibitors. Ro 31-8220, calphostin C, rottlerin, genistein, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl) pyrazolo(3,4-D)pyrimidine (PP1), 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo(3,4-D)pyrimidine (PP2), piceatannol, LY294002, wortmannin, dolastatin 15, colchicine, U73122, cytochalasin D, latrunculin A, and vinblastine sulfate were purchased from Biomol (Plymouth Meeting, PA). All of the preceding agents were dissolved in either dimethyl sulfoxide (DMSO) or water and stored at −20°C. Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma-Aldrich and maintained as a stock solution in DMSO. Lipopolysaccharide (LPS) from *Escherichia coli* O55:B5, recombinant gamma interferon (IFN-γ) from mouse and *N*-formyl-Met-Leu-Phe (fMLP) were obtained from Sigma-Aldrich. Each agent was used at the concentrations indicated for each experiment in 200 μl of culture medium per well. Accounting for dilution by culture medium, the final concentration of DMSO for those agents where DMSO was used as a solvent was ≤0.5%. Control experiments showed that DMSO in concentrations of ≤2.5% had no effect on the accumulation of GXM by elicited macrophages. Monolayers were prepared as described above for assessment of GXM binding by ELISA and preincubated with inhibitors or activators for the times indicated for each experiment. Unless otherwise indicated, macrophages were preincubated for 30 min with each inhibitor. GXM was then added in a 20-μl volume to achieve a final concentration in the medium of 80 μg/ml, and the incubation was continued for an additional 2 h or an alternative time as required for a given experiment. The cells were then washed, fixed, and permeabilized as described above, and the amount of total bound GXM was determined by use of HRPO-MAb 3C2 as described above.

All experiments that examined the effects of pharmacologic activators or inhibitors were accompanied by control experiments that assessed the effects of the inhibitors on cell viability. Conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) by living cells was used as a quantitative measure of cell metabolic activity and viability as described previously (13). Assessment of MTT reduction in the presence of various inhibitors was done in parallel with GXM binding studies under identical conditions, including the presence of GXM. MTT activity was measured after the cells were washed to remove GXM.

Western blot analysis of PI3K activity. The abilities of wortmannin and LY294002 to inhibit phosphatidylinositol 3-kinase (PI3K) were confirmed by analysis of the effects of the inhibitors on LPS-induced phosphorylation of Akt by PI3K, which is sensitive to wortmannin and LY294002. Elicited macrophages were prepared in 96-well plates as described above. The macrophages were unstimulated or stimulated for 30 min with LPS (10 μg/ml) in the presence or absence of wortmannin (400 nM) or LY294002 (10 μM). Cell lysates were prepared by use of mammalian protein extraction reagent (Pierce), and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Phosphorylation of Akt at Ser473 was assessed by use of a phosphorylated Akt antibody (catalog no. 9276; Cell Sig-

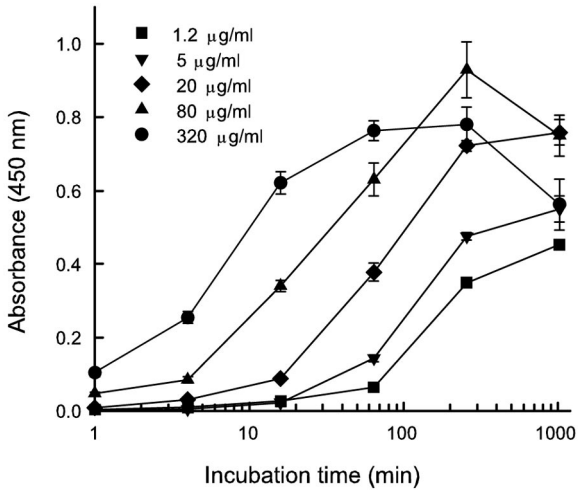

FIG. 1. Time course for accumulation of GXM by elicited peritoneal macrophages. Monolayers of macrophages were incubated with the indicated concentrations of GXM for 1, 4, 16, 64, 256, or 1,024 min, and the total amount of bound GXM was determined. Results are reported as the means ± SDs (error bars) of four replicates for each data point. Results shown are from one of three independent experiments with similar results.

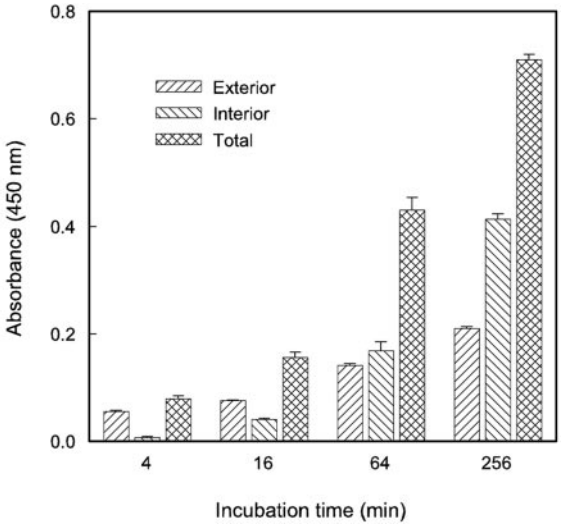

FIG. 2. Time course for (i) total binding of GXM to elicited peritoneal macrophages, (ii) binding of GXM to the macrophage exterior, or (iii) accumulation of GXM in the cell interior. Macrophages were incubated with GXM at 80 μg/ml for the indicated times, and the amount of total, exterior, or interior binding was assessed as described in Materials and Methods. Results are reported as the means ± SDs (error bars) of four replicates for each data point. Results shown are from one of four independent experiments with similar results.

naling Technology, Inc., Beverly, MA) followed by incubation with alkaline phosphatase-labeled rabbit anti-mouse secondary antibody and alkaline phosphatase substrate (Promega, Madison, WI). An antibody that bound to both phosphorylated and nonphosphorylated Akt (catalog no. 9272; Cell Signaling Technology, Inc.) was used as a control for sample loading.

Statistics. Comparisons of individual treatment effects with the medium control were made by analysis of variance with a post hoc assessment of individual treatment effects done by the Tukey test. Each experiment was done at least two times with four replicates per experiment. Data were combined and reported as the means ± standard deviations (SDs) when the experimental design was identical for replicate experiments and the same set of reagents was used. Data from a representative experiment are reported in those instances where replication of the experiment involved slight variation in the experimental design, e.g., incubation times or doses of reagents, or when different lots of reagents were used, e.g., different batches of HRPO-MAb.

RESULTS

Binding and uptake of GXM by elicited macrophages. An ELISA was used to assess binding of GXM to macrophages. Monolayers of macrophages were incubated for 1, 4, 16, 64, 256, or 1,024 min with 200 μl of GXM at concentrations of 1.2, 5, 20, 80, or 320 μg per ml of culture medium. The results (Fig. 1), expressed as the OD_{450}, showed gradual binding of GXM over time for all doses of GXM. Accumulation was most rapid at the highest concentration of GXM, approaching a maximum after 16 to 64 min of incubation. Monolayers incubated with GXM at 20 and 80 μg/ml reached maximum levels of binding similar to those observed with GXM at 320 μg/ml but required longer incubation times (64 to 256 min and 256 to 1,024 min, respectively).

The results shown in Fig. 1 reflected the total amount of GXM bound to macrophages but did not indicate whether GXM was located on the exterior or in the interior of the cells. As a consequence, an experiment was done to assess the relative amounts of GXM bound to the surface or found in the interior of the cells. In this experiment, monolayers of macrophages were incubated for 4, 16, 64, or 256 min with 200 μl of GXM at 80 μg per ml. The results of assays that examined total GXM binding (Fig. 2) were similar to the results shown in Fig. 1. A comparison of GXM bound to the cell surface and cell interior showed that most bound GXM was at the cell exterior after 4 min of incubation. After 64 min of incubation, approximately equal amounts of GXM were found on the cell surface and interior. After 256 min of incubation, the majority of the GXM was intracellular; however, an appreciable amount of GXM was also bound to the cell surface.

The results in Fig. 2 demonstrate that GXM is found at both the exterior and interior of macrophages. However, these results do not provide qualitative information as to the pattern of GXM binding. An experiment to assess the sites of GXM binding utilized an experimental design in which macrophage monolayers were incubated for 4, 16, 64, or 256 min with GXM at 80 μg/ml. The cells were fixed with paraformaldehyde, and the sites of exterior binding were identified by incubation with Alexa Fluor 555-MAb 3C2 (red). A high concentration of antibody was used (5 μg/ml) in an effort to completely block all GXM located on the cell surface. The cells were then permeabilized with saponin, and the sites of interior binding were identified by incubation with Alexa Fluor 488-MAb 3C2 (green). The results (Fig. 3) largely paralleled the results shown in Fig. 2. GXM was primarily located on the cell surface after 4 min of incubation. With increasing incubation time, there was an increase in the amount of GXM found in the cell interior such that the majority was intracellular after 256 min. Perhaps the most striking feature of the sites of GXM binding was the punctate pattern of localization. This discontinuous punctate pattern was observed for both extracellular and intracellular GXM. In no instance was there a continuous distribution of GXM either on the surface or in the cell interior. Many cells showed sites for accumulation of GXM that were

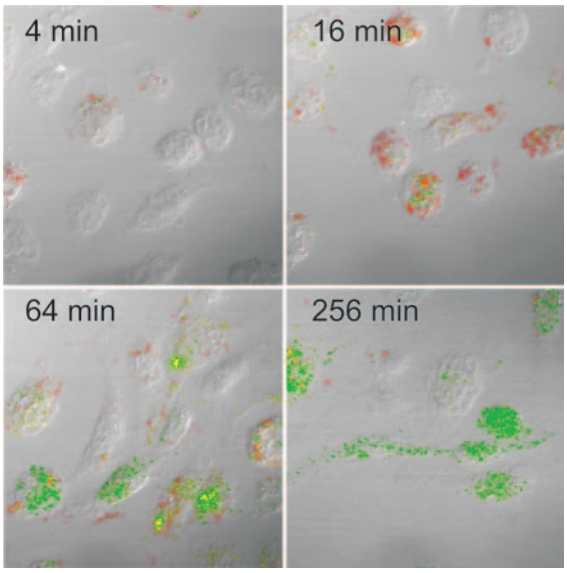

FIG. 3. Cellular sites for GXM bound to the exterior and interior of elicited peritoneal macrophages. Macrophages were incubated with GXM at 80 μg/ml for the indicated times, and GXM bound to the exterior or interior of the cells was determined as described in Materials and Methods. GXM bound to the exterior of the cells is shown by binding of Alexa Fluor 555-MAb 3C2 (red). GXM bound to the interior of the cells is shown by binding of Alexa Fluor 488-MAb 3C2 (green).

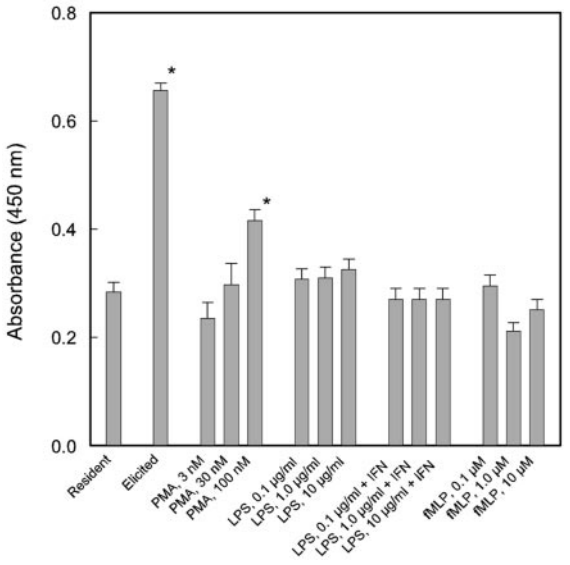

FIG. 4. Effect of macrophage activation on accumulation of GXM by macrophages. Thioglycolate-elicited or resident peritoneal macrophages were collected. Resident macrophages were preincubated for 18 h with LPS, LPS in combination with IFN-γ, or fMLP. Pretreatment time with PMA was 45 min (32). Following pretreatment with each potential activator or with medium alone, GXM was added to produce a final concentration of 80 μg/ml and incubated for an additional 2 h at 37°C. Bound GXM was assessed by an ELISA, and the results are reported as OD_{450}. Data are reported as the means ± SDs (error bars) from three independent experiments. Values that are significantly different ($P < 0.05$) from the value for untreated resident peritoneal macrophages (*) are indicated.

considerably larger than the GXM deposited in the punctate pattern. Finally, there was considerable cell-to-cell variability in the relative amount of bound GXM; some cells showed considerable amounts of bound GXM, while other cells showed little or no bound GXM.

Contribution of macrophage activation. Results from experiments shown in Fig. 1 to 3 were done with thioglycolate-elicited macrophages. Intraperitoneal injection of thioglycolate broth induces an influx of cells from a pool of preformed monocytes. The biological activities of thioglycolate-elicited macrophages differ from resident peritoneal macrophages in several respects, including increased metabolic activity and phagocytosis via complement receptors. As a consequence, we examined differences in GXM accumulation between resident macrophages, elicited macrophages, and resident macrophages that were activated by treatment in vitro with various doses of PMA, LPS, IFN-γ in combination with LPS, and fMLP. The results (Fig. 4) showed that elicited macrophages accumulated a significantly larger amount of GXM (ca. twofold) than did resident macrophages. Stimulation of resident macrophages with PMA produced a significant dose-dependent increase in GXM accumulation. LPS, LPS in combination with IFN-γ, and fMLP had no significant effect on accumulation of GXM by resident macrophages.

Requirement for cytoskeleton in accumulation of GXM by elicited macrophages. Inhibitors of actin and tubulin were used to assess the requirements for cytoskeleton in the accumulation of GXM by elicited macrophages. We evaluated the effects of two inhibitors of actin filament function, cytochalasin D and latrunculin A, and one inhibitor of microtubule activity, vinblastine sulfate. Inhibitory activity was examined over a broad range of inhibitor concentrations. The results (Fig. 5) show a near complete inhibition of GXM accumulation by both cytochalasin D and latrunculin A in a dose-dependent fashion. Substantial inhibition of GXM accumulation was noted with vinblastine; however, the level of inhibition was less than that observed for cytochalasin D and latrunculin A. Evaluation of the effects of each inhibitor on macrophage viability by use of the MTT assay showed no significant ($P > 0.05$) loss of MTT reduction activity by any inhibitor at the concentrations used in the study (data not shown).

There was only partial inhibition of GXM accumulation by the tubulin inhibitor vinblastine sulfate. As a consequence, we evaluated additional tubulin inhibitors in an effort to determine whether partial inhibition was unique to vinblastine or a feature that is shared by the general group of tubulin inhibitors. The results (Fig. 6) showed that vinblastine, dolastatin 15, and colchicine all produced a significant ($P < 0.05$) inhibition of GXM accumulation, but the level of inhibition never exceeded 50%. Evaluation of the effect of each inhibitor on cell metabolic activity/viability in the MTT assay showed no significant effect by any inhibitor (data not shown).

Effects of pharmacologic inhibitors of signal transduction on accumulation of GXM by elicited macrophages. Internalization of exogenous materials by cells may involve multiple signal transduction pathways. As a consequence, we examined the effects of inhibitors of protein kinase C (PKC), PI3K, phospholipase C, and selected protein tyrosine kinase (PTK) inhibitors on the accumulation of GXM by elicited macro-

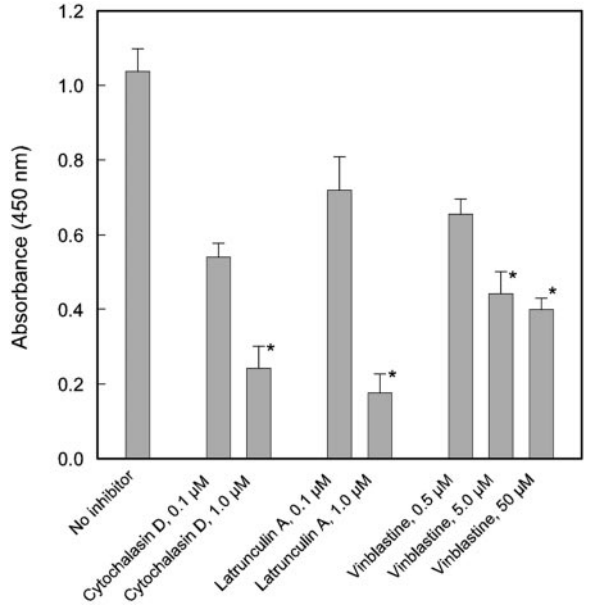

FIG. 5. Effects of inhibitors of cytoskeleton function on accumulation of GXM by elicited macrophages. Elicited macrophages were preincubated with each inhibitor at the indicated concentration or medium alone for 30 min, and GXM was added to produce a final concentration of 80 μg/ml and incubated for 2 h at 37°C. Bound GXM was assessed by an ELISA, and the results are reported as OD_{450}. Results are the combined data from two independent experiments and are reported as the means ± SDs (error bars). Values that are significantly different ($P < 0.05$) from the value for the control with no inhibitor (*) are indicated.

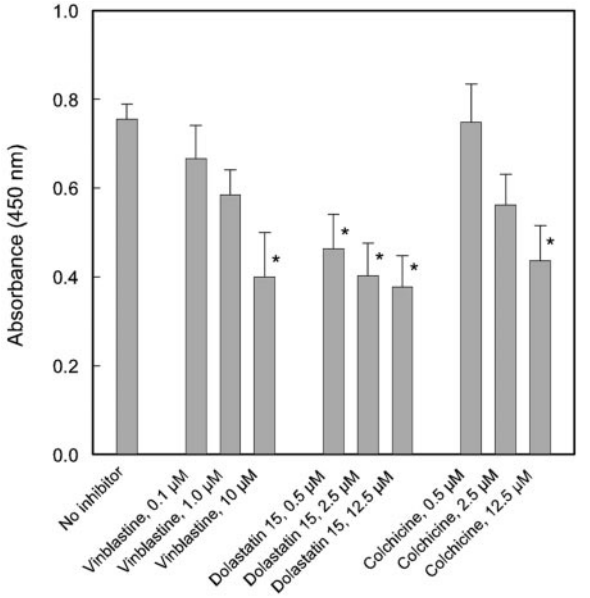

FIG. 6. Effects of inhibitors of tubulin function on accumulation of GXM by elicited macrophages. Macrophages were preincubated with each inhibitor at the indicated concentration or medium alone for 30 min, and GXM was added to produce a final concentration of 80 μg/ml and incubated for 2 h at 37°C. Bound GXM was assessed by an ELISA, and the results are reported as OD_{450}. Results are the combined data from two independent experiments and are reported as the means ± SDs (error bars). Values that are significantly different ($P < 0.05$) from the value for the control with no inhibitor (*) are indicated.

phages. All of the PKC inhibitors produced near complete inhibition of GXM accumulation in a dose-dependent manner (Fig. 7). Evaluation of the effects of each PKC inhibitor on macrophage viability by use of the MTT assay showed no significant loss of MTT reduction activity at any inhibitor concentration that was used ($P > 0.05$; data not shown).

The protein tyrosine kinase inhibitors selected for study showed significant ($P < 0.05$) inhibition of GXM accumulation (Fig. 8). The Src family inhibitors PP1, PP2, and piceatannol were particularly potent inhibitors, producing a near complete inhibition at the highest inhibitor concentrations. Evaluation of the effects of each inhibitor on macrophage viability by use of the MTT assay showed a significant reduction in activity in the presence of 20 μM PP1 (24%) and 40 μM PP1 (42%) (data not shown). As a consequence, a portion of the inhibitory activity of PP1 may be attributed to a loss of cell activity or viability. No other inhibitors produced a significant inhibition; however, a significant increase in MTT activity was noted for macrophages incubated with GXM in the presence of 100 μM and 400 μM genistein.

Results from three independent experiments found that the PI3K inhibitors LY294002 and wortmannin had little or no effect on the accumulation of GXM by elicited macrophages (data not shown). Given this negative result, we examined the effects of the inhibitors on constitutive and LPS-induced phosphorylation of Akt, which is on a wortmannin- and LY294002-sensitive pathway involving PI3K. The results showed a near complete absence of constitutive or LPS-induced phosphorylation of Akt at Ser473 in the presence of wortmannin (400 nM) or LY294002 (10 μM).

Finally, we examined the effect of the phospholipase C inhibitor U-73122 on the accumulation of GXM by elicited macrophages. The results of three independent experiments showed that U-73122 at concentrations of 1, 3, and 9 μM reduced GXM accumulation by 35%, 48%, and 73%, respectively, relative to uninhibited control macrophages. Examination of the effect of U-73122 on cell viability using the MTT assay showed no significant effect at any concentration of U-73122 that was used in the experiment.

DISCUSSION

In vivo and in vitro studies have provided abundant evidence for the binding of GXM to macrophages. Moreover, the interaction of GXM with macrophages has biological consequences. The goal of our study was to examine selected cellular mechanisms for binding and uptake of GXM. We found that macrophages gradually accumulate GXM over time and that binding of GXM by macrophages is followed by ingestion, confirming results reported by Monari et al. who examined the interaction between GXM and monocyte-derived macrophages (21). The primary new findings of our study are as follows. (i) Cellular sites for GXM binding exhibit a discontinuous punctate pattern. (ii) There was considerable cell-to-cell variability in binding and uptake of GXM. (iii) Thioglycolate-elicited macrophages bound much more GXM than resident peritoneal macrophages. (iv) The accumulation of GXM was blocked by inhibitors of actin and to a lesser extent by inhibi-

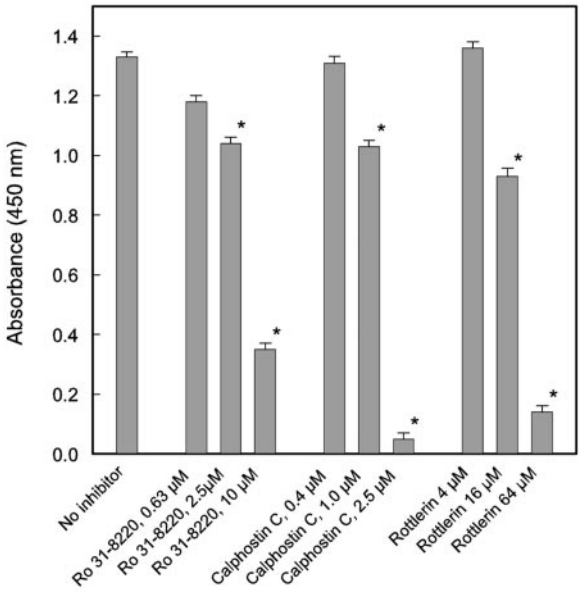

FIG. 7. Effects of protein kinase C inhibitors on accumulation of GXM by elicited macrophages. Macrophages were preincubated for 30 min with each inhibitor at the indicated concentration or medium alone, and GXM was added to produce a final concentration of 80 μg/ml and incubated for 2 h at 37°C. Bound GXM was assessed by ELISA, and the results are reported as OD_{450}. Results are the combined data from three independent experiments and are reported as the means ± SDs (error bars). Values that are significantly different ($P < 0.05$) from the value for the control with no inhibitor (*) are indicated.

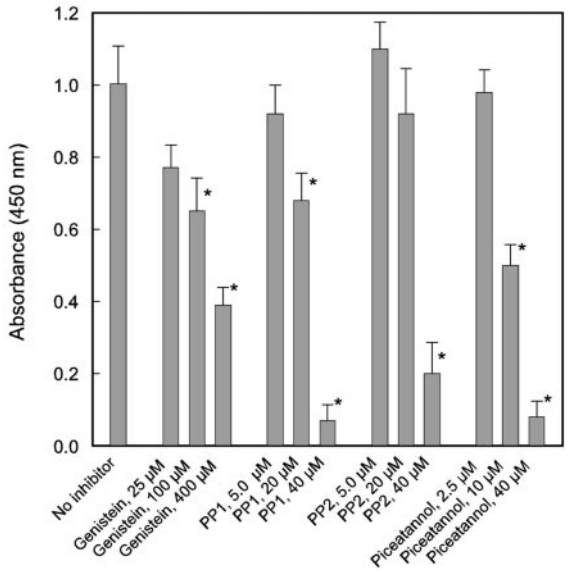

FIG. 8. Effects of tyrosine kinase inhibitors on accumulation of GXM by elicited macrophages. Macrophages were preincubated with each inhibitor at the indicated concentration or medium alone for 30 min, and GXM was added to produce a final concentration of 80 μg/ml and incubated for 2 h at 37°C. Bound GXM was assessed by ELISA, and the results are reported as OD_{450}. Results are the combined data from two independent experiments and are reported as the means ± SDs (error bars). Values that are significantly different ($P < 0.05$) from the value for the control with no inhibitor (*) are indicated.

tors of tubulin. (v) The accumulation of GXM was blocked by inhibitors of tyrosine kinase, protein kinase C, and phospholipase C, but not inhibitors of PI3K.

The rate of accumulation of GXM varied with the GXM concentration; a faster rate of accumulation was observed with higher doses. The gradual accumulation of GXM by elicited macrophages resembles the gradual accumulation of GXM by monocyte-derived macrophages that was reported by Monari et al. (21) and was slower than the very rapid accumulation (minutes) found with neutrophils. There was a maximum level for accumulation that was not exceeded by extended incubation time or by use of higher concentrations of GXM. This threshold effect suggests that potential receptors may have been saturated or depleted.

Although the accumulation of GXM by resting peritoneal macrophages was readily measurable, there was a twofold increase in GXM accumulation if thioglycolate-elicited macrophages were used. Intraperitoneal injection of thioglycolate broth induces an influx of cells from a pool of preformed monocytes (15). A mixture of exudate cells with resident peritoneal macrophages is consistent with our observation that the population of thioglycolate-elicited macrophages was quite heterogeneous in the uptake of GXM (Fig. 3). One of the features of thioglycolate-elicited macrophages is mobilization of C3 receptors for phagocytosis (20). Stimulation with PMA produced a modest but significant enhancement of GXM accumulation by resident peritoneal macrophages (Fig. 4). PMA causes phosphorylation of CD18 (7, 9), activates CD11b/CD18 for phagocytosis (31, 32), and produces clustering of CD11b/CD18 beneath bound particles (1). Activation of macrophages for increased microbicidal and tumoricidal activity can be accomplished by priming with IFN-γ and triggering with LPS (19). However, such activation produced no increase in GXM uptake by resident peritoneal macrophages. Notably, treatment of resident macrophages with IFN-γ does not stimulate complement receptor-mediated phagocytosis (25).

Drugs that target actin filaments and microtubules were used to assess cytoskeletal requirements for accumulation of GXM. Actin filaments are highly concentrated just beneath the plasma membrane and influence cell shape and locomotion. In contrast, microtubules determine the positions of membrane-enclosed organelles and direct intracellular transport. Cytochalasin D and latrunculin A inhibit actin function via distinct mechanisms. However, the effects of cytochalasin D and latrunculin A on accumulation of GXM were similar; both agents produced a near complete inhibition of GXM accumulation in a dose-dependent fashion, indicating that uptake occurs via either phagocytosis or pinocytosis. In contrast, microtubule-specific drugs produced a partial inhibition of approximately 50%. One explanation for the partial effect of microtubule-specific agents is the possibility that these drugs do not block GXM attachment but inhibit transport to intracellular depots. This explanation is consistent with the apparent saturation of GXM accumulation that occurred with extended incubation times or use of high GXM doses.

The involvement of cytoskeleton in GXM binding is consistent with the punctate pattern of binding that was observed on the surfaces of the macrophages. Such punctate binding suggests the aggregation of potential receptors or binding sites.

This would be made possible by the large, presumably multivalent, nature of the GXM molecule. The affinity and identity of the GXM receptor(s) are not known, but involvement of multiple receptors would allow for high-avidity interaction. The requirement for cytoskeleton suggests an active process on the part of the cell. Such an active involvement is also suggested by the sensitivity of the process to blockade of signal transduction pathways. Notably, one of the consequences of receptor-mediated induction of intracellular signaling can be targeting of cytoskeletal proteins for altered cell shape or movement.

Tyrosine kinase, protein kinase C, and phospholipase C have roles in several signaling cascades. Our data do not provide information regarding the specific roles of these signaling proteins in GXM accumulation. This is an area that has received very little attention. In this regard, Shoham et al. found that nuclear translocation of NF-κB occurred after GXM stimulation of CHO cells that expressed CD14 and Toll-like receptor 2, human peripheral blood mononuclear cells, and the murine macrophage cell line RAW 264.7 (27). In contrast, GXM stimulation of peripheral blood mononuclear cells and RAW 264.7 cells does not lead to stimulation of mitogen-activated protein kinase pathways leading to phosphorylation of extracellular signal-regulated kinase 1/2, stress-activated protein kinase/Jun N-terminal protein kinase, or p38. This report of GXM-induced NF-κB translocation coupled with results from the present study suggest that GXM has the potential to stimulate multiple signaling cascades; however, there is much to be learned regarding the intracellular targets and the cellular consequences of such signaling.

Our study used an in vitro model for study of GXM uptake by macrophages. The cell-to-cell variability in uptake suggests that there is a subset of macrophages with the ability to accumulate GXM. Several studies have shown that parenteral administration of GXM leads to accumulation of GXM in Kupffer cells of the liver and in the marginal zone macrophages of the spleen (12, 14). In contrast, there was little or no GXM accumulation in lung or brain tissue. Despite the absence of GXM in the lung or brain, it is possible that GXM could accumulate in alveolar macrophages or microglial cells at the site of infection. Notably, Lipovsky et al. reported that GXM induces interleukin 8 (IL-8) production by human microglia, raising the possibility for binding and/or uptake of GXM by this biologically relevant cell type (18).

The interaction of GXM with macrophages can impact the pathogenesis of cryptococcosis at several levels. First, ligation of capsular GXM by macrophages can directly mediate uptake of the yeast by macrophages and facilitates complement receptor and Fcγ receptor-dependent phagocytosis (23, 28). Second, incubation of phagocytic cells with GXM or exposure to encapsulated cryptococci has potent immunoregulatory effects. GXM suppresses LPS-induced secretion of tumor necrosis factor alpha (TNF-α) (30). In contrast, GXM stimulates release of the proinflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α by human neutrophils (24). Administration of GXM in vivo leads to increased production of macrophage inflammatory protein 1α (MIP-1α), MIP-2, monocyte chemotactic peptide 1, and IL-1β, IL-6, and TNF-α in liver (17). Finally, cells of the mononuclear phagocyte system play an active role in clearance of GXM in vivo (14). The results of our study demonstrate that it is possible to experimentally enhance or suppress binding of GXM to macrophages, raising the possibility for regulation of the interaction between this essential virulence factor and binding sites on cells that are central to host resistance.

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