Ingestion of Acapsular *Cryptococcus neoformans* Occurs via Mannose and β -Glucan Receptors, Resulting in Cytokine Production and Increased Phagocytosis of the Encapsulated Form

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Cryptococcus neoformans is a pathogenic yeast and a major cause of opportunistic infection in AIDS patients. It is commonly found in an acapsular form in the environment, and infection is likely to occur by inhalation. The lung provides a suitable environment for capsule synthesis, and once encapsulated, *C. neoformans* becomes resistant to phagocytosis. A stable acapsular mutant of the organism is readily ingested by murine macrophages in vitro, indicating entry via constitutively competent receptors. We demonstrate in this report that this process is inhibitable by particles derived from *Saccharomyces cerevisiae* that are rich in mannan and β -glucan, as well as more purified forms of these glycans. Furthermore, ingestion of the acapsular form of *C. neoformans* induces a range of proinflammatory cytokines, including tumor necrosis factor alpha and granulocyte-macrophage colony-stimulating factor, which, as we have previously shown, enhance ingestion of serum-opsonized encapsulated *C. neoformans* in vitro. We demonstrate that ingestion of the acapsular form of the organism also enhances ingestion of the pathogenic encapsulated form. This is dependent on the production of tumor necrosis factor alpha and granulocyte-macrophage colony-stimulating factor by the macrophages, since addition of neutralizing antibodies to both cytokines inhibited the observed increase in ingestion. Together, these data demonstrate that ingestion of acapsular *C. neoformans* is mediated via mannose and β -glucan receptors on the macrophage surface and that this process activates macrophages for enhanced phagocytosis of the encapsulated form via production of macrophage-derived cytokines.

Infection with *Cryptococcus neoformans* occurs following inhalation of acapsular or poorly encapsulated yeast cells from the environment (29). It is thought that upon entry into the alveolar space, *C. neoformans* rapidly synthesizes a polysaccharide capsule that confers resistance to phagocytosis (19). Despite this, immunocompetent individuals rarely succumb to clinical disease, suggesting that initial exposure to the acapsular organism activates macrophages for effective clearance of the antiphagocytic encapsulated form. Expression of a polysaccharide capsule is an important virulence determinant (6) and prevents induction of T-cell-mediated responses in vitro (3). The antiphagocytic properties of the polysaccharide capsule can be partially overcome by prior opsonization with a specific antibody or serum (5). We have recently shown that encapsulated *C. neoformans* opsonized with heat-labile serum components is ingested by murine macrophages in vitro by a complement receptor type 3 (CR3)-dependent mechanism and that the level of ingestion can be increased four- to fivefold by activating the macrophages in vitro with recombinant cytokines tumor necrosis factor alpha (TNF- α) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (4).

In contrast to the extensive literature describing the interaction of encapsulated *C. neoformans* with host macrophages, few data are available on the mechanisms of ingestion of the acapsular form which, together with basidiospores, probably initiate infection in the host. While encapsulated yeast cells are

resistant to phagocytosis, stable acapsular mutants of the organism are readily ingested by macrophages in vitro in the absence of either exogenous opsonins or macrophage-activating cytokines, indicating entry via constitutively competent receptors. Furthermore, the cell wall of *C. neoformans* expresses carbohydrates such as glucan, *N*-acetylglucosamine, and mannose in the form of mannoproteins (27, 39). Endocytosis of mannose ligands and phagocytosis of complex organisms containing mannosyl residues, such as the pathogens *Candida albicans* (8), *Leishmania* sp. (40), *Mycobacterium* sp. (30), and *Pneumocystis carinii* (9), can be mediated via the mannose receptor. Other candidate receptors include the β -glucan receptor, which has also been implicated in ingestion of *P. carinii* (14). Although *C. neoformans* has exposed carbohydrate residues on its cell wall suitable for interaction with these constitutively active receptors, neither the macrophage receptors used for ingestion of this form of the organism nor the downstream effects of receptor ligation are known.

In this study, we addressed three issues. Firstly, which receptors mediate ingestion of a potentially infective form of *C. neoformans*, secondly, does binding and ingestion of acapsular *C. neoformans* trigger a cytokine response; and thirdly, are there any functional consequences of this activation? We found that ingestion of acapsular *C. neoformans* by resting macrophages, in the absence of exogenous opsonins, is inhibitable by the glycans mannan and β -1 $\rightarrow$ 3 glucan. This ingestion results in the production of a range of cytokines, including TNF- α and GM-CSF, which enhance phagocytosis of the pathogenic encapsulated form. This mechanism of macrophage activation may be relevant to the host, enabling clearance of encapsulated yeast cells before the advent of T-cell-derived cytokines.

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MATERIALS AND METHODS

Target particles for phagocytosis. An encapsulated strain (B3501) of the pathogenic yeast *C. neoformans* and an acapsular mutant strain (B4131) were kindly provided by K. J. Kwon-Chung, National Institutes of Health, Bethesda, Md. Both strains were cultured on Sabouraud dextrose agar plates at 37°C. Before use, yeast cells were scraped from the plates and acapsular *C. neoformans* was heat killed at 80°C for 1 h and washed three times in pyrogen-free saline. Live encapsulated *C. neoformans* was serum opsonized by incubation of 1×10^7 to 5×10^7 yeast cells with 200 μ l of normal mouse serum (from uninfected CBA mice housed under specific-pathogen-free conditions at the London School of Hygiene and Tropical Medicine) for 90 min at 37°C and then washed as outlined above. We have previously demonstrated (i) that this opsonization is abolished by heat inactivation at 56°C, (ii) that it is observed with serum essentially devoid of any immunoglobulin (obtained from mice with severe combined immunodeficiency), and (iii) that ingestion of yeast cells opsonized in this manner is blocked by anti-CR3 antibodies (4). Zymosan (Sigma, Dorset, United Kingdom [U.K.]), a cell wall extract from *Saccharomyces cerevisiae*, and 3- μ m latex beads (Sigma) were routinely washed in complete growth medium before use. Acapsular *C. neoformans* and zymosan particles were directly conjugated to tetramethyl rhodamine isothiocyanate and fluorescein isothiocyanate respectively, by incubating up to 10^8 particles for 2 h at room temperature with 0.5 mg of the fluorochrome per ml in buffer containing 0.1 M NaHCO₃, 0.01 M Na₂CO₃, and 0.9% NaCl. The concentration of zymosan particles was estimated to be 2×10^7 /mg.

Recombinant cytokines and anticytokine monoclonal antibodies. Recombinant murine TNF- α (specific activity, 1.2×10^7 U/mg) was a kind gift from G. Adolf, recombinant GM-CSF (specific activity, 7.8×10^7 U/mg) was purchased from Genzyme (Kent, U.K.), and both cytokines were used at 1 to 100 U/ml. Neutralizing antibodies to TNF- α (TN3.19-12; a kind gift from R. D. Schreiber, St. Louis, Mo.) and GM-CSF (MP1.22E9.11) and isotype-matched control antibodies (L2 and GL117, respectively; kind gifts from J. Abrams, DNAX, Palo Alto, Calif.) were used at 50 μ g/ml.

Preparation of macrophage monolayers and phagocytosis assay. Specific-pathogen-free female CBA/CA and BALB/c mice were purchased from Harlan Olac and used at 12 to 18 weeks of age. Peritoneal exudate cells were harvested by peritoneal lavage with RPMI 1640 containing glutamine (100 mM), penicillin (100 IU/ml), streptomycin (100 μ g/ml), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (10 mM), and 1% heat-inactivated fetal calf serum. Cells were washed once and resuspended at a final concentration of 2×10^6 /ml in medium prepared as described above but containing 10% heat-inactivated fetal calf serum (complete growth medium). Cells were plated in 300- μ l aliquots into wells of an eight-well glass Labtek chamber (Gibco, Paisley, Scotland). After 2 h at 37°C in 5% CO₂, cell monolayers were washed three times in complete growth medium to remove nonadherent cells. Assessment of phagocytic activity was done at different times as described in Results but was done essentially as previously described (4). In summary, cell monolayers were incubated for 1 to 2 h with target particles previously washed three times in complete growth medium. After ingestion, cell monolayers were washed three times to remove unbound particles and the slides were fixed in absolute methanol for 10 min at room temperature, washed in phosphate-buffered saline (PBS), and mounted in antifade mountant (2) under glass coverslips. Ingestion was quantitated by visualizing fluorescent particles in four or five distinct fields and expressed as the mean number of particles ingested per 100 macrophages $\pm$ the standard deviation. Previous studies in our laboratory with double staining techniques have demonstrated that >90% of macrophage-associated *C. neoformans* cells are internalized (4).

Preparation of mannan- or laminarin-coated Labtek chambers. Mannan (derived from *S. cerevisiae*; Sigma) laminarin (β -1 $\rightarrow$ 3 glucan; derived from *Laminaria digitata*; Sigma), and bovine serum albumin (BSA; Sigma) were cross-linked onto glass surfaces by the method of Sung et al. (34). Labtek chambers were initially coated for 30 min at room temperature with 0.1% poly-L-lysine (Sigma). After four washes with sterile PBS, various concentrations of mannan or laminarin in PBS containing 10 mg of ethyldimethylaminopropylcarbodiimide hydrochloride (Sigma) per ml were added to wells in 300- μ l aliquots and incubated at 4°C overnight. Wells were washed twice with PBS and then in complete growth medium before addition of peritoneal exudate cells (2×10^6 /ml).

Mannose-binding protein (MBP) binding assay. Acapsular and encapsulated *C. neoformans* cells were washed in PBS containing 1.5 mM calcium chloride and 1% BSA (PBS/C) and incubated in the presence or absence of EDTA (5 mM final concentration) with 1 to 30 μ g of human recombinant MBP (a kind gift from R. A. B. Ezekowitz, Boston, Mass.) for 1 h at 4°C. After three washes in PBS/C, the yeast cells were incubated for 1 h at 4°C with a rabbit anti-human MBP antibody (a kind gift from P. D. Stahl) diluted 1:2,000 in PBS/C and incubated with a fluorescein isothiocyanate-labelled anti-rabbit immunoglobulin (diluted 1:400 in PBS/C; Sigma). Collectin binding to *C. neoformans* was assessed with a FACScan flow cytometer.

Reverse transcription-PCR for detection of mRNA for cytokines. Total RNA was prepared from approximately 2×10^6 adherent macrophages by the RNazol extraction method (Biogenesis, Bournemouth, U.K.). After in vitro stimulation, cell monolayers (cultured at 10^6 /ml in a 24-well tissue culture dish [Costar]) were washed once in RPMI 1640 with no supplements, 200 μ l of RNazol solution was added per well, and after cell lysis the solution was transferred to an RNase-free

Eppendorf tube. One-tenth of a volume of chloroform was added, and the tubes were incubated on ice for 15 min and then centrifuged at $10,000 \times g$ for 25 min at 4°C. The aqueous phase was removed while avoiding the interface and transferred to a clean, RNase-free tube. RNA was precipitated by addition of an equal volume of isopropanol and then mixed and incubated on ice for a further 15 min. RNA was pelleted by centrifugation at $10,000 \times g$ for 25 min at 4°C, washed in ice-cold 75% aqueous alcohol, and centrifuged at $2,000 \times g$ for 10 min at 4°C. The pellet was resuspended in 20 μ l of diethyl pyrocarbonate-treated water. The amount of RNA isolated was estimated by determining the optical density at 260 nm, and 1 μ l was electrophoresed on a 1% agarose gel to confirm its integrity.

Reverse transcription was performed by using total RNA, Superscript R/T enzyme, and the Superscript Protocol 1 from Gibco (Paisley, Scotland). In summary, 3 μ g of RNA was made up to 13 μ l with diethyl pyrocarbonate-treated water and 1 μ l of oligo(dT) was added to each tube. After 10 min at 70°C, the tubes were transferred to ice and 2 μ l of 10 $\times$ buffer, 1 μ l of deoxynucleoside triphosphates, 2 μ l of 0.1 M dithiothreitol, and 1 μ l of Superscript enzyme were added. After 10 min at room temperature, the tubes were incubated at 42°C for 50 min to allow first-strand cDNA synthesis and then for 15 min at 70°C to terminate the reaction. The tubes were then incubated on ice for a further 10 min, after which single-stranded RNA was removed by incubation at 37°C for 20 min in the presence of 1 μ l of RNase H solution. The resulting cDNA was diluted 1:2 in diethyl pyrocarbonate-treated water, and 5 μ l was added to each PCR. For each set of previously described (23, 24, 26) primers to be used for cDNA amplification, a reaction mixture was prepared containing 5 μ l of 10 $\times$ buffer, 1 μ l of a 10 mM deoxynucleoside triphosphate mixture, 1 μ l of each primer from a 10 OD₂₆₀ stock, 37 μ l of sterile distilled water, 0.2 μ l of *Taq* polymerase, and 0.2 μ l of dimethyl sulfoxide. cDNA was amplified in a Perkin Elmer Cetus 9600 GeneAmp PCR machine under the following conditions: initial denaturation for 2 min at 95°C and then five cycles of denaturation for 30 s at 94°C, primer annealing for 30 s at 61°C, and extension for 40 s at 72°C. This was followed by 25 cycles at slightly lower temperatures with shorter incubation times: denaturation at 93°C for 20 s, annealing at 61°C for 25 s, and extension at 72°C for 25 s. The tubes were then incubated for 10 min at 72°C, and 10 μ l of the amplified product from each sample was visualized on a 1.5% agarose gel. Hypoxanthine phosphoribosyltransferase (HPRT) is a noninducible enzyme, the message for which is produced by cells at levels similar to that of the cytokine message (23). cDNA for HPRT was amplified alongside test samples and used as an internal control. Amplified products for HPRT were normalized by diluting each sample and estimating visually the dilution at which the band intensity was equivalent between samples. These adjusted volumes of the test samples were then electrophoresed on a 1.5% agarose gel.

Enzyme-linked immunosorbent assay (ELISA) for detection of TNF- α . Plastic 96-well microtiteration plates (Immulon 2; Dynatech, Sussex, U.K.) were coated with a monoclonal antibody to TNF (TN319.12; 10 μ g/ml) in 0.1 M carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C. Plates were washed eight times with ELISA wash buffer (PBS containing 0.1% [vol/vol] Tween 20 [pH 7.4]) before addition of test samples and serial dilutions of the samples in complete growth medium. All subsequent dilutions were made in complete growth medium. Dilutions of TNF- α at known concentrations (0 to 50 U/ml) were added to each plate as standards, and the plates were incubated for 2 h at 37°C. The plates were washed eight times with ELISA wash buffer, and a polyclonal rabbit anti-TNF- α antibody (1:1,000 dilution, kindly provided by P. Kaye, London School of Hygiene and Tropical Medicine, London, U.K.) was added to each well and incubated for 1 h at 37°C. After being washed as outlined above, goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (1:10,000 dilution; Tago, Oxford, U.K.) was added. Specifically bound TNF- α was visualized with a TMB (3,3',5,5'-tetramethylbenzidine) substrate prepared in accordance with the manufacturer's instructions (Dynatech), and the reaction was stopped with 1 M phosphoric acid. Optical densities were read at 450 nm with a Dynatech MR5000 plate reader. Units of TNF- α per milliliter were estimated with a standard curve of known cytokine concentrations against optical density at 450 nm. The lower limit of detection by this assay was 2 U/ml.

RESULTS

Inhibition of ingestion of acapsular *C. neoformans* by zymosan and β -glucan particles. The acapsular form of *C. neoformans*, unlike the encapsulated form, is readily ingested by murine macrophages in vitro, indicating that these two forms of the organism enter macrophages by different sets of receptors. The cell wall of *C. neoformans* is rich in saccharides and glycans, including glucose and *N*-acetylglucosamine, and possibly also mannoproteins (16, 39), and our initial experiments assessed the role of mannose and β -glucan receptors in the ingestion of acapsular *C. neoformans* by murine macrophages in vitro. Zymosan and glucan particles (derived from *S. cerevisiae* and rich in glucan-mannan and glucan, respectively) were readily ingested by macrophages and, at equivalent challenge

FIG. 1. Concentration-dependent inhibition of macrophage ingestion of acapsular *C. neoformans* by zymosan and β -glucan particles. Macrophage monolayers were challenged with either 10^7 acapsular *C. neoformans* cells per ml (closed symbols) or 2×10^6 latex beads per ml (open symbols) together with increasing concentrations of *S. cerevisiae*-derived zymosan (■) or glucan (●) particles. Zymosan at 100 μ g/ml is equivalent to approximately 2×10^6 particles per ml. Results are presented as mean numbers of acapsular *C. neoformans* cells or latex beads ingested per 100 macrophages $\pm$ the standard deviation and are representative of four separate experiments.

ratios, were ingested more efficiently than acapsular *C. neoformans* (data not shown). Acapsular *C. neoformans* was added to macrophage monolayers, together with increasing concentrations of zymosan or β -glucan particles, and ingestion was assessed by fluorescence microscopy. At an infection ratio of approximately one zymosan particle for every two cryptococci, ingestion of *C. neoformans* was inhibited by more than 90% (Fig. 1). Internalization of similar numbers of latex beads had no effect on ingestion of acapsular *C. neoformans*, confirming that ingestion of these particles did not nonspecifically affect

phagocytosis or cell viability (data not shown). Also, although both zymosan and glucan particles were both equally efficient at inhibiting ingestion of acapsular *C. neoformans*, when competed against each other, increasing concentrations of glucan (up to 200 μ g/ml) did not inhibit the ingestion of zymosan particles (20 μ g/ml; data not shown). The direct competition between acapsular *C. neoformans* and zymosan and glucan particles suggests the involvement of mannose and/or β -glucan receptors in the ingestion of this organism.

Effect of soluble and immobilized mannan and β -1 $\rightarrow$ 3 glucan on ingestion of acapsular *C. neoformans*. To investigate further the involvement of mannose and β -glucan receptors in the ingestion of the acapsular yeast, we examined the effects on macrophage phagocytic activity of purified mannan (from *S. cerevisiae*) and β -1 $\rightarrow$ 3 glucan (laminarin, from *L. digitata*) in either soluble or insoluble form. Ingestion of acapsular *C. neoformans* was effectively inhibited by both soluble mannan and β -1 $\rightarrow$ 3 glucan, with 50% inhibition at 10 μ g/ml and 90% inhibition at 2 mg/ml (Fig. 2A). Immobilized mannan and β -1 $\rightarrow$ 3 glucan were more effective inhibitors than in solution, and at a coating concentration of 0.2 μ g/ml, both glycans caused 50% inhibition with maximal inhibition of >90% at 2 mg/ml (Fig. 2B). No inhibition of yeast ingestion was observed in wells treated with carbodiimide in the absence of glycan or with BSA-carbodiimide (data not shown). In all cases, ingestion of latex beads was unaffected by the presence of either soluble or immobilized glycans (see Fig. 2 legend for details). Together, these data suggest that there is a role for carbohydrate-binding receptors, including both the mannose and β -glucan receptors, in the ingestion of acapsular *C. neoformans*.

Binding of MBP to *C. neoformans*. MBP is a member of the newly characterized collectin family, which exhibits a range of ligand specificities similar to that of the mannose receptor and binds to mannan, galactose, and *N*-acetylglucosamine residues in a calcium-dependent manner (15, 37). To determine whether this protein binds to either the acapsular or encapsulated form of *C. neoformans*, binding assays were done with

FIG. 2. Concentration-dependent inhibition of ingestion of acapsular *C. neoformans* by soluble and solid-phase glycans. (A) Acapsular *C. neoformans* (10^7 organisms per ml) was added, together with increasing concentrations of *S. cerevisiae*-derived mannan (■) or *L. digitata*-derived laminarin (●) (0.02 to 2,000 μ g/ml), to macrophage monolayers, and levels of ingestion were assessed after 1 h. Both soluble mannan and glucan were effective inhibitors of yeast ingestion but had no effect on the ingestion of latex beads (774 $\pm$ 129 beads per 100 macrophages in the absence of glycan versus 761 $\pm$ 68 [with laminarin at 2,000 μ g/ml] or 839 $\pm$ 292 [with mannan at 2,000 μ g/ml] beads per 100 macrophages). (B) Labtek chambers were coated with increasing concentrations of mannan (■) or laminarin (●) by cross-linking with carbodiimide. Macrophage monolayers were established on these substrates and challenged with 10^7 acapsular *C. neoformans* cells per ml or 2×10^6 latex beads per ml, and ingestion levels were assessed as previously described. Ingestion of latex beads was unaffected by the presence of the glycans (721 $\pm$ 100 beads per 100 macrophages in the absence of glycans versus 691 $\pm$ 58 [with laminarin at 20,000 μ g/ml] or 821 $\pm$ 208 [with mannan at 20,000 μ g/ml] beads per 100 macrophages). The results shown are representative of three separate experiments.

FIG. 3. Concentration-dependent binding of MBP to acapsular *C. neoformans*. (A) Acapsular *C. neoformans* was incubated with 0, 1, 10, 20, or 30 μg of MBP per ml, and specific binding of all groups was assessed with a monoclonal anti-MBP antibody and is expressed as the mean peak channel of fluorescence. (B) Histograms: a, encapsulated *C. neoformans* incubated as described above with 20 μg of MBP per ml; b, acapsular *C. neoformans* without MBP; c, acapsular *C. neoformans* incubated with 20 μg of MBP per ml in the presence of 5 mM EDTA; d, acapsular *C. neoformans* incubated with 20 μg of MBP per ml.

recombinant human MBP and specific antibodies. One microgram of MBP (10 μg/ml) was sufficient to demonstrate binding of the collectin to acapsular *C. neoformans* as assessed by flow cytometry (Fig. 3A). In contrast, no binding was seen when encapsulated *C. neoformans* was incubated under the same conditions with 2 μg of MBP (20 μg/ml). When calcium ions were removed from the medium by chelation with EDTA, no shift in fluorescence intensity was seen, confirming the cation dependency of the interaction (Fig. 3B). These data support the view that the acapsular form of *C. neoformans* has exposed carbohydrate residues on its surface that are suitable for interaction with lectin-like receptors which are masked by the polysaccharide capsule in the encapsulated form of the organism.

Induction of mRNA for cytokines in macrophages following in vitro activation with acapsular *C. neoformans*. Ligation of lectin-like receptors has previously been demonstrated to result in triggering of a cytokine response and production of proinflammatory cytokines, including TNF-α and interleukin 1β (14, 32). We wanted to determine whether binding and/or ingestion of acapsular *C. neoformans* by macrophages in vitro results in macrophage activation. Macrophage monolayers were challenged with acapsular *C. neoformans* for 1 h before removal of unbound organisms and then incubated for 1, 4, 24, or 30 h, after which time supernatants were taken and RNA was isolated. As a positive control for TNF-α induction, equivalent monolayers were treated with 50 ng of lipopolysaccharide per ml and 100 U of gamma interferon per ml for the whole incubation period, and as a negative control, macrophages were cultured without a stimulus. Total RNA prepared at each time point was reverse transcribed, cDNAs for TNF-α, GM-CSF, interleukin 1β, and macrophage inflammatory protein-1α (MIP-1α) amplified by PCR with previously published primer sequences (23, 24, 26) were normalized for HPRT expression, and the products were visualized on a 1.5% agarose gel. Transcription of mRNA for all of the cytokines under test was upregulated following challenge with acapsular *C. neoformans* compared with that of macrophages incubated for the same time period without a stimulus (Fig. 4). However, levels of cytokine induction varied depending on the exact experimental conditions. For example, our initial experiments showed that live acapsular *C. neoformans* was more efficient for induction of mRNA for TNF-α than were heat-killed organisms (data

not shown). In addition, TNF-α gene transcription was much greater in peritoneal macrophages derived from BALB/c mice than in those from CBA mice following challenge with acapsular *C. neoformans* (data not shown). mRNAs for GM-CSF and interleukin 1β were strongly induced as early as 1 h following activation with acapsular *C. neoformans*. TNF-α and MIP-1α were detectable by 4 h, and although band intensity had decreased by 24 h, indicating downregulation of transcription, the message was still detectable at 30 h by dot blot hybridization (data not shown). Together, these data demonstrate that ingestion of acapsular *C. neoformans* is a potent stimulus for expression of proinflammatory cytokines.

Detection of TNF-α protein produced by macrophages following ingestion of acapsular *C. neoformans*. To determine

FIG. 4. Induction of mRNA for cytokines following ingestion of acapsular *C. neoformans*. Macrophage monolayers were cultured without a stimulus or stimulated with 10⁸ acapsular *C. neoformans* cells for 1 h (CAP- CN), or 50 ng of lipopolysaccharide-100 U of gamma interferon (LPS/IFN γ) and then cultured for a further 1, 4, 24, or 30 h. Following reverse transcription-PCR, products for cytokine mRNA were electrophoresed on 1.5% agarose gels after normalization of the product for HPRT activity. The results shown are representative of three separate experiments.

FIG. 5. Secretion of TNF- α by macrophages in vitro following activation with acapsular *C. neoformans*. Supernatants from macrophage cultures incubated either without a stimulus (■) or with stimulation for 1 h with acapsular *C. neoformans* (▨) and then incubated for various time periods or incubated for the whole culture period with 50 ng of lipopolysaccharide-100 U of gamma interferon (▨) per ml were assayed for TNF- α protein by ELISA. Results are presented as mean units of TNF- α protein per milliliter in duplicate wells $\pm$ the standard deviation.

whether the increase in TNF- α gene transcription following macrophage challenge with acapsular *C. neoformans* resulted in secretion of TNF- α protein, we tested supernatants taken from cells in the above-described experiment prior to the preparation of RNA. TNF- α protein was detectable by ELISA in the supernatants of cells stimulated with acapsular *C. neoformans* within 4 h and reached maximum levels by 24 h (Fig. 5). Following our initial observation that TNF- α gene expression was only slightly upregulated in CBA mice compared with that in BALB/c mice, we compared the amounts of TNF- α released from these two strains following stimulation with acapsular *C. neoformans* cells (10^8 /ml). Twenty-four hours following stimulation, macrophages derived from BALB/c mice secreted significantly more TNF- α than did those from CBA mice under the same conditions (BALB/c, 95 ± 2 U/ml; CBA, 21 ± 4 U/ml; medium-only controls, <2 U/ml). This was not related to the number of acapsular organisms ingested, because after removal of unbound organisms, equal numbers of acapsular *C. neoformans* cells were ingested by both CBA and BALB/c mouse-derived macrophages (data not shown).

Ingestion of acapsular *C. neoformans* activates macrophage phagocytic activity for enhanced ingestion of opsonized encapsulated *C. neoformans*. We have previously demonstrated that TNF- α and GM-CSF act synergistically to activate macrophages for enhanced ingestion of encapsulated *C. neoformans*. Since ingestion of acapsular *C. neoformans* by macrophages in vitro induces mRNAs for both cytokines, we investigated whether this process activates macrophages for enhanced ingestion of the encapsulated yeast form. Heat-killed acapsular *C. neoformans* was used in these experiments because intracellular growth of the live organism occurs during the subsequent incubation period. Macrophage monolayers derived from CBA mice were incubated with increasing concentrations of heat-killed acapsular *C. neoformans* for 1 h at 37°C, and uningested organisms were removed by thorough washing. This typically led to the ingestion of 0.25 to 12 organisms per macrophage

FIG. 6. Enhanced ingestion of serum-opsonized encapsulated *C. neoformans* by murine macrophages following ingestion of acapsular *C. neoformans*. Macrophage monolayers were incubated for 1 h in medium alone (ratio of 0) or with acapsular *C. neoformans* at a yeast-macrophage ratio of 1:1 or 10:1. At ratios of 1:1 and 10:1, 0.25 and 3 to 6 acapsular yeast cells were ingested per macrophage, respectively. At the end of the incubation period, uningested yeast cells were washed off and the cell monolayers were incubated for a further 72 h. Control wells were incubated for the whole 72 h with either medium alone or 50 U of TNF- α and GM-CSF per ml. Macrophage monolayers were then challenged with fluorescein isothiocyanate-labelled, serum-opsonized encapsulated *C. neoformans* (10^7 organisms per ml). The mean number of ingested particles per 100 macrophages $\pm$ the standard deviation is presented.

(infection ratios of 1:1 and 100:1, respectively). Monolayers were incubated for a further 72 h, and ingestion of serum-opsonized encapsulated *C. neoformans* was assessed. Acapsular *C. neoformans* was able to activate the macrophages in a dose-dependent manner for enhanced ingestion of serum-opsonized encapsulated yeast cells at a cryptococcus-macrophage infection ratio as low as 1:1 (Fig. 6). The number of encapsulated yeast cells ingested by macrophages activated in this way was 2 to 2.5-fold greater than that of those ingested by resting macrophages. This increase in ingestion was reproducible in four separate experiments.

Ingestion of serum-opsonized encapsulated *C. neoformans* added immediately after ingestion of acapsular *C. neoformans* was not enhanced (data not shown), indicating that the increased level of ingestion was not due to a direct effect on the receptors used for ingestion. This is supported by the observation that 24 h of incubation of the cells was required, following ingestion of the acapsular organism, before increased ingestion of encapsulated *C. neoformans* could be demonstrated (data not shown). To determine whether macrophage-derived TNF- α and GM-CSF, produced following activation with acapsular *C. neoformans*, were indeed responsible for the observed enhanced ingestion of encapsulated yeast cells, neutralizing antibodies to TNF- α and GM-CSF were added to macrophage monolayers following 1 h of incubation with acapsular *C. neoformans*. Neutralizing antibodies to either TNF- α or GM-CSF were individually able to reduce the number of serum-opsonized encapsulated *C. neoformans* cells to that of resting macrophages (Fig. 7). Isotype-matched control antibodies had no effect on the levels of ingestion of encapsulated organisms. These results indicate that in CBA mice, both cytokines may act synergistically at low levels for enhanced ingestion of serum-opsonized encapsulated *C. neoformans* so

FIG. 7. Neutralizing antibodies to TNF and GM-CSF downregulate the enhanced ingestion of serum-opsonized encapsulated *C. neoformans* by macrophages activated with acapsular *C. neoformans*. Macrophage monolayers were incubated without a stimulus (a) or were activated (b) as described in the legend to Fig. 6 at a yeast-macrophage challenge ratio of 10:1, with the exception that neutralizing antibodies to either TNF (TN33.19.12; ▩), GM-CSF (MP1.22E9.11; ▨), or isotype-matched control antibodies (L2 [▩] or GL117 [▨], all added at 50 µg/ml) or no antibodies (□) were added following the removal of excess acapsular organisms. Seventy-two hours later, monolayers were challenged with serum-opsonized *C. neoformans* and ingestion was assessed as previously described. The results shown are representative of three independent experiments. Similar results were obtained at challenge ratios of 1:1 (mean numbers of acapsular *C. neoformans* organisms per 100 macrophages $\pm$ the standard deviation: medium alone, 359 $\pm$ 113; GL117, 360 $\pm$ 41; L2, 372 $\pm$ 61; TN3, 210 $\pm$ 71; MP1, 150 $\pm$ 54) and 100:1 (medium alone, 423 $\pm$ 68; GL117, 454 $\pm$ 59; L2, 430 $\pm$ 80; TN3, 243 $\pm$ 20; MP1, 252 $\pm$ 53).

that removal of one of the cytokines is sufficient to prevent the activation events required for enhanced ingestion.

DISCUSSION

In this study, we used a stable acapsular mutant of *C. neoformans* to investigate the mechanisms by which a commonly found environmental form of *C. neoformans* is ingested by murine macrophages in vitro and the consequences of this ingestion. We demonstrated that ingestion of acapsular *C. neoformans* is inhibitable by soluble and immobilized glycans and that the serum lectin MBP binds to carbohydrate residues on the surface of acapsular *C. neoformans* which are subsequently masked by synthesis of the polysaccharide capsule. Also, ingestion of this organism causes macrophage activation, resulting in cytokine production and increased phagocytosis of the encapsulated organism.

Our initial experiments examined the involvement of the mannose and β -glucan receptors in vitro with resident peritoneal macrophages which express these receptors. By using particles enriched for either glucan and mannan (zymosan particles) or β -glucan (glucan particles) as competitors, we demonstrated that ingestion of acapsular *C. neoformans* involves constitutively competent carbohydrate-binding receptors. In addition, more purified, soluble forms of both glycans (mannan and β -1 $\rightarrow$ 3 glucan [laminarin]) were also able to inhibit ingestion of the organism significantly. Although less effective than zymosan or glucan particles, cross-linking of mannan and laminarin onto a glass surface increased the efficiency of their inhibitory effect in comparison with each glycan in solution. The concentrations required to inhibit ingestion were remarkably similar for both glycans, raising the possibility

of blocking-capping of a single receptor by both glucan and mannan. However, this is not supported by the observation that increasing concentrations of glucan particles did not inhibit ingestion of zymosan. The data therefore suggest that different receptors are employed for ingestion of these two particles and implicate both the mannose and β -glucan receptors in ingestion of acapsular *C. neoformans*. Expression of the mannose receptor, found on differentiated macrophages but not circulating monocytes (31), can be modulated by cytokines and glucocorticoids (13, 33). As well as endocytosing mannosylated ligands, it has been shown to act as a phagocytic receptor for complex ligands (8) and requires both transmembrane and cytoplasmic domains for efficient phagocytosis (20). Less is known about the β -glucan receptor, although glucocorticoids and cytokines have been demonstrated to regulate its expression and function (7, 10, 17, 18). Furthermore, in other systems, both the mannose and β -glucan receptors have been shown to be constitutively active and to cooperate for ingestion of the opportunistic pathogen *P. carinii* (14). The involvement of these receptors in the binding of acapsular *C. neoformans* is consistent with its rapid ingestion by resting macrophages without a requirement for prior opsonization or activation of the cells.

It is likely that ingestion of a complex organism such as *C. neoformans* involves multiple receptor interactions, and involvement of additional receptors cannot be ruled out. Studies have shown that glycan-rich structures, including zymosan, can bind to CR3 in an opsonin-independent manner (28, 36), suggesting that binding occurs via a lectin-like binding domain distinct from that which binds iC3b. Another study has demonstrated that saccharides, including mannan, can inhibit the cocapping of CR3 with Fc γ receptor III on neutrophils (41). However, with affinity chromatography it has recently been shown that isolated CR3 molecules from activated neutrophils did not bind zymosan or β -glucan, although binding of other established CR3 ligands, such as gp63 from *Leishmania mexicana* and lipopolysaccharide from *Escherichia coli*, was demonstrated (38). The ingestion of acapsular *C. neoformans*, unlike that of serum-opsonized encapsulated *C. neoformans*, is complement receptor independent, as demonstrated with anti-CR3 monoclonal antibody 5C6, and is efficiently ingested without prior opsonization. This does not, however, rule out the binding of acapsular *C. neoformans* to CR3 at a site distinct from that recognized by 5C6 and used for binding of iC3b. Antibodies which specifically block binding to the receptors of interest, including the mannose and β -glucan receptors, will provide a more direct means of assessing the involvement of these various receptors.

We have also presented data here demonstrating binding of the collectin MBP to the acapsular, but not to the encapsulated, form of *C. neoformans*. MBP is a serum collectin thought to be involved in early defense mechanisms against bacterial and viral pathogens by acting as a primitive opsonin (35). MBP has ligand-binding specificities similar to those of the mannose receptor and has even been described as a soluble form of the receptor (8), binding mannose and *N*-acetylglucosamine. Although the exact molecular architecture of the *C. neoformans* cell wall is not clearly defined, Reiss and colleagues have demonstrated the presence of *N*-acetylglucosamine, but not mannan, in extracts of acapsular *C. neoformans* (27), whereas mannan residues have more recently been reported as a nonstructural component of the cell wall (39). Our observations here not only confirm the presence of exposed carbohydrate residues on the cell wall of the acapsular organism, which are masked by the polysaccharide capsule in the encapsulated form, but may potentially represent an additional ligand-re-

ceptor interaction between this organism and macrophages. In other experiments, we have recently demonstrated specific binding of other members of the collectin family, namely, lung surfactant proteins SP-A and SP-D, to acapsular *C. neoformans*. Like that of MBP, this binding is blocked by the polysaccharide capsule of the organism (29a). Binding of pulmonary surfactant proteins to the organism may be particularly relevant in the ingestion of this lung pathogen by alveolar macrophages at the initial site of infection. The alveolar macrophage is the key cell type involved in the initial control of cryptococcal infection in the lungs, and we are currently analyzing the receptors used by alveolar macrophages versus peritoneal macrophages for ingestion of acapsular *C. neoformans*. When bound to a ligand, collectins such as MBP interact with the C1q receptor (collectin receptor), which is expressed on a variety of cell types, including macrophages and polymorphonuclear cells (22). Although the functional significance of the binding of collectins to *C. neoformans* has yet to be elucidated, interaction of C1q with the collectin receptor induces the respiratory burst (11) and cross-linking of MBP to plastic tissue culture dishes enhances ingestion of encapsulated *C. neoformans* (21). These observations indicate that there may be a role for collectins in the ingestion and effective clearance of pathogens such as *C. neoformans*.

Ligation of macrophage cell surface receptors results in the release of soluble mediators such as TNF- α and other macrophage-activating cytokines (7, 14, 25, 32). Independent studies have demonstrated that although the mannose receptor is sufficient for ingestion of the pathogen *P. carinii*, during its interaction with macrophages, the β -glucan receptor is also engaged and a consequence of this ligation is secretion of TNF- α (14). We have shown here that ingestion of acapsular *C. neoformans* by murine macrophages *in vitro* induces a range of proinflammatory cytokines, including GM-CSF, TNF- α , interleukin 1 β , and MIP-1 α . These cytokines exert pleiotropic effects on host cells, including upregulation of adhesion molecules, recruitment of lymphocytes into sites of inflammation, and enhanced major histocompatibility complex class I expression. In particular, TNF- α acts as a cofactor in the induction of nitric oxide, which is a key mediator of macrophage microbistatic activity against *C. neoformans* (12). MIP-1 α is a member of the newly characterized chemokine family which acts as a chemoattractant on basophils and eosinophils, as well as monocytes (1). Together, these cytokines, released rapidly and at the site of infection, could initiate an influx of effector cells crucial for effective clearance of *C. neoformans* from the lungs. To understand the mechanisms of innate immunity to *C. neoformans* further, we are currently studying the cytokines released in the lungs following infection with acapsular *C. neoformans*.

The data presented here demonstrate that macrophage infection with acapsular *C. neoformans* in the absence of exogenous opsonins has functional relevance since it results in the activation of murine macrophages for enhanced ingestion of the antiphagocytic encapsulated form of the organism. This enhanced phagocytosis is dependent on the production of TNF- α and GM-CSF by macrophages, since the effect is ablated by addition of neutralizing antibodies to these cytokines. These observations are consistent with our data previously obtained with recombinant cytokines (4) and demonstrate a novel mechanism of macrophage activation leading to enhanced phagocytosis and TNF- α and GM-CSF production. The importance of these two cytokines is highlighted by our previous demonstration that neutralization *in vivo* with specific monoclonal antibodies to both TNF- α and GM-CSF converts a chronic infection with *C. neoformans* into a rapidly lethal

event, with an increase in the onset and severity of meningitis (4). These results suggest that, together with gamma interferon, TNF- α and GM-CSF are key cytokines which mediate resistance to infection with *C. neoformans* *in vivo*. In conclusion, ligation of macrophage lectin-like receptors during ingestion of acapsular *C. neoformans* and subsequent macrophage activation could be an important defense mechanism against infection with this organism prior to the expression of T-cell-derived cytokines.

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