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Differential Localization of Complement Component 3 within the Capsular Matrix of *Cryptococcus neoformans*

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The polysaccharide capsule of *Cryptococcus neoformans* is a powerful activator of the complement system. The goal of the present study was to assess serum and cellular variables that influence the sites for C3 binding within the capsular matrix. Confocal microscopy using fluorophore-labeled polyclonal anti-C3 and anticapsular monoclonal antibodies and rosetting of fluorescent microspheres coated with anti-C3 were used to identify sites of C3 binding relative to the capsular edge. The results showed that the source of serum was a major variable influencing localization of C3. C3 bound at or very near the capsular edge in the case of human serum. C3 deposition was further from the capsule edge with guinea pig and rat sera; in the case of mouse serum, there was no binding of C3 in the outer region of the capsule. Addition of human C3 to mouse serum led to deposition of the C3 at the capsular edge, indicating that distinct properties of mouse and human C3 account for the differential localization of C3. Finally, the density of the capsular matrix was an important variable in determining sites for C3 deposition. Yeast cells with a high concentration of polysaccharide near the capsule edge supported deposition of mouse C3 at or near the capsular edge, whereas cells with a low matrix density showed deposition well beneath the edge. Taken together, these results indicate that the spatial deposition of C3 within the capsular matrix is a complex process that is influenced by the serum source and the density of the capsular matrix.

Capsules composed of polysaccharides or polypeptides are essential virulence factors for many bacteria and the encapsulated yeast *Cryptococcus neoformans*. Acapsular mutants of *C. neoformans* are typically avirulent (7, 25). Although the cryptococcal capsule has a variety of biological activities, the property of the capsule that is probably most important to virulence is inhibition of phagocytosis by macrophages and neutrophils (4, 5, 20, 21). Despite the absolute requirement of the capsule for virulence of a broad spectrum of microbes, the cellular and/or molecular mechanisms by which a capsule renders a yeast or bacterium resistant to phagocytosis are poorly understood.

One mechanism that has been proposed for inhibition of phagocytosis by some microbial capsules is a blockade of the action of potentially opsonic fragments of the complement cascade that might bind to the cell, most notably fragments of C3. Encapsulation may interfere with activation of the complement system such that incubation of the microbes in serum leads to an absence of bound C3 fragments. This blockade of complement activation may occur by (i) facilitating binding of the regulatory protein factor H to C3b that has bound to the capsule, (ii) creating an environment that promotes inefficient binding of factor B to C3b on the capsule, or (iii) presenting a microbial surface that is incapable of serving as an acceptor for covalent C3 deposition (reviewed in reference 19). Alternatively, incubation of encapsulated microbes in serum may lead to binding of potentially opsonic C3 fragments to the cell, but the fragments may bind to the cell wall or other sites within the capsule but not at the surface of the capsule where these opsonic proteins could interact with phagocyte complement receptors. Examples of organisms with deposition of C3 fragments beneath the capsular surface include *Staphylococcus aureus* (10, 34) and *Streptococcus pneumoniae* (2).

The encapsulated yeast *C. neoformans* is ideally suited for study of capsular localization of C3 fragments because the capsule is quite large, enabling an evaluation of C3 binding sites by use of optical microscopic techniques such as confocal microscopy. The primary constituent of the cryptococcal capsule is glucuronoxylomannan (GXM), a high-molecular-weight polysaccharide with an α-1,3-linked mannose backbone that is O acetylated and decorated with single side chains of xylose and glucuronic acid (9). Importantly, several GXM monoclonal antibodies (MAbs) that vary in epitope specificity and allow for localization of capsular boundaries relative to sites of C3 deposition have been produced (14).

A seminal report by Zaragoza et al. found that incubation of small-capsule cryptococci in normal mouse serum led to deposition of C3 fragments at sites throughout the capsule that extended to the capsular edge. In contrast, incubation of large-capsule cryptococci in serum deposited C3 within the capsule but not at the capsular edge (36). We recently examined the molecular architecture of the cryptococcal capsule and found that the capsule is a matrix of variable porosity and concentration (14). The GXM concentration is greatest and the capsular porosity is most restricted in the region near the cell wall. However, there is a gradual decrease in GXM concentration and an increase in porosity as the capsular edge is approached. In addition, matrix density is influenced by yeast growth conditions. Matrix density is greatest in yeast cells harvested from infected tissue and is markedly less if cells are grown in vitro under capsule induction conditions, despite the fact that capsule widths are similar for the two cell types.

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The goal of our study was to evaluate the factors that determine the sites for deposition of C3 onto and into the capsules of cryptococci incubated in serum. Our results showed that the position of C3 binding relative to the capsule edge is determined by the species of the serum source used for the study and the matrix density of GXM within the capsule.

MATERIALS AND METHODS

Reagents, sera, and MAbs. Fluorescein-conjugated goat immunoglobulin G (IgG) fractions of antibodies to rat, guinea pig, and mouse C3 were purchased from ICN/Cappel (Irvine, Calif.). Fluorescein-conjugated goat gamma globulin fraction of anti-human C3 was purchased from Kent Laboratories (Bellingham, Wash.). Evaluation of the binding activity of anti-human or -mouse C3 sera by probing of a Western blot of human or mouse C3 showed that the anti-human and anti-mouse C3 both bound primarily to the C3 alpha chain, with much less binding to the beta chain. MAb 3C2 is an IgG1 antibody that is reactive with GXM, the major capsular polysaccharide of *C. neoformans* (27). Fab fragments of MAb 3C2 were prepared by ficin digestion using the Immunopure IgG1 Fab and $F(ab')_2$ preparation kit (Pierce, Rockford, Ill.) according to the manufacturer's directions and were separated from intact IgG and Fc fragments by protein A affinity chromatography (Pierce) and molecular sieve chromatography. MAb 3C2 and its Fab fragments were labeled with Alexa Fluor 555 (Molecular Probes, Eugene, Oreg.) according to the manufacturer's directions. The goat IgG fraction of antibodies to mouse (ICN/Cappel) or human (ICN/Cappel) C3 was coupled to fluorescent microspheres (yellow-green, 0.2 μm; Molecular Probes) according to the manufacturer's directions.

Human factor B and factor H were purchased from Calbiochem (La Jolla, Calif.). Human C3 was either purchased (Calbiochem) for use in the experiments shown in Fig. 6 or isolated from a pool of human plasma (Reno Blood Services, Reno, NV) for the experiment shown in Fig. 1E. Mouse C3 was isolated from mouse plasma (Harlan Bioproducts, Indianapolis, Ind.). Human and mouse C3 were isolated from plasma by differential precipitation with polyethylene glycol, plasminogen depletion by affinity chromatography with lysine-Sepharose, ion-exchange chromatography on DEAE-Sepharose, and molecular sieve chromatography on Superdex 200, followed by depletion of IgG with protein A (1, 16, 33). Purified human or mouse C3 was labeled with Alexa Fluor 488 (Molecular Probes) according to the manufacturer's directions. Most experiments that used human serum utilized a pool of sera obtained from blood of at least 10 healthy adult donors. One experiment used sera collected from individual donors. Collection of blood from adult donors was approved by the University of Nevada, Reno, Biomedical Institutional Review Board. Mouse serum was a pool of sera obtained from blood collected from BALB/c mice. Rat and guinea pig sera were purchased from Harlan Bioproducts.

***C. neoformans* cells.** *C. neoformans* serotype A strain CN6 (ATCC 62066) was used throughout. The cells were induced for production of large capsules by incubation for 4 days at 37°C with 5% CO_2 in synthetic medium (8) supplemented with 24 mM sodium bicarbonate and 25 mM HEPES (15). Yeast cells were also grown in the absence of capsule induction conditions by culture at 30°C in synthetic medium without sodium bicarbonate, HEPES, and CO_2. All yeast cells were killed by treatment overnight with 1% formaldehyde, washed with sterile phosphate-buffered saline (PBS), and stored at 4°C. The capsule width for cells grown in the absence of capsule induction conditions was 1.1 ± 0.2 μm (mean ± standard deviation [SD]); the capsule width for cells grown under induction conditions was 3.6 ± 0.8 μm (14). One experiment examined yeast cells that were harvested from the brains of infected mice. In this instance, BALB/c mice were challenged via the intravenous route with 1.5×10^6 cells of strain CN6. Once hydrocephalus was observed (7 to 10 days after challenge), the mice were sacrificed, brain tissue was collected, and tissue-derived yeast cells were isolated as described previously (14). The capsule width of the cells that were harvested from tissue was 4.1 ± 0.6 μm (14).

Treatment of yeast cells with serum. Unless otherwise indicated, *C. neoformans* cells (1.2×10^5) were incubated for 32 min at 37°C with 40% serum in PBS in a total reaction volume of 250 μl. Previous studies have shown that deposition of C3 onto encapsulated cryptococci incubated in 40% normal human serum is largely complete after 15 to 20 min of incubation (23). In some cases, incubation with serum was done in the presence of 5 mM EGTA and 5 mM $MgCl_2$ or with higher or lower concentrations of serum. Treatment of serum with Mg-EGTA was used to chelate the Ca^{2+} that is needed for classical pathway activity while leaving sufficient amounts of Mg^{2+} required for the alternative pathway (3, 13, 30). Another experiment was done using mouse serum that had been adsorbed by incubation of the serum for 60 min at 0°C with 10^8 yeast cells (grown in vitro under capsule induction conditions). Previous studies have shown that adsorption of serum with yeast cells in this manner will remove antibodies reactive with the yeast surface but has no effect on the ability of the serum to support activation of C3 and binding to encapsulated cryptococci and has no effect on the ability of the absorbed serum to support classical complement activation via an irrelevant system, i.e., classical pathway 50% hemolytic complement (22, 35). The effects of adsorption on IgG and IgM antibodies to GXM were determined by enzyme-linked immunosorbent assay (ELISA) using GXM in the solid phase (12). Finally, one experiment used serum that had been heat inactivated by incubation for 30 min at 56°C. In all cases, the reaction was stopped by the addition of 1 ml of ice-cold 10 mM EDTA in PBS, and the cells were washed three times with PBS.

Evaluation of C3 binding in the capsular matrix. Four methods were used to determine the position of C3 in the capsular matrix. In the first approach, serum-treated yeast cells (1.2×10^5) were incubated with fluorescein isothiocyanate (FITC)-conjugated antibody to human, rat, guinea pig, or mouse C3 (1/50 in PBS with 1% bovine serum albumin), washed, and incubated with Alexa 555-conjugated MAb 3C2 or its Fab fragments (50 μg/ml). Binding was evaluated by confocal microscopy with a Nikon confocal microscope C1 unit that was fitted to a Nikon Eclipse E800 microscope equipped with differential interference contrast (DIC) optics (100× objective). Confocal images were processed with Nikon EZ-C1 software, version 1.70. Merging and cropping of images were done with SimplePCI software (Compix, Cranberry Township, Pa.). Unless otherwise noted, all images were acquired using identical conditions for illumination and image acquisition, e.g., instrument gain, scan time, and averaging number. For preparation of figures, at least five images were captured for each treatment condition and a single representative image was chosen. Quantitative determinations of the outer boundaries of the capsule or sites of C3 binding were done by modifications of previously described procedures (14, 36). Briefly, a line of fluorescence intensity (pixel intensity) was acquired across the equator of a cell for binding of antibody to C3 (green) or antibody to GXM (red). Pixel intensity and position were imported into SigmaPlot (SPSS Inc., Chicago, Ill.) for further analysis. A quantitative determination of the distance between the capsule edge and the site of C3 localization was done by measuring the distance between the peaks of fluorescein (due to complement) and Alexa 555 (outer edge of the capsule) as described previously (36). For quantitative determinations, at least 10 cells were examined, and data are reported as the mean ± SD.

In the second method for identification of the proximity of C3 deposition to the capsule edge, serum-treated cells (1.2×10^5) were incubated with fluorescent microspheres coated with anti-human or -mouse C3 IgG. Yeast cells were separated from free microspheres by centrifugation through 20% sucrose. Rosetting of the microspheres was assessed by confocal microscopy.

In the third approach, trace amounts (7.5 μg/ml serum) of Alexa 488-labeled human or mouse C3 were added back to human or mouse serum, respectively. The optimal amount of fluorophore-labeled C3 needed for identification of the site of C3 binding was based on a preliminary dose-response titration. Encapsulated cryptococci were incubated with the trace-labeled serum as described above and washed, and the position of the cryptococcal capsule was determined by use of Alexa 555-labeled MAb 3C2.

Finally, previous studies found that incubation of encapsulated cryptococci in serum leads to binding of C3 within the capsular matrix to such an extent that the additional protein causes a change in the refractive index that can be seen by DIC microscopy (14). As a consequence, the capsular quellung-type reaction was used as an additional measure of complement activation. It should be noted that treatment of yeast cells with capsular MAb also produces a quellung reaction (27), so evaluation of sites of C3 binding by DIC can be done with accuracy only with yeast cells that have not been treated with antibody. As a consequence, the capsular boundary was identified by India ink negative staining.

Statistical analysis. Statistical analysis involving multiple treatment groups was done by one-way analysis of variance with all post hoc pairwise multiple comparisons by the Holm-Sidak method. In two instances, the data failed a test for equality of variance. In this case, the analysis was done by the Mann-Whitney rank sum test. Statistical analysis was done with the assistance of SIGMASTAT 3.0 (SPSS Inc.).

RESULTS

Dependence of C3 localization on the serum donor species. An initial experiment evaluated the impact of the serum donor species on the sites for deposition of C3 within the cryptococcal capsule. Cryptococci grown in vitro under capsule induction

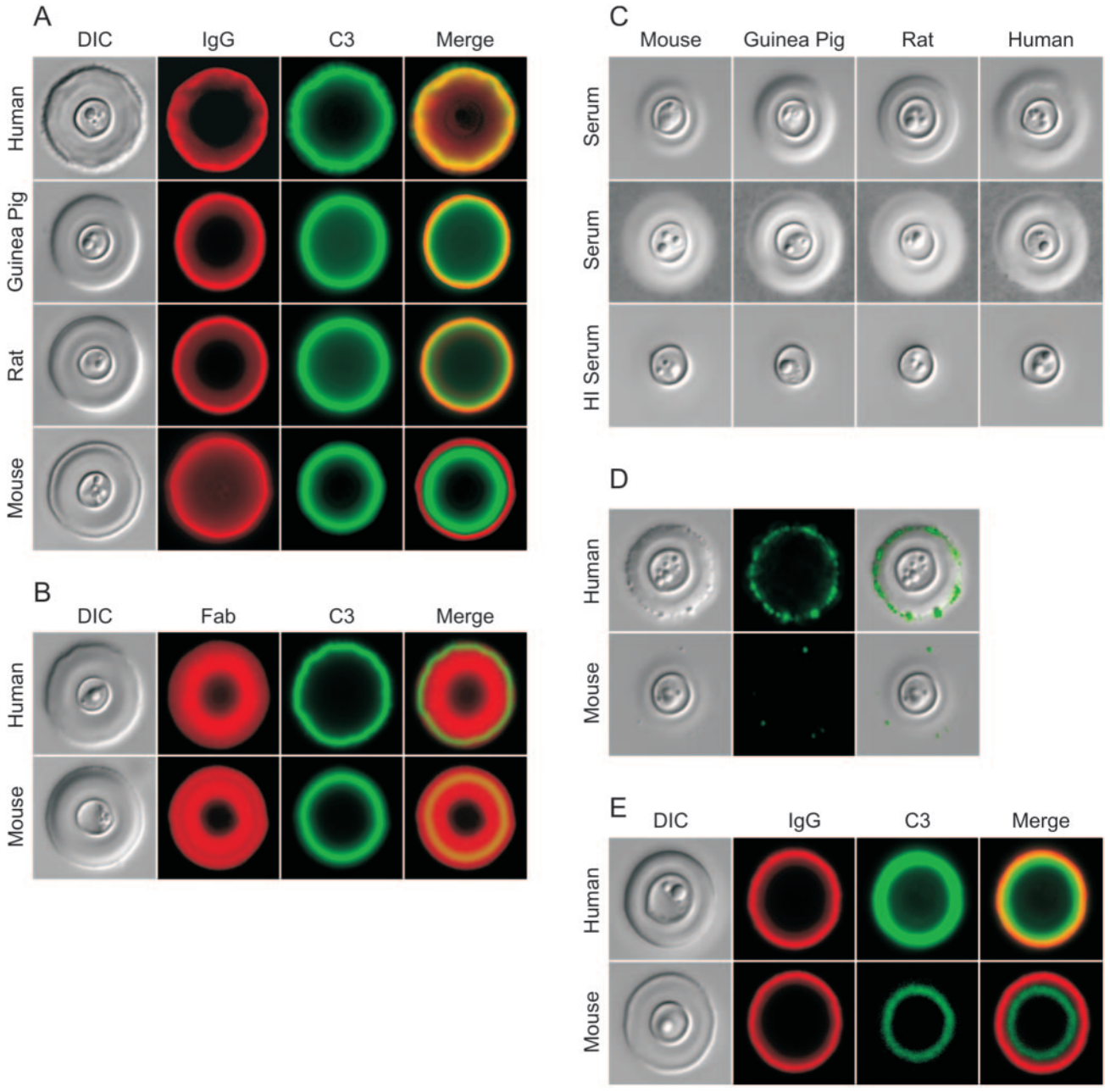

FIG. 1. Effect of serum source on sites for C3 deposition relative to the capsular edge of yeast cells grown in vitro under capsule induction conditions. Panel A, binding following incubation of cryptococci for 32 min in 40% human, guinea pig, rat, or mouse serum. The capsular edge was identified with Alexa 555-labeled MAb 3C2; binding of C3 was identified with FITC-labeled anti-human, -guinea pig, -rat, or -mouse C3. Panel B, use of Alexa 555-labeled Fab fragments of MAb 3C2 to identify capsule boundaries; C3 deposition following incubation with human or mouse serum was identified with FITC-labeled anti-human or -mouse C3. Panel C, effect of C3 deposition on capsule architecture as shown by DIC microscopy. Cryptococci were incubated with untreated or heat-inactivated (HI) mouse, guinea pig, rat, or human serum and examined by DIC microscopy alone (top and bottom rows) or were negatively stained with India ink and examined by DIC microscopy (middle row). Panel D, proximity of C3 to the capsular edge as shown by incubation with polystyrene beads coated with antibody to C3. Cryptococci were incubated with human or mouse serum, washed, and incubated with fluorescent beads coated with anti-human or -mouse C3. Panel E, proximity of C3 to the capsular edge as shown by incubation of cryptococci in human or mouse serum containing Alexa 488-labeled C3 (7.5 μg/ml serum) that had been isolated from human or mouse serum, respectively. The cells were washed, and the capsular edge was identified with Alexa 555-labeled MAb 3C2.

conditions were incubated with human, guinea pig, rat, or mouse serum. The capsular edge was identified with Alexa 555-labeled MAb 3C2; sites of C3 deposition were identified with FITC-labeled polyclonal anti-C3 for each species. The results (Fig. 1A) showed that C3 bound at or very near the capsular edge in the case of human serum (mean distance of C3 from capsule edge ± SD, 0.29 ± 0.14 μm). The sites of C3 localization were progressively further from the capsule edge

with rat (0.48 ± 0.06 μm; $P = 0.007$ versus human serum), guinea pig (0.56 ± 0.08 μm; $P < 0.001$ versus human serum), and mouse (1.1 ± 0.16 μm; $P < 0.001$ versus human, guinea pig, or rat serum) sera. Similar results for human and mouse sera were produced when Fab fragments of GXM MAb 3C2 were used to identify the capsular edge. The use of Fab fragments has the advantage of not dramatically perturbing the capsular architecture, and Fab fragments of MAb 3C2 penetrate to the capsule interior and allow for labeling throughout the capsule (14). The results (Fig. 1B) showed that C3 was bound at the capsule edge of cryptococci incubated in human serum but was localized well beneath the capsule edge of cryptococci incubated in mouse serum (Fig. 1B).

The use of antibodies for identification of the capsule edge or sites of C3 binding has the potential to cross-link the capsule and might perturb capsule architecture (14). As a consequence, three additional approaches that did not require fluorescently labeled antibodies were used to assess the position of C3 binding within the capsule. The first approach is based on our observation that incubation of encapsulated cryptococci in serum produces a massive deposition of C3 in the capsule that can be visualized by DIC microscopy (14). For this experiment, cryptococci grown under capsule induction conditions were incubated with mouse, guinea pig, rat, or human serum. The results confirmed the observation that cryptococci incubated in human serum accumulate C3 near the capsule edge, whereas cryptococci incubated in mouse serum accumulate C3 well beneath the capsule edge (Fig. 1C). Incubation of cryptococci in heat-inactivated serum produced no visible capsule reaction, demonstrating that the capsule reaction was dependent on heat-labile proteins.

In a third approach, cryptococci were incubated with mouse or human serum, washed, and incubated with fluorescent microspheres coated with anti-mouse or -human C3. The results showed a striking rosetting of the microspheres around cryptococci that were incubated with human serum and little or no rosetting around cryptococci incubated in mouse serum (Fig. 1D).

Finally, since the polyclonal anti-human and -mouse C3 used for identification of the position of C3 within the capsular matrix might have different epitope specificities, Alexa 488-labeled, purified human and mouse C3 were added in trace amounts to human and mouse sera. Cryptococci were incubated with the trace-labeled sera, and the position of the capsular edge was determined by use of Alexa 555-MAb 3C2. The results (Fig. 1E) showed human C3 to be located at or near the capsular edge and to extend into the capsular matrix, whereas the outer edge of mouse C3 was located well beneath the capsular surface.

Several additional experiments were done to exclude alternative explanations for the differential positions of C3 deposition following incubation in human or mouse serum. First, an experiment evaluated the effect of serum concentration on the position of C3 binding. Cryptococci were incubated in 10, 20, 40, or 80% serum, and the positions of human and mouse C3 were determined as described for Fig. 1A. The results showed little or no C3 deposition in the presence of 10% human or mouse serum (not shown). Twenty percent human serum failed to support deposition of C3. The positions of human C3 relative to the capsule edge were similar in the cases of 40 and 80% serum (Fig. 2A) ($P = 0.46$). The positions of mouse C3 relative to the capsule edge were similar in the cases of 20, 40, and 80% serum (Fig. 2A) ($P = 0.64$).

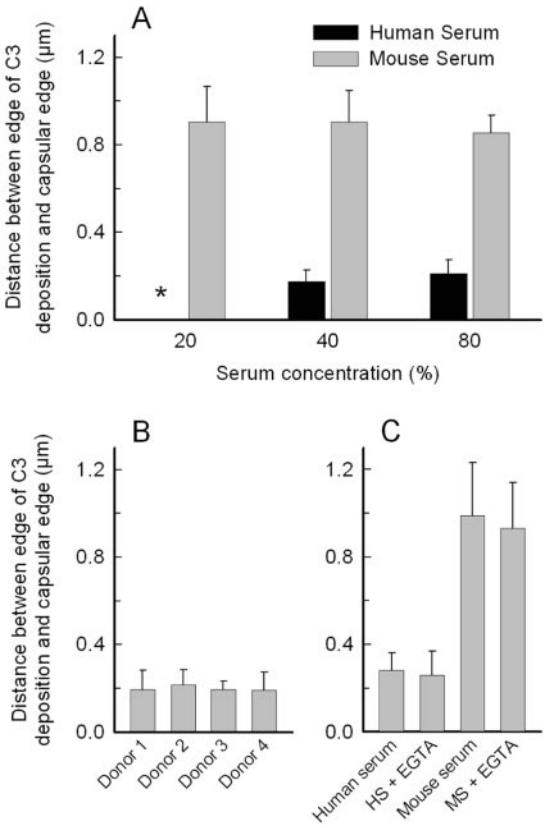

FIG. 2. Effect of serum variables on sites for deposition of C3 within the capsule matrix. In all instances, the capsular edge was identified with Alexa 555-labeled MAb 3C2; sites of C3 binding were assessed with FITC-labeled anti-human or -mouse C3. The relative positions of the two labels were determined by inspection of 10 confocal images of cells for each treatment condition. Results are reported as the mean ± SD of the distance between the capsule edge and the outer edge of C3 deposition. Panel A, encapsulated cryptococci were incubated for 32 min with 20%, 40%, or 80% human or mouse serum. *, 20% human serum failed to support deposition of C3 into the capsule. Panel B, encapsulated cryptococci were incubated for 32 min with 40% serum from two individual donors with high anti-GXM IgG titers (donors 1 and 2; ELISA titers, 1/825 and 1/970, respectively) or low IgG titers (donors 3 and 4; ELISA titers, 1/150 and 1/160, respectively). Panel C, encapsulated cryptococci were incubated for 32 min with 40% human serum (HS) or mouse serum (MS) in the presence or absence of Mg-EGTA.

The studies shown in Fig. 1 used pooled human sera and did not reflect potential donor variability. Moreover, human sera contain variable amounts of IgG antibody to GXM (18). In an effort to evaluate the impact of donor and/or IgG antibody titer on variability of the site of C3 deposition, we examined the position of C3 deposition relative to the capsule edge following incubation in sera from two donors having relatively high levels of anti-GXM IgG (ELISA titers of >1/800) and two donors having relatively low GXM IgG titers (ELISA titers of <1/200). The results (Fig. 2B) showed no difference in the effects of sera from the four donors ($P = 0.88$).

Finally, we examined the effect of chelation of the sera with

EGTA to block the action of the classical pathway on the position of C3 deposition following incubation in human or mouse serum. The results (Fig. 2C) showed no difference between the positions following incubation in untreated serum and serum treated with Mg-EGTA ($P = 0.56$).

Species variability in the kinetics of C3 deposition in the cryptococcal capsule. The species variability in localization of C3 in the capsule suggested that sera from different species may be fundamentally different in their ability to support complement activation by the cryptococcal capsule. We previously demonstrated that activation of the human complement system by *C. neoformans* and deposition of C3 in the cryptococcal capsule are mediated solely by the alternative pathway (23, 24). Such activation is delayed and is characterized by the asynchronous formation of initiation sites of bound C3 that expand to fill the capsule. As a consequence, we evaluated the sites of C3 binding in the capsule as a function of incubation time with human, guinea pig, rat, or mouse serum. Cryptococci grown under capsule induction conditions were incubated for 1, 2, 4, 8, 16, and 32 min with 40% serum, and the sites of C3 binding were identified by use of FITC-labeled anti-C3 and confocal microscopy. Incubation of cryptococci in human serum led to the delayed, asynchronous accumulation of C3 that we have observed previously (Fig. 3A). Guinea pig serum produced a similar pattern; however, accumulation was more rapid than was observed with human serum. Accumulation of C3 from rat serum was also focal at early incubation times (1 and 2 min), but the capsule was uniformly filled with C3 after 4 min. Mouse serum showed a markedly different pattern. C3 binding appeared as a dense speckled pattern after 1 min of incubation and increased in intensity after 2 min of incubation; the process was largely complete after 4 min of incubation.

The rapid accumulation of C3 that occurs on cryptococci incubated with mouse serum suggested activation of the classical complement pathway (23, 35, 37). Consequently, an experiment was done to assess the contribution of the classical pathway to activation and binding of C3 from human and mouse sera. The sera were absorbed with whole cryptococci in an effort to remove any potential initiators or treated with Mg-EGTA to chelate Ca^{2+} and block the classical pathway such that any observed deposition of C3 would be due to the action of the alternative pathway (13, 30). The results showed that serum absorption had no effect on the rate of accumulation of C3 from human serum (Fig. 3B) relative to what is observed with untreated serum (Fig. 3A). Treatment of human serum with Mg-EGTA produced a slight acceleration in accumulation of C3, but the pattern of accumulation remained asynchronous and focal. Absorption of mouse serum with encapsulated cryptococci also had no effect on the pattern of accumulation of mouse C3 on the yeast. However, treatment of mouse serum with Mg-EGTA changed the pattern of C3 binding to the delayed, focal, asynchronous pattern observed with human serum. Assays of the absorbed and unabsorbed mouse serum by ELISA for anti-GXM IgG or IgM were negative (not shown).

Mechanism for differential localization of human and mouse C3. Early deposition of C3 on yeast cells incubated in mouse serum and the apparent role of the classical pathway in this process suggested that mouse serum might contain an initiator that is not found in human serum. Conversely, the

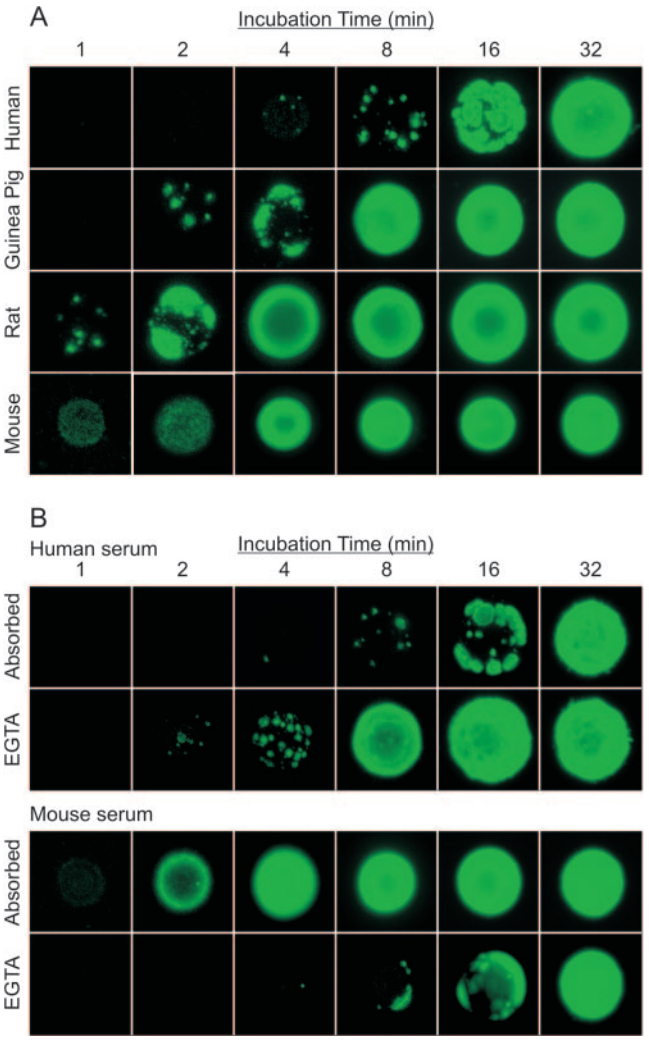

FIG. 3. Influence of serum source on sites and kinetics of C3 accumulation on cryptococci grown in vitro under capsule induction conditions. Panel A, pattern of C3 deposition onto cryptococci incubated in human, guinea pig, rat, or mouse serum. Cryptococci were incubated with 40% serum for various times, and the sites of C3 deposition were determined by immunofluorescence microscopy. Panel B, effect of serum absorption or treatment with Mg-EGTA on the kinetics and sites of deposition of C3 on cryptococci incubated in human or mouse serum.

inability of mouse serum to support C3 deposition near the capsule edge raised the possibility that mouse serum might contain an inhibitor that suppresses C3 binding at the capsular periphery. As a consequence, an experiment was done in which human and mouse sera were mixed and the ability of the mixed sera to support activation of human and mouse C3 and binding to encapsulated cryptococci was assessed.

An initial experiment evaluated the specificity of the human and mouse C3 antisera. Encapsulated cryptococci were incubated for 1 to 16 min with human serum, and binding of human C3 was assessed by use of FITC-labeled anti-mouse C3. The results (Fig. 4A) showed no binding of the anti-mouse C3 to cryptococci incubated with human serum. In contrast, cryptococci incubated with mouse serum showed staining when in-

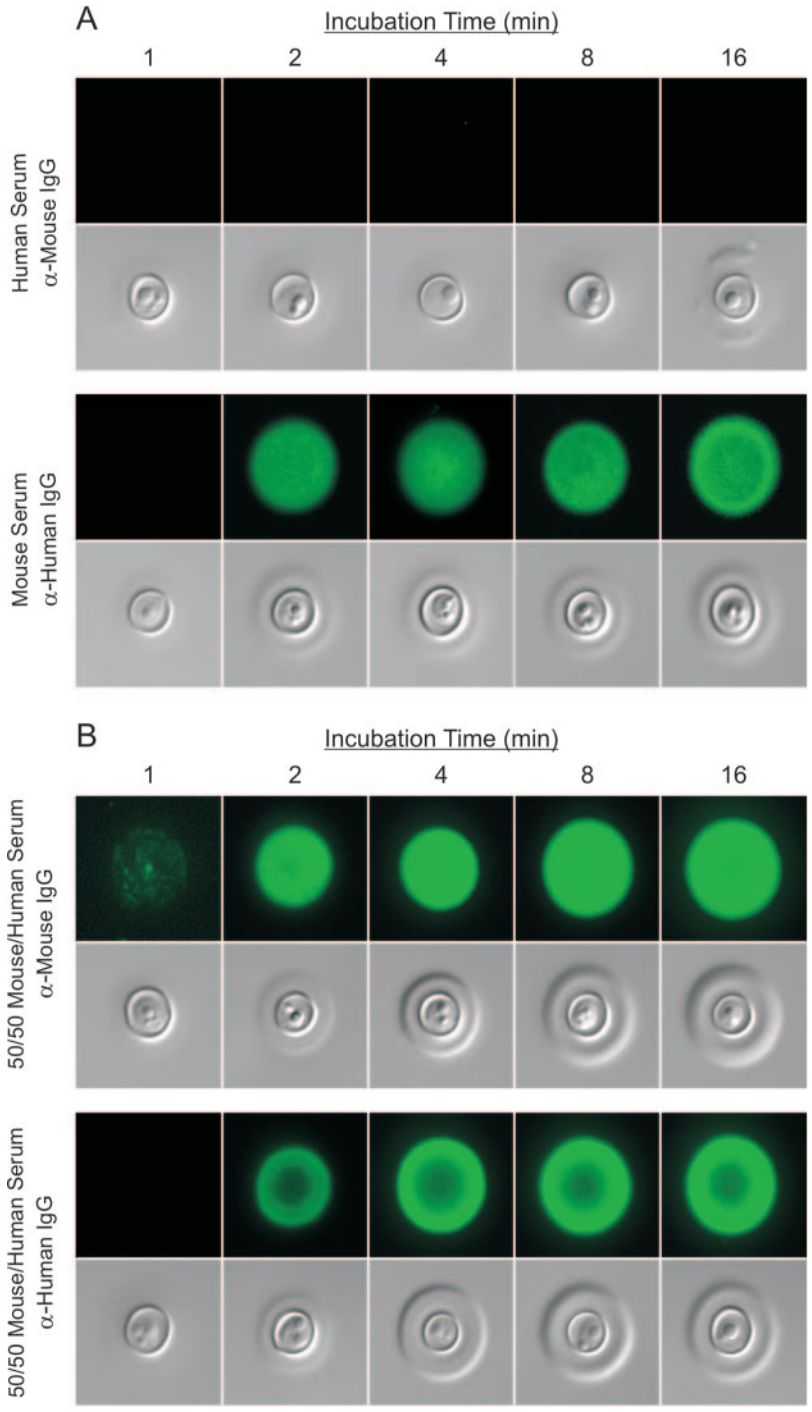

FIG. 4. Ability of mouse serum to accelerate the kinetics of deposition of human C3 into the capsule. Panel A, specificity of anti-human and anti-mouse C3. Upper panels, cryptococci were incubated for various times with 40% human serum, and binding was assessed with FITC-labeled anti-mouse C3. Lower panels, cryptococci were incubated with 40% mouse serum, and binding was assessed with FITC-labeled anti-human C3. Panel B, encapsulated cryptococci were incubated for various times with 40% of a 50/50 mixture of human and mouse sera. Binding of human and mouse C3 was determined with FITC-labeled anti-human or -mouse C3, respectively.

cubated with FITC-labeled anti-human C3; however, the intensity of staining was less than was observed when FITC-labeled anti-human C3 was used to stain cryptococci that were incubated with human serum (Fig. 3). These results showed that anti-mouse C3 is not reactive with human C3; however, anti-human C3 shows limited, but demonstrable (by confocal microscopy), cross-reactivity with mouse C3.

Having determined the specificity of the anti-human and -mouse C3 antisera used for these studies, we examined the kinetics and patterns of binding of human and mouse C3 following

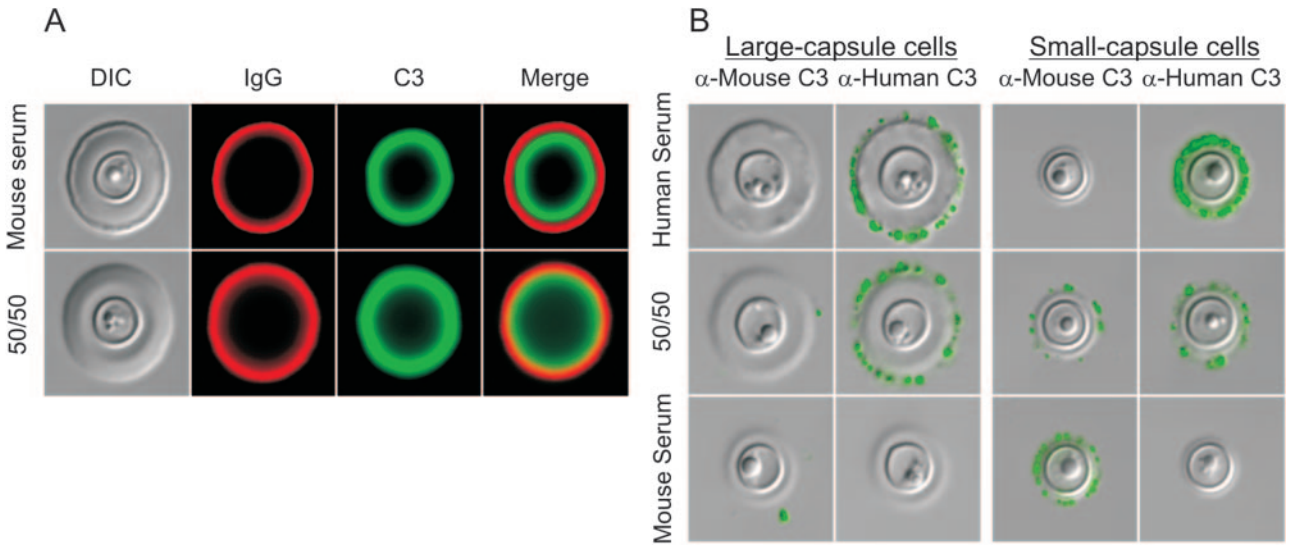

FIG. 5. Inability of human serum to alter the position of binding of mouse C3 relative to the capsule edge and vice versa. Panel A, cryptococci grown in vitro under capsule induction conditions were incubated for 32 min with 40% mouse serum or 40% of a 50/50 mixture of human and mouse sera. The capsular edge was identified with Alexa 555-labeled MAb 3C2; sites of C3 binding were assessed with FITC-labeled anti-human or -mouse C3. Panel B, surface location of human or mouse C3 following incubation of cryptococci in 40% of a 50/50 mixture of human and mouse sera. The proximity of human or mouse C3 to the capsular surface was determined by incubation with polystyrene beads coated with anti-human or -mouse C3. Left panels, cryptococci grown in vitro under capsule induction conditions. Right panels, cryptococci grown in the absence of capsule induction conditions.

incubation in a 50/50 mixture of human and mouse sera. The results shown in Fig. 4B showed that mixing of human and mouse sera converted the kinetics for deposition of human C3 to the kinetics observed with mouse serum. The observed fluorescence was more intense than was found with binding of the anti-human C3 to mouse C3 (Fig. 4A), indicating that the apparent rapid, diffuse binding of human C3 to the capsule was due to staining for human C3 rather than to a cross-reaction with mouse C3. In contrast, the kinetics for deposition of murine C3 remained rapid and synchronous, indicating that the presence of human serum did not influence the kinetics for deposition of mouse C3.

A second experiment was done to determine the extent to which mixing of human and mouse sera might change the position for deposition of mouse C3 within the capsular matrix. The results (Fig. 5A) showed the mouse C3 to remain at sites beneath the capsular surface with the mouse-human serum mixture; however, there was some broadening or diffusion of the zone of C3 binding toward the capsular edge.

The effect of serum mixing on the surface localization of C3 was examined further by evaluation of rosetting of microspheres coated with anti-human or -mouse C3. The results (Fig. 5B) showed rosetting of microspheres coated with anti-human C3 around cells incubated with human serum or with a mixture of human and mouse sera but not with cells incubated with mouse serum. In contrast, beads coated with anti-mouse C3 failed to produce rosettes with cells incubated with human or mouse serum or a mixture of the two. Taken together, the results in Fig. 5 indicate that mixing of the sera did not appreciably alter the surface or subsurface location of C3 from human or mouse serum, respectively.

Previous studies by Zaragoza et al. showed that the position of C3 following incubation in mouse serum is influenced by capsule size; yeast cells with small capsules localized C3 at the capsule surface, whereas yeast cells with large capsules localized C3 beneath the capsule surface (36). As a consequence, we evaluated rosette formation following incubation of small-capsule cells (yeast cells grown in the absence of capsule induction conditions) with human or mouse serum. The results (Fig. 5B) showed rosette formation by beads coated with anti-mouse C3 with cells incubated with mouse serum or a mixture of human and mouse sera but not with cells treated with human serum. In contrast, beads coated with anti-human C3 produced rosettes with yeast cells treated with human serum or the mixture but not with cells treated with mouse serum. These results demonstrate the specificity of the assay for human or mouse serum; the limited cross-reactivity between anti-human C3 and anti-mouse C3 observed by immunofluorescence (Fig. 4A) is not reflected in the rosetting assay. In addition, these results verify by an independent approach the conclusions of Zaragoza et al. and provide an important positive control for our study in which the ability of beads coated with anti-mouse C3 to produce rosettes is shown.

One explanation for the differential localization of human and mouse C3 is the possibility that one or more of the murine complement proteins might be fundamentally different than its human counterpart. C3 shows considerable specificity in its binding to potential ligands (31). Factors B and H can also influence species specificity in activation of the alternative pathway (17). To address this possibility, purified human C3, factor B, or factor H was individually added to mouse serum, and the sites of C3 deposition were determined following incubation of encapsulated cryptococci in this chimeric mixture. The results (Fig. 6A and B) showed that addition of human C3 to mouse serum led to binding of the human C3 at or near the capsular edge in a manner similar to that of cryptococci incu-

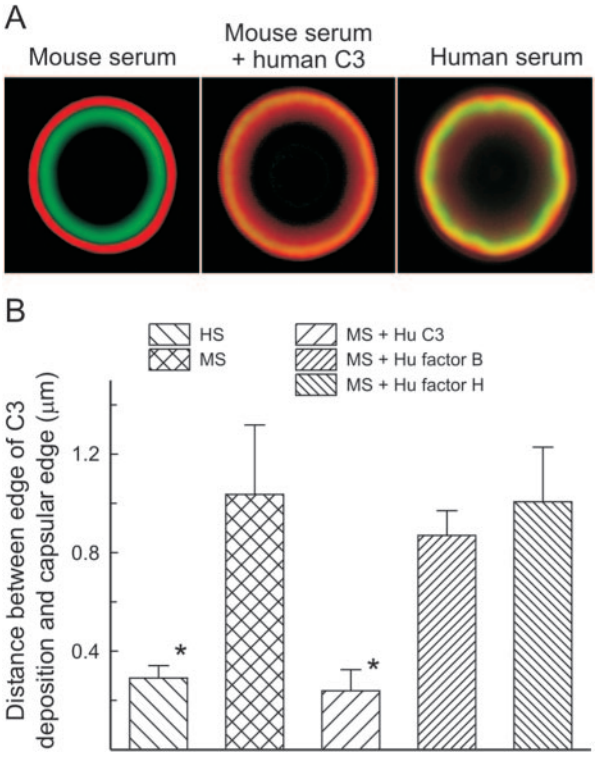

FIG. 6. Addition of human C3 to mouse serum leads to deposition of human C3 near the capsular edge. Panel A, left, position of mouse C3 relative to the capsule edge following incubation for 32 min in 40% mouse serum. Panel A, right, position of human C3 following incubation in human serum. Panel A, center, position of human C3 following incubation in mouse serum supplemented with human C3 (1,200 μg/ml). The capsular edge was identified with Alexa 555-labeled MAb 3C2; sites of C3 binding were assessed with FITC-labeled anti-human or -mouse C3. Panel B, analysis of the distance between the capsule edge and the outer edge for deposition of mouse C3 following incubation for 32 min with 40% mouse serum (MS), mouse serum plus human (Hu) factor B (200 μg/ml), or mouse serum plus human factor H (400 μg/ml) or of the distance between the capsule edge and the outer edge for deposition of human C3 following incubation with 40% human serum (HS) or mouse serum plus human C3 (1,200 μg/ml). Values are means ± SDs; $n = 10$ cells/treatment; *, $P < 0.001$ versus cells incubated with mouse serum.

bated in human serum alone. It should be noted that fluorescence following staining of the bound human C3 from the chimeric mixture was clearly evident but of a lesser intensity than was found by use of human serum alone, possibly reflecting a reduced activity of the commercially obtained C3 preparation. In contrast, addition of human factor B or factor H had no influence on the position of murine C3 in the capsule (Fig. 5B).

Influence of *C. neoformans* growth conditions on sites of C3 deposition. As noted above, Zaragoza et al. found that incubation of small-capsule cryptococci in mouse serum led to deposition of C3 at or near the capsular edge (36). The molecular basis for this differential localization is not known. A notable difference between cryptococci grown in vitro for production of small or large capsules is the concentration of GXM within the capsular matrix; GXM achieves much higher concentrations in the capsules of cells grown for production of small capsules than it does in capsules of cells grown under capsule induction conditions (14). Cryptococci harvested from infected tissue share selected properties with cryptococci grown in vitro for production of either large or small capsules; the tissue-derived cells have large capsules that are similar in size to the capsules of yeast cells grown under capsule induction conditions; however, the concentration of GXM at the periphery of tissue-derived cells is much higher than that in the capsules of cells grown under capsule induction conditions. Thus, it is possible that the differences between cells grown in vitro for production of large or small capsules are due to differences in GXM concentration rather than differences in capsule width. To address this question, cryptococci (i) grown in vitro in the absence of capsule induction conditions (small), (ii) grown in vitro under capsule induction conditions (large), or (iii) derived from infected brain tissue (tissue) were incubated for 32 min in 40% mouse or human serum, and the positions of C3 binding were determined. The results (Fig. 7) showed that the position of human C3 with small-capsule cells was slightly, but significantly ($P < 0.05$), closer to the capsule edge than that with large-capsule cells or tissue-derived cells, but there was no difference in the position of human C3 in large-capsule versus tissue-derived cells ($P > 0.05$). In contrast, the position of mouse C3 in the capsules of tissue-derived cryptococci was significantly ($P < 0.001$) closer to the capsule edge than the sites for binding of C3 within the capsules of cryptococci grown in vitro under capsule induction conditions (Fig. 7C). The proximity of mouse C3 to the capsule edge of tissue-derived cells was apparent both by confocal microscopy (Fig. 7B) and by rosetting with microspheres coated with anti-mouse C3 (Fig. 7D).

DISCUSSION

Previous studies by Zaragoza et al. found that incubation of small-capsule cryptococci in mouse serum leads to deposition of C3 at or very near the capsular surface (36). In contrast, incubation of large-capsule cryptococci in mouse serum leads to deposition of C3 within the capsule but demonstrably beneath the capsular surface. In the present study, we confirmed this observation regarding the behavior of mouse serum and further found that the position of bound C3 within the capsular matrix is dramatically influenced by the species of the serum source. Incubation of large-capsule cryptococci in human serum leads to deposition of C3 at or very near the capsular edge; incubation in mouse serum leads to C3 binding well beneath the capsule perimeter. Incubation in rat or guinea pig serum produces a result that is intermediate between the results found with human and mouse sera. This differential localization was demonstrated by confocal microscopy that used antibodies to C3 and the GXM capsule to identify the sites of C3 binding and the capsule edge, respectively; by use of fluorophore-labeled C3 to directly identify the sites of C3 binding; and by a rosetting assay in which fluorescent beads coated with antibodies to human or mouse C3 were used to assess the proximity of bound C3 to the capsular surface. To our knowledge, this is the first use of fluorophore-labeled C3 to follow sites of C3 binding. Taken together, the primary findings of this report were demonstrated by three fundamentally different experimental approaches.

Activation of C3 and binding to cryptococci incubated in

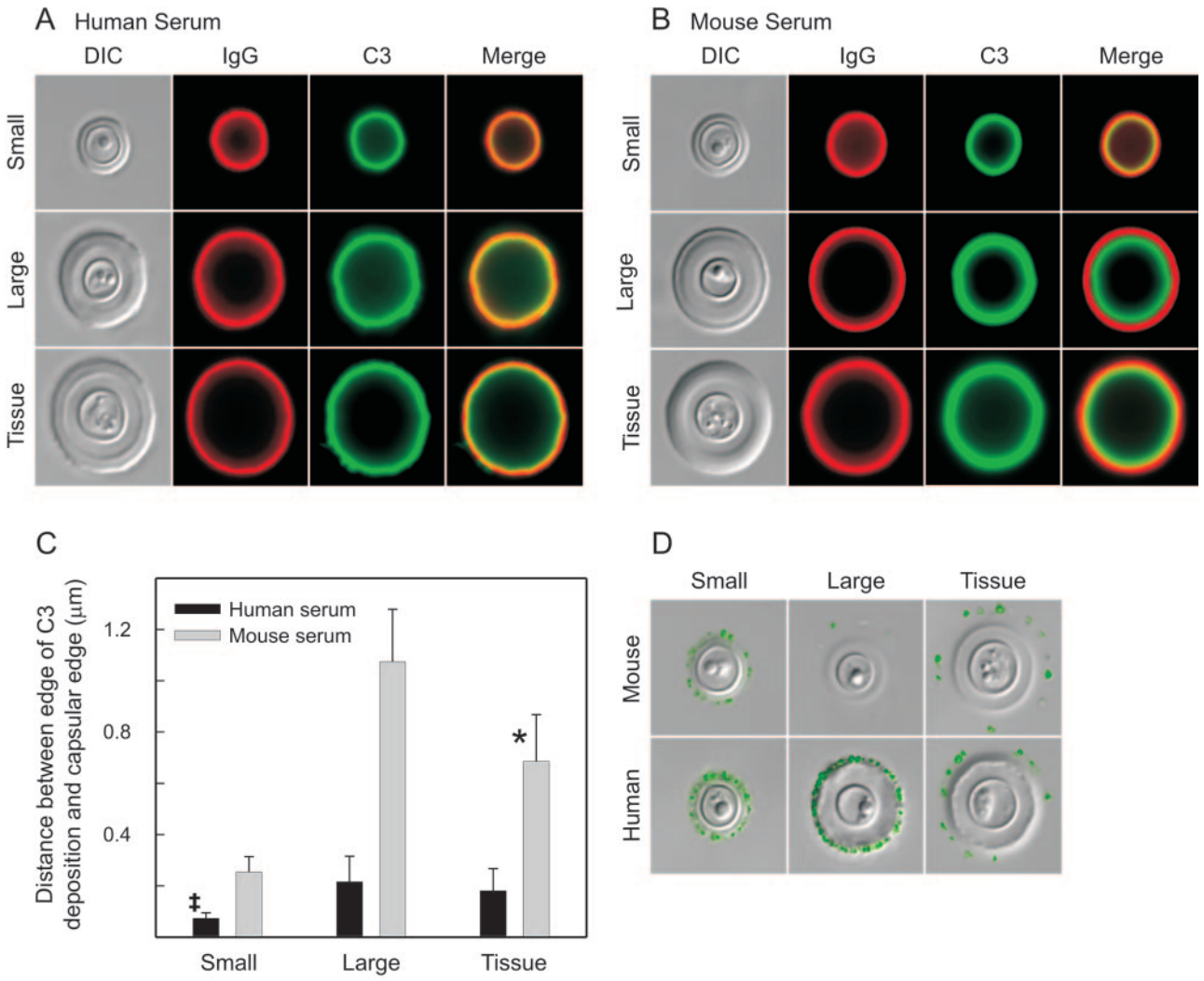

FIG. 7. Influence of manner of cell growth on the sites for binding of human or mouse C3 to the capsular matrix. Panels A and B, cryptococci (i) grown in vitro in the absence of capsule induction conditions (small), (ii) grown in vitro under capsule induction conditions (large), or (iii) derived from infected brain tissue (tissue) were incubated for 32 min in 40% mouse (panel A) or human (panel B) serum. The capsular edge was identified with Alexa 555-labeled MAb 3C2; C3 binding was assessed with FITC-labeled anti-human or -mouse C3. Panel C, analysis of the distance between the outer edge of C3 deposition and the capsular edge. Values are means ± SDs; $n = 10$ cells/treatment; ‡, $P < 0.05$ versus large or tissue cells; *, $P < 0.001$ versus large cells. Panel D, cryptococci were grown as described for panel A and treated with mouse or human serum. Proximity of C3 to the capsular surface was determined by incubation with polystyrene beads coated with anti-human or -mouse C3.

human serum occur solely via the action of the alternative pathway (24). In contrast, the results in Fig. 3 show that early deposition of C3 during incubation in mouse serum is mediated by the classical pathway. As a consequence, one explanation for the differential localization of C3 following incubation in human and mouse sera is the possibility that the mouse classical and alternative pathways differ in the sites for C3 deposition. We have eliminated this possibility because the positions for deposition of C3 in the capsule following incubation in mouse serum (classical pathway) or Mg-EGTA-treated mouse serum (alternative pathway) were identical. Moreover, deposition of C3 following incubation of cryptococci in rat or guinea pig serum, a process that is mediated by the alternative pathway (Fig. 3), was significantly beneath the position of C3 following incubation in human serum. Thus, even for activation that occurs solely via the alternative pathway, there are species-specific differences in the sites for C3 deposition.

Given the importance of C3, factor B, and factor H in recognition by the alternative pathway (17, 26, 29, 31), we added the human proteins to mouse serum and evaluated the effects of the proteins on the position of C3 binding relative to the capsule edge. Addition of factor B or factor H had no effect on the position of mouse C3; it remained bound well beneath the capsular surface. In contrast, addition of human C3 to mouse serum led to binding of the human C3 at or very near the capsular surface at a position that was indistinguishable from that with human serum alone. As a consequence, we conclude that differences in the actions of human and mouse C3 account for the differential localization of C3 following incubation in human and mouse sera. We cannot exclude the possibility that

other proteins of the alternative pathway from the two species may have differential activities with regard to activation of the complement system; however, the primary component that influences the observed positional effect is C3.

There are at least four potential mechanisms for the abilities of human and mouse C3 to produce C3 accumulation at different sites within the capsule. First, serum concentration could influence the position of C3 binding. The results shown in Fig. 2A indicate that this is not the case. The position of C3 binding from mouse serum was similar over a range of 20 to 80% serum, whereas C3 binding from human serum was similar at 40 and 80% serum; 20% human serum failed to support C3 deposition. Second, the capsular matrix is a molecular sieve with variable porosity (14). If human C3 were of a larger molecular size than murine C3, it is possible that the human C3 would be unable to penetrate into the matrix to the extent of mouse C3. This is an unlikely explanation because the alpha and beta chains of human and mouse C3 are quite similar (1). Third, human and mouse C3 could show different specificities in binding sites. If the cryptococcal capsule presented potential ligands for binding of activated C3 that differed as a function of the position within the capsule, C3 from the two donor species might exhibit consequent preferences for binding sites within the capsular architecture. Notably, there has been evolutionary divergence in the binding specificities of C4 in lines leading to primates, rodents, and ungulates (11); such species-specific differences may also exist for C3.

An alternative explanation is the possibility that human and mouse metastable C3b differ in their lifetimes. Human metastable C3 has a lifetime of approximately 60 μs, which limits the distance for diffusion of activated C3b to 20 to 40 nm (28, 32). The cryptococcal capsule presents a matrix of variable porosity, with the concentration of GXM becoming very low as the capsular edge is approached (14). With a decrease in the concentration of potential acceptors at the capsule edge, metastable C3 may become inactivated before binding to the acceptor. If activated mouse C3b showed a shorter lifetime than activated human C3b, the mouse C3b might require a higher matrix density to allow for acceptor binding before inactivation occurs.

Although it is difficult to determine molecular distances within the capsule with certainty, some calculations are possible. For example, pore sizes within the capsule can be determined by sieving of dextrans having a known molecular size (14). Based on exclusion of dextrans having known molecular sizes at variable distances from the cell wall (14), one can extrapolate the results to the capsule edge and calculate that pores at the edge of the capsule would have diameters of approximately 55 nm (calculation not shown). Thus, the diffusion distances that must be overcome near the capsule edge approach or exceed limitations imposed by the lifetime of human C3 (28, 32). To our knowledge, the lifetime of activated murine C3 has not been described.

If the concentration of GXM within the capsular matrix is an important variable for support of complement activation, then the position for accumulation of mouse C3 should differ in cells showing differences in matrix density near the periphery of the capsule. We previously reported that the concentration of GXM in the capsular matrix of yeast cells grown for production of small capsules is markedly higher than that in yeast cells grown under capsule induction conditions (14). This difference could account for the observation by Zaragoza et al. that small-capsule cryptococci deposit mouse C3 near the capsule edge while large-capsule cryptococci deposit C3 well beneath the capsule edge (36). Our previous studies also showed that cryptococci harvested from infected brain tissue have a capsular matrix with a higher GXM concentration than cells grown in vitro under capsule induction conditions. Such differences allowed for a testing of the hypothesis that matrix density influences the position for accumulation of mouse C3. The results (Fig. 7) showed that incubation of tissue-derived cryptococci in mouse serum led to deposition of C3 at a distance that was significantly closer to the capsule edge than occurred with cells grown in vitro under capsule induction conditions. Notably, the capsule widths of tissue-derived cells and cells grown under capsule induction conditions are similar (14). Taken together, these results suggest that matrix density is a more important determinant of the site of C3 deposition than capsule width.

Our studies identified a second substantive difference between human and mouse sera in activation of C3 and binding to the cryptococcal capsule. Incubation of cryptococci in mouse serum leads to a sudden and synchronous activation and binding of C3. Such a pattern and the early binding are characteristic of what we have observed previously with the classical pathway (23, 35, 37). Mg-EGTA-treated mouse serum produced the delayed, asynchronous pattern observed with human serum. Mixing of human and mouse sera produced no alteration in the rapid pattern for accumulation of mouse C3 in the capsule. These results suggest that mouse serum contains an initiator that is not found in human serum. However, assay of the mouse serum for antibodies to GXM showed an absence of detectable antibody. Similarly, absorption of the serum to remove a potential activator failed to alter the pattern and kinetics for binding. As a consequence, the identity of the putative classical pathway initiator in mouse serum is not known.

Of the various animal models for cryptococcosis, mice are generally regarded as the most susceptible to *C. neoformans* (6). Given the link between a failure in opsonization of large-capsule cryptococci and deposition of murine C3 beneath the capsular surface (36), it is possible that this opsonic failure may account, in part, for the high susceptibility of mice to cryptococcosis. On the other hand, it is also possible that such an opsonic failure would not occur in vivo or would be minimized due to the high density of the capsular matrix found in cryptococci obtained from infected mouse tissue. An examination of the functional consequences of differential localization of human and murine C3 within the capsule for phagocytosis of variously encapsulated cryptococci is in progress.

Taken together, our studies add a new dimension to our understanding of the interactions between microbial capsules and the complement system. Bacterial capsules are generally regarded as inhibitors of complement activation or, at the very least, sites where complement activation does not occur. The cryptococcal capsule appears to be an exception because dense accumulation of C3 occurs in the capsule following incubation in human serum. Mechanisms that have been shown to inhibit complement activation by bacterial capsules include enhanced binding of the regulatory protein factor H, poor binding of factor B, or a failure to act as an acceptor for covalent C3 deposition (for a review, see reference 19). Our studies indi-

cate that matrix density may also be a factor that influences complement activation by bacterial capsules. Little is known about the molecular architectures of bacterial capsules or the concentrations of polysaccharides that occur within the capsules. As a consequence, the role of matrix density in complement activation by microbial capsules is a rich area for future study.

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