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Monoclonal antibodies can affect complement deposition on the capsule of the pathogenic fungus *Cryptococcus neoformans* by both classical pathway activation and steric hindrance

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Summary

The capsule of the human pathogenic fungus *Cryptococcus neoformans* presents the immune system with a formidable problem for phagocytosis. Capsule-mediated activation of the alternative complement (C) pathway results in component 3 (particularly, C3) binding to the capsule near the cell wall surface. Hence, for cells with large capsule, C3 cannot interact with the complement receptor (CR) and is not opsonic. However, C activation in either immune serum or in the presence of monoclonal antibody (mAb) to capsular polysaccharide localizes C3 to the capsular edge. When *C. neoformans* cells were coated with both C and antibody (Ab) opsonins, Ab bound first and promoted C3 deposition at the edge of the capsule. The mechanism for the Ab-mediated change in C3 localization to the capsule edge involved both classical C pathway activation and steric hindrance preventing C3 penetration into the capsule. The change in C3 localization changed the mode of phagocytosis in macrophages, such that localizing C3 at the edge of the capsule allowed phagocytosis through C3–CR3 and C3–CR4 interactions, which did not occur in serum without Ab. These findings reveal a new mechanism of Ab action whereby Abs affect the location of C3 and its interaction with its receptor in macrophages depending on the immunoglobulin concentration.

Introduction

The two major opsonins in the serum of mammalian hosts are antibodies (Abs) and complement (C), and mediate phagocytosis through distinct receptors. Abs induce phagocytosis by binding to the Fc receptors of the phagocytic cells. The C system is activated by proteolysis in the presence of either Ag–Ab complexes (classical pathway), foreign particles (alternative pathway) or the lectin pathway (which depends on the mannose-binding protein). C proteins mediate phagocytosis through the complement receptors (CR) (see reviews in Kozel, 1993; 1998). The two systems interact because C can influence Ab responses and Abs activate the C system through the classical pathway.

Cryptococcus neoformans is the yeast that causes cryptococcosis (see review in Casadevall and Perfect, 1998). *C. neoformans* is unique among the fungi because it has a polysaccharide capsule composed mainly by glucuronoxylomannan (GXM) surrounding the cell that is a major virulence factor (Chang and Kwon-Chung, 1994; Casadevall and Perfect, 1998; McFadden and Casadevall, 2001). The capsule makes *C. neoformans* resistant to phagocytosis such that in the absence of opsonins, phagocytic cells do not ingest yeast cells (Mitchell and Friedman, 1972; Kozel and Gotschlich, 1982; Kozel *et al*., 1988a; Small and Mitchell, 1989). However, for *C. neoformans*, Abs also promote phagocytosis through CR3 and CR4 in the absence of C3 by a mechanism that involves a lectin-like interaction between the capsular polysaccharide and CR3 and CR4 (Dong and Murphy, 1997; Netski and Kozel, 2002; Taborda and Casadevall, 2002). The cryptococcal polysaccharide capsule activates the alternative C pathway directly, although this mechanism is slower than that mediated by Ab (Kozel *et al*., 1998). Unfortunately, the capsular polysaccharide is poorly immunogenic and high Ab titer responses are seldom elicited during infection (Murphy and Cozad, 1972; Kozel *et al*., 1977). In fact, when Ab responses are made these have characteristics of non-protective Abs (Zaragoza and Casadevall, 2004a). Hence, the alternative pathway of the activation of C is believed to make a critical contribution to phagocytosis of the yeast *in vivo*. This is supported by the observation that hosts depleted of C are highly susceptible to experimental *C. neoformans* infection (Diamond *et al*., 1973;

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Shapiro *et al*., 2002). However, the effect of C pathway can be also enhanced by the presence of specific mAbs to GXM (Zhong and Pirofski, 1998), which means that both pathways interplay to ensure a proper response to the pathogen (Diamond *et al*., 1974; Casadevall *et al*., 1998).

It has been shown that C3 binds to the cryptococcal capsule in the form of iC3b (Kozel and Pfrommer, 1986), and recent studies indicate that location of this protein in the *C. neoformans* polysaccharide capsule can have profound effects on its opsonic efficacy, and this process is strongly influenced by the size of the capsule (Zaragoza *et al*., 2003). When C3 localizes deep inside the capsule, as occurs for cryptococcal cells with large capsules, C3 is not accessible to the receptor and the process is inefficient in promoting phagocytosis (Zaragoza *et al*., 2003). Consequently, the efficiency of C-mediated phagocytosis is critically dependent on the size of the capsule of the *C. neoformans* strain (Mitchell and Friedman, 1972; Kozel *et al*., 1988b; Small and Mitchell, 1989; Yasuoka *et al*., 1994; Zaragoza *et al*., 2003). Recent studies show that another factor that influences C3 localization is the source of serum, with serum from different mammalian species in different regions of the capsule (Gates and Kozel, 2006).

Antibodies affect C3 localization on microbial capsules by activating the classical pathway. For example, in the case of *Streptococcus pneumoniae*, Abs to the capsule and cell surface promote deposition of C3 on the capsule and cell surface respectively (Brown *et al*., 1983), but only Abs to the capsule increase adherence (Brown *et al*., 1982). In contrast, for *Neisseria gonorrhoeae*, blocking non-protective Abs promote the deposition of C3 in the microbial surface at sites that do not promote serum killing (Joiner *et al*., 1985). However, the changes in C3 localization in that system are mediated by Abs of different specificity and we are not aware of a precedent where a single Ab can alter C3 localization as a function of concentration. Here we show that a single Ab can alter C3 localization on a microbial capsule as a function of immunoglobulin concentration.

Results

Immune serum changes C3 localization

When C3 binds to the capsule following alternative pathway activation, C3 is deposited close to the cell wall, and depending on the thickness of the capsule, it cannot interact with the CR in phagocytic cells (Zaragoza *et al*., 2003). In this study we examined whether Abs to *C. neoformans* elicited by cryptococcal infection could also change C3 localization. For this purpose, we incubated heat-inactivated immune serum with *C. neoformans* cells with large capsule. The same experiment was also done using non-immune sera. Afterwards, the cells were washed and incubated in fresh mouse serum. Incubation of cells in non-immunized serum produced C3 localization close to the cell wall (Fig. 1). However, when the cells were incubated with immune serum, a significant proportion of cells changed C3 localization. In some cells, C3 localized throughout the capsule whereas in others the C3 localized to a thin annulus at the outer edge of the capsule. We

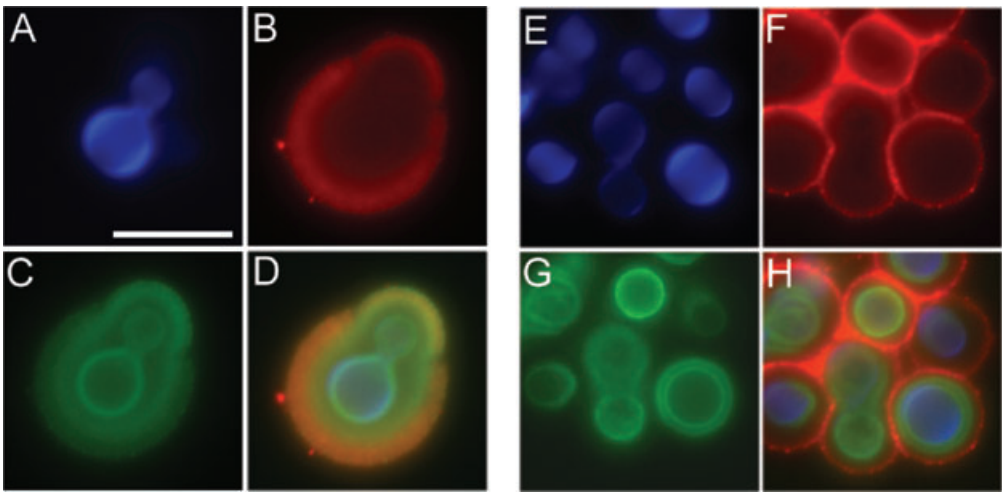

Fig. 1. Polyclonal serum changes C3 localization in the cryptococcal capsule. C57BL/6J mice were intratracheally injected with 10^6 yeast cells (H99 strain) or PBS. After 7 days, the serum of the infected (immunized) and non-infected (non-immunized) mice was collected. This serum was used to promote C3 deposition on the *C. neoformans* capsule. *C. neoformans* cells had large capsules, which had been induced by incubation of the yeast cells in MOPS 50 mM pH 7.3 with 10% Sab medium (see *Experimental procedures*). After 1 h incubation at 37°C, the cells were washed, and C3 and Ab were detected by immunofluorescence. In the case of non-immunized serum, 18B7 was added after the serum incubation to detect the capsule. A–D, immunized serum; E–H, non-immunized serum. A and E, cell wall (calcofluor); B and F, mAb to the capsule (rhodamine); C and G, complement (fluorescein); D and H, merge of the rhodamine and fluorescein fluorescences. Scale bar, 10 microns.

repeated this experiment incubating the cells directly in immunized serum, without heat inactivating, and we found similar results (result not shown).

mAb binding to C. neoformans *capsule affects the localization of C3*

Given that immune sera affected C3 localization we investigated whether this effect could be reproduced by mAbs to GXM. Yeast cells with large capsules were first incubated with mAb 18B7, and then placed in mouse serum to promote C3 deposition. Localization of C3 in the capsule depended on Ab concentration (Fig. 2). At low mAb concentration (10 $\mu g\ ml^{-1}$), C3 localized inside the capsule. However, at higher mAb concentration (100 $\mu g\ ml^{-1}$), C3 localized in the capsule edge. Intermediate concentrations gave a mixed result (data not shown). At very high Ab concentrations (1 $mg\ ml^{-1}$) the capsule architecture had an irregular edge, and C3 localized at the capsule edge. Hence, mAb changes the C3 localization in the capsule depending on immunoglobulin concentration.

Antibody-coated capsules are less permeable

To investigate capsule permeability after Ab binding, yeast cells were coated with mAb and then incubated with rhodamine-labelled dextrans of different molecular weight and Stokes radius. Dextran penetration in the capsule was measured by fluorescence microscopy. In non-Ab-coated cells, the low molecular weight dextrans (3000 and 10 000 Da) penetrated deep into the capsule (Fig. 3A). However, when the cells were treated with mAb 18B7, a significant amount of exclusion was observed. Both Ab-coated and native cells excluded the high-molecular-weight dextrans, but in Ab-coated cells there was a clearer area of exclusion. In contrast, in native cells there was always some penetration of high-molecular-weight dextrans into the capsule. This result is consistent with a reduction in porosity in the capsule by Ab binding.

We considered that blocking of the capsule by mAb could prevent the penetration of the anti-C3-FITC (fluorescein-isothiocynate) reagent, which would prevent detection of C3 molecules that penetrated the capsule. To test this possibility, we incubated cells with induced capsule in serum before treatment with mAb 18B7, and then labelled them with anti-C3-FITC to detect the C3 molecules inside the capsule. Then we coated the cells with mAb 18B7, and incubated again in serum. Finally, we detected C3 and mAb 18B7 with the secondary Ab conjugated to FITC or tetramethyl-rhodamine-isothiocynate (TRITC) respectively. As shown in Fig. 3B, when the cells were incubated in serum without mAb and then detected with the secondary Ab, C3 was detected inside the capsule. When Ab and serum were added afterwards, two rings of fluorescence were detected, one inside the capsule, and another one at the capsule edge (Fig. 3B1). However, when the cells were incubated in the same way, but without adding the secondary anti-C3-FITC Ab after the first serum incubation, C3 was visualized only at the capsule edge, and not inside (Fig. 3B2). This indicates that mAb 18B7 blocks the capsule, and avoids the penetration of the secondary Ab (GAM-C3-FITC). This result further confirmed that Ab seals the capsule and avoids penetration of large molecules. Furthermore, in another experiment mAb 18B7-labelled cells were incubated in serum, then stripped of mAb with acid solution and then labelled with Ab to C3. That experiment showed little or no C3 inside the capsule consistent with the view that Ab seals the capsule and prevents C3 penetration to the capsular inside where it binds preferentially in the absence of specific Ab (data not shown). To explore the possibility that C3 was directly bound to mAb, in the same experiment we tried to detect C3 bound to 18B7 by capture enzyme-linked immunosorbent assay (ELISA), but did not find any interaction (result not shown).

C3 and Ab binding to the C. neoformans *capsule when both opsonins are exposed to the yeast cells at the same time*

Ab–Ag complexes activate the classical C pathway, and this results in faster C3 deposition in the *C. neoformans* capsule than the alternative pathway (Kozel *et al*., 1991). Consequently we studied how C3 and Ab bound to the cryptococcal capsule when both were present simultaneously. When yeast cells were incubated in mouse serum containing mAb 18B7, C3 bound only to the cells that had Ab (Fig. 4A and B). In cells without Ab, C3 was only observed in the capsule after 5 min of incubation, and at shorter periods, such as 1.5 min, there was no signal at all (Fig. 4A). Even after 5 min C3 fluorescence was very weak, and localized as small dots inside the capsule, close to the cell wall (Fig. 4B). In the absence of Ab, C is activated by *C. neoformans* by the alternative pathway, which is slower as shown previously (Kozel *et al*., 1991; 1998), and results in C3 deposition inside the capsule, close to the cell wall. To confirm this result, we studied the kinetics of mAb and C3 binding in the presence of EGTA, which inhibits the classical C activation pathway. As expected, EGTA delayed C3 deposition, and C3 was only detected after 15 min of incubation (Fig. 5). We observed a granular pattern of C3 binding, most probably due to a slow C3 binding to the capsule due to a suboptimal activation of the alternative pathway in the presence of EGTA, with extensive C3 deposition taking a significantly longer time. We noticed in our conditions a longer delay when C3 deposition occurs after activation of the alternative

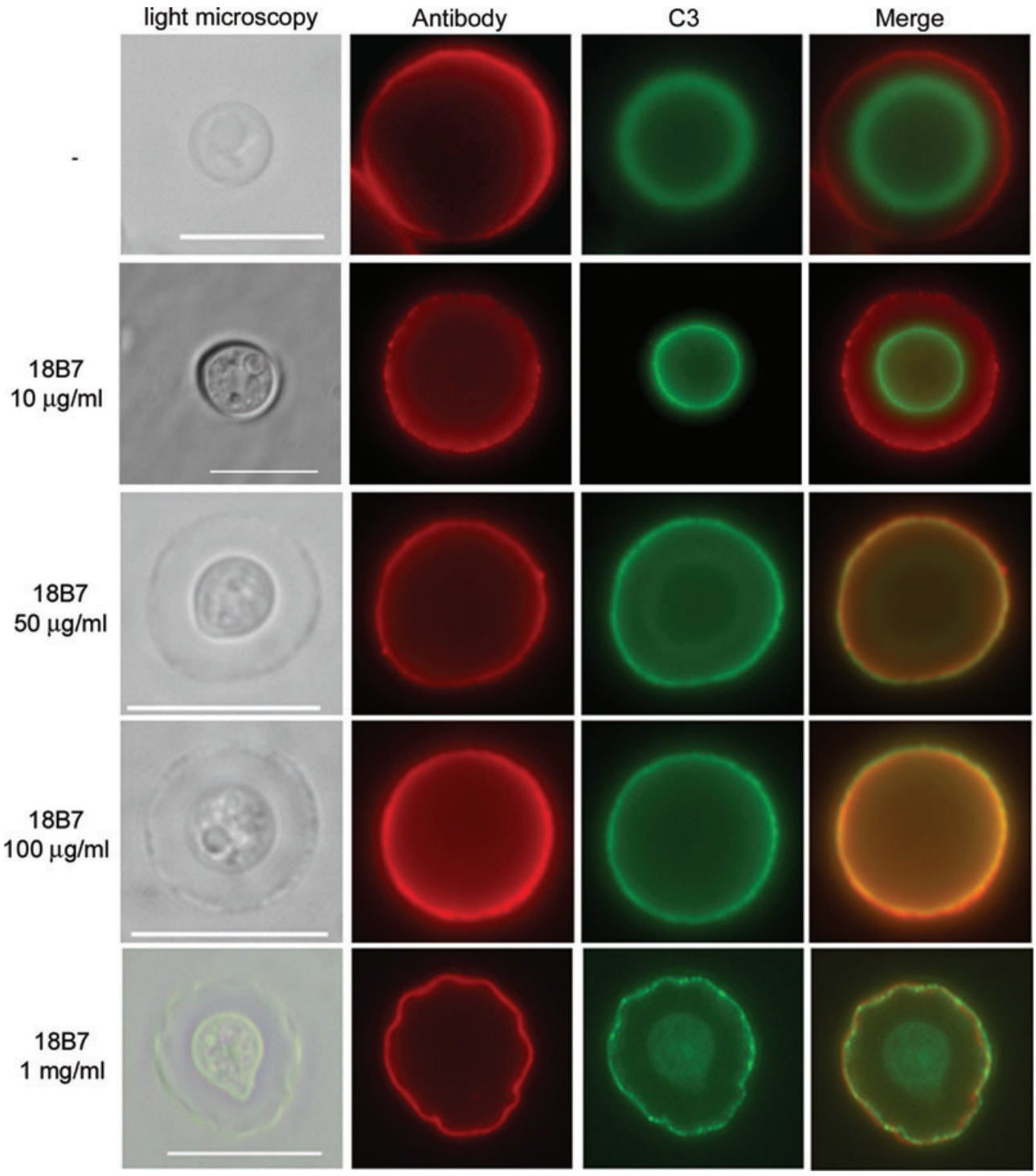

Fig. 2. Antibody affects C3 localization on the capsule of *C. neoformans*. The size of the capsule yeast cells from strain H99 was induced in diluted Sabouraud in MOPS buffer. The cells were then placed on blocking solution (upper row), or incubated with either 10, 50, 100 or 1000 μg ml^{-1} of mAb 18B7. After 1 h, the cells were suspended in mouse serum to allow C3 deposition. Finally, mAb 18B7 was added after the serum incubation to the control not treated with Ab (upper row). The Ab (rhodamine) and C3 (fluorescein) bound to the capsule were detected with secondary conjugated Abs, and representative pictures are shown, as well as a composite generated by merging the two images. Scale bar in the first panel denotes 10 microns.

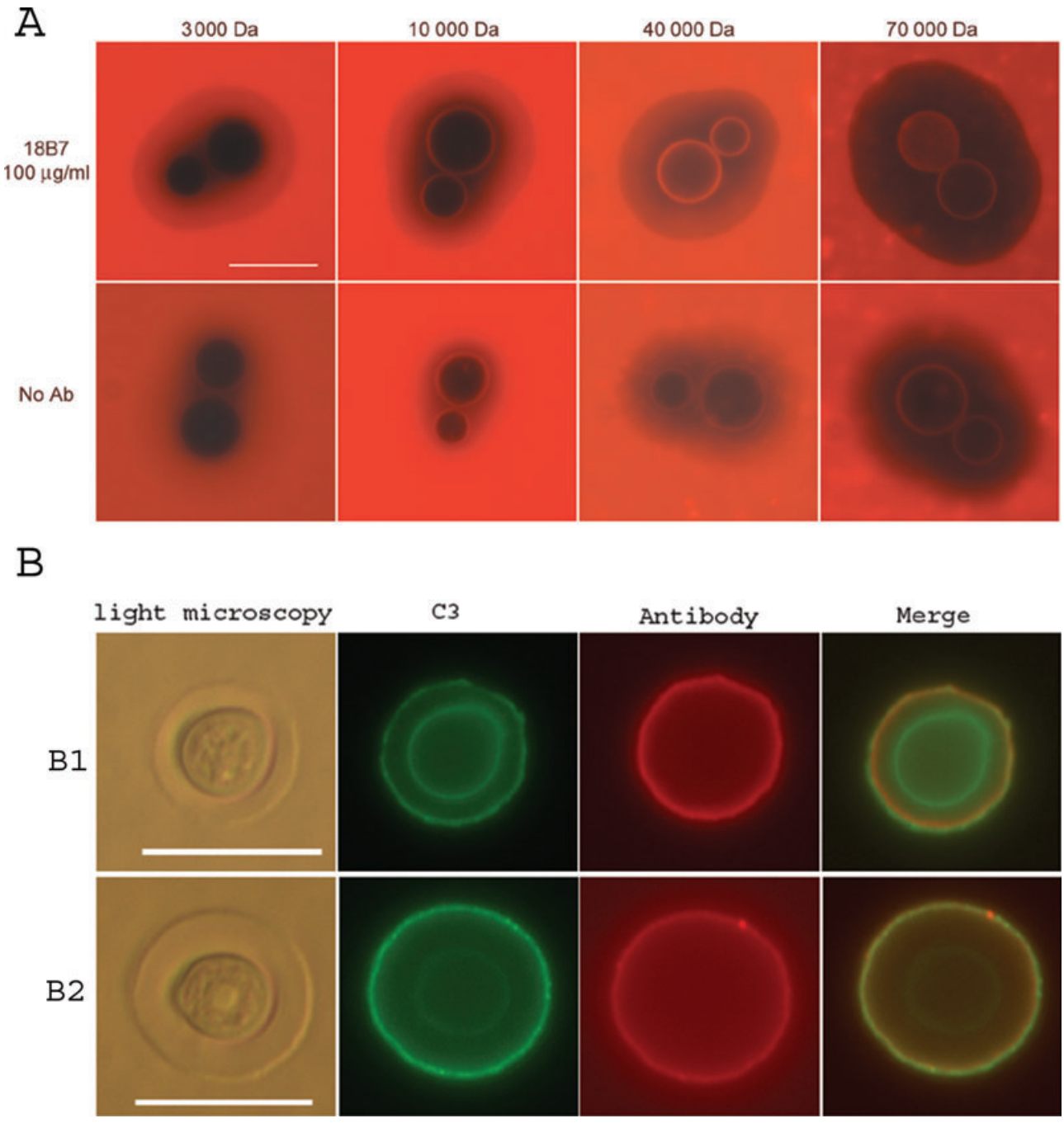

Fig. 3. Blocking of the capsule by mAb 18B7.
A. Exclusion of fluorescent dextrans by the cryptococcal capsule in the presence and absence of mAb 18B7. H99 cells with induced capsule were incubated with mAb 18B7 100 μg ml^{-1}, incubated for 1 h, then washed with PBS and placed in a suspension with rhodamine-labelled dextrans of different molecular weights (3000, 10 000, 40 000 and 70 000 Da). The exclusion of the dextrans by the capsule was observed under the fluorescent microscope. Bar, 10 μm.
B. Penetration of Ab to C3 into the cryptococcal capsule after mAb 18B7 treatment. H99 strain were grown as in (A), incubated in 100 μl of mouse serum for 1 h at 37°C, then incubated with goat anti-mouse-C3-FITC conjugated Ab (B1) or PBS (B2) for 1 h at 37°C, and finally incubated with mAb 18B7 (100 μg ml^{-1}, 1 h at 37°C), mouse serum (100 μl, 1 h at 37°C). mAb and C3 were detected with secondary conjugated Abs as described in *Experimental procedures*. Scale bar denote 10 microns.

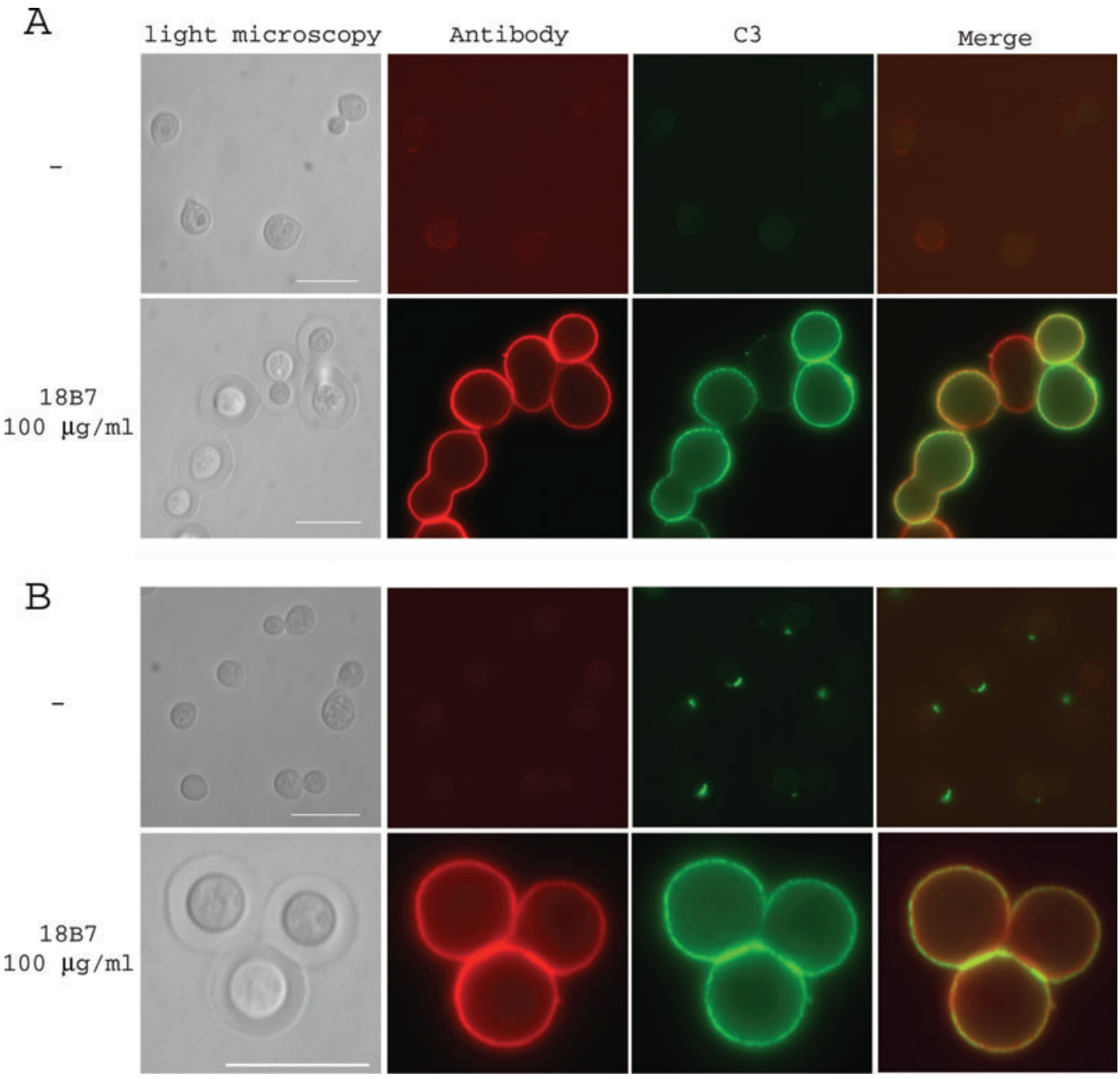

Fig. 4. Kinetics of binding of C3 and mAb 18B7 to *C. neoformans*. H99 cells with induced capsule were incubated with serum, or 100 µg ml^{-1} of mAb 18B7. C3 and Ab deposition was observed by immunofluorescence with specific Abs conjugated to fluorescein (C3) or rhodamine (Ab) after 1.5 min (A) or 5 min (B) of incubation at 37°C. The corresponding light microscopy picture and merge of both fluorescent images is also shown. Scale bars in the left panel denote 10 microns.

pathway than reported previously (Kozel *et al*., 1991; 1998). This small difference may reflect interlaboratory experimental differences such as different serum sources, or the use of Mg-EGTA in the experiments. However, in these conditions, C3 localized at the outer edge of the capsule, which confirms that mAb localizes C3 at the edge without activation of the C classical pathway and that this localization is not dependent on the C-activation pathway. When cells were exposed to both opsonins in the presence of EGTA for longer periods, such as 1 h, there was a localization of C3 and Ab to the edge of the capsule in an annular pattern (result not shown).

Change in C3 localization affects the mechanism of phagocytosis

To investigate whether the Ab-mediated localization of C3 correlated with an increase in C3-mediated phagocytosis as described earlier (Zaragoza *et al*., 2003), we compared the phagocytosis of cells opsonized in serum previously

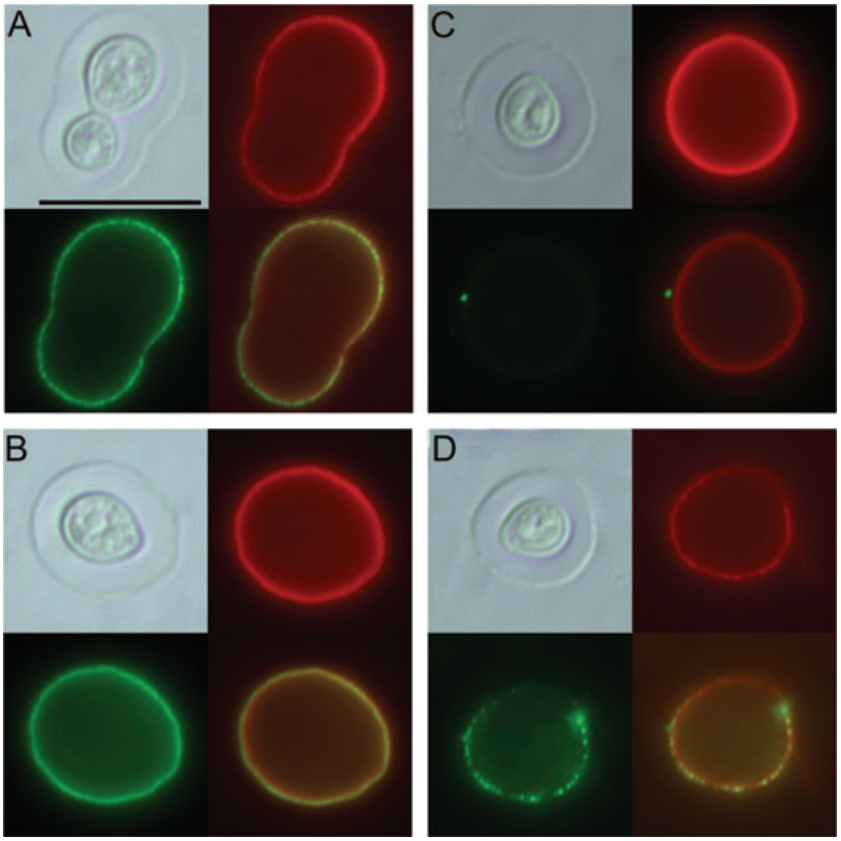

Fig. 5. Effect of EGTA on C3 and mAb 18B7 deposition in *C. neoformans*. Yeast cells were treated as described in Fig. 4, but also incubated with 10 mM EGTA (C, D). The reaction was stopped after either 5 (A, C) or 15 min of incubation (B, D). Complement and Ab were detected by immunofluorescence. In all the panels, light microscopy (upper left), capsule edge (rhodamine, red, upper right), complement (fluorescein, green, lower left) and the merge of both fluorescent images (lower right) is shown. Scale bar in panel A denotes 10 microns, and applies to all the pictures.

treated with mAb 18B7 by a macrophage-like cell line (J774.16). IgG1 mAb was opsonic by itself, and mAb binding followed by incubation in serum produced a slight increase of the phagocytosis, although there was not a statistical significant difference (Fig. 6, $P = 0.2$). To investigate the mechanism of the phagocytosis, we blocked C3-dependent phagocytosis with $F(ab)_2$ to C3. We used $F(ab)_2$ fragments to block phagocytosis produced by this Ab. When the cells were incubated with mAb 18B7, the Ab to C3 slightly increased the phagocytosis, although this increase was not statistical significant ($P = 0.11$). We believe that this small effect of the $F(ab)_2$ Ab on phagocytosis could be due to the presence of Abs in the polyclonal serum that could promote opsonization cells, or to capsular structural changes that could allow other type of interactions, such as IgG–FcRI/II/III or GXM–CR interactions (Taborda and Casadevall, 2002; Zaragoza *et al*., 2003). In this regard, it is noteworthy that GXM–CD18, GXM–CD14 and GXM–TLR2,4 interactions have been described (Shoham *et al*., 2001; Taborda and Casadevall, 2002; Zaragoza *et al*., 2003). However, addition of $F(ab)_2$ to C3 significantly reduced the phagocytosis of cells incubated first with mAb 18B7 and then in serum ($P = 0.0015$), indicating that in these cells, where mAb 18B7 localizes C3 at the capsular edge, a significant proportion of the phagocytosis was C3-mediated. When cells were first incubated with serum and then with mAb 18B7, C3 localized deep within the capsule (Zaragoza *et al*., 2003). In these conditions, the anti-C3 $F(ab)_2$ Ab did not reduce the phagocytosis, but slightly increased it in the same way that in the case of cells incubated only with mAb 18B7. This suggests that when C3 localizes deep in the capsule, phagocytosis was mediated through IgG–FcI/II/III receptors, and not through the CR, which explains why the anti-C3 Ab does not have a deleterious effect on the phagocytosis. Finally, the anti-C3 Ab was not opsonic by itself.

Antibodies vary on their effects on C3 localization

We studied the effect of Abs differing in affinity and specificity on C3 localization. The IgG1 mAbs 18B7, 2H1 and 3E5 are each protective against *C. neoformans* (Yuan *et al*., 1995; Casadevall *et al*., 1998). mAb 2H1 had only a small effect on C3 deposition but at high concentration, and no effect was observed after incubation of the cells with mAb 3E5 (Fig. 7). The fact that mAb 18B7 was the most efficient Ab in localizing C3 outside the capsule is correlated with the localization of this mAb in the capsule. It bound at the edge in an annular pattern surrounding the capsule. mAbs 2H1 and 3E5 were found in a more diffused pattern throughout the capsule (Fig. 7).

We also studied the role of IgM on C3 localization, as

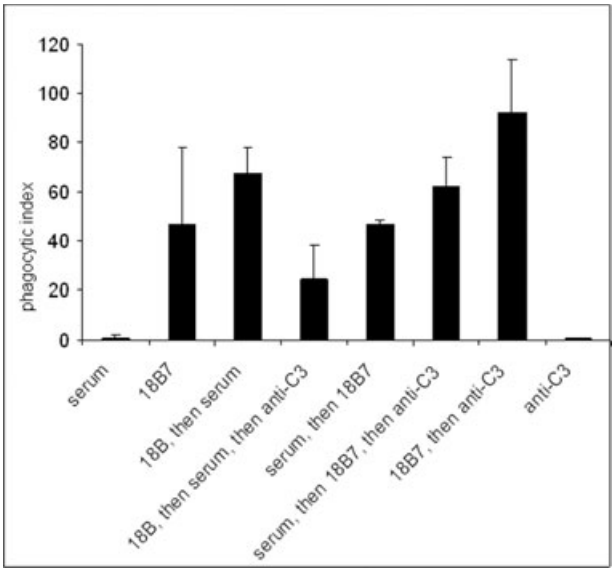

Fig. 6. Change in C3 localization by mAb 18B7 treatment induces C-mediated phagocytosis. *In vitro* phagocytosis was performed using macrophage-like cell line J774.16 and yeast cells in which capsule size was increased by incubation in diluted Sabouraud in MOPS buffer pH 7.3. The yeast cells were treated with mAb 18B7, serum or $F(ab)_2$ GAM-C3 in the following order: serum; mAb 18B7 (100 μg ml^{-1}); mAb 18B7 (100 μg ml^{-1}) and then incubated in serum (18B7 + serum); mAb 18B7, serum, and then with GAM-C3 (18B7 + serum + anti-C3); in serum and then with mAb 18B7 (serum + 18B7); serum followed by 18B7 and by GAM-C3 (serum + 18B7 + anti-C3); mAb 18B7 followed by GAM-C3 (18B7 + anti-C3); and finally with GAM-C3 (anti-C3). The cells prepared in these conditions were added to the macrophages to a ratio 1:1, and the suspension incubated for 1 h. Measurements were done in triplicate for each experiment. Bars denote the average and bracket the standard deviation of the mean. See text for statistical comparisons using the Kruskal–Wallis test.

they are pentameric, its mechanism of protection is not well established, and in the case of mAbs 13F1 and 12A1, the fluorescence pattern differs in serotype D strains, being punctuate and annular respectively (Cleare and Casadevall, 1998). The effect of the IgM was highly variable (Fig. 8). At high concentration mAb 2D10 produced a mixed result and promoted a change in C3 localization such that it was partially outside (Fig. 8). mAb 12A1 had no effect on C3 localization, although the fluorescence pattern of this Ab was clearly annular (Fig. 8). mAb 13F1 also produced an annular pattern, and C3 localized exclusively at the edge of the capsule (Fig. 8). Finally, mAb 21D2, which has a lower affinity for O-acetylated GXM, produced a punctate pattern, and also did not have any effect on C3 localization (Fig. 8). Interestingly, mAbs 12A1 and 13F1 produced a highly visible capsular reaction apparent by light microscopy, but their behaviour in changing C3 localization was different. As these mAbs presumably have different fine specificity, this result indicates that the effect of Ab on C3 localization is also a function of specificity.

Moreover, we studied dextran penetration with Abs that did not localize C3 at the capsular edge, such as 12A1, 3E5, 2H1 and 21D2, and observed that cells coated with these Abs excluded the fluorescently labelled dextrans in the same way as unlabelled cells (result not shown). Hence, Ab-mediated C3 localization to the capsule surface correlated with their ability to exclude dextran which presumably reflects their ability to seal the capsule surface.

Discussion

Antibodies to the *C. neoformans* capsule are known to affect the timing and amount of capsular C3 deposition (Kozel *et al*., 1998). These findings indicate that some mAbs to GXM can accelerate C3 deposition after activation of the classical pathway while reducing the total amount of C3 on the capsule while others do not affect the rate or the amount of C3 deposited on the capsule. These differences in Ab effect appeared related to their epitope specificity and isotype (Kozel *et al*., 1998). Here we demonstrate that Ab binding to yeast cells prior to C3 binding may also induce a change in the localization of C3, and that this effect is a function of Ab amount, isotype and specificity. In the absence of mAb 18B7 or in the presence of mAb 3E5, C3 localized inside the capsule, at a site where it is not expected to be an efficient opsonin based on prior studies (Zaragoza *et al*., 2003). However, for mAb 18B7, Ab binding was associated with C3 localization at the edge of the capsule, where it promoted phagocytosis through the CR. The mechanism by which the binding of certain mAbs changes the location of subsequent C3 binding is likely to be a consequence of both a physical effect of the Abs on the capsule and their effect on C activation. The density of the polysaccharide capsule is a function of its radial density, being less dense at the capsular edge (Pierini and Doering, 2001; Gates *et al*., 2004), which allows the penetration of C3 into non-Ab-coated cells. For some Abs such as mAb 18B7, binding to the capsule reduces its porosity, and this interferes with C3 penetration into the deeper regions of the capsule where C3 naturally binds. Consistent with this explanation, Ab 18B7 binding to the capsule resulted in a differential exclusion of fluorescent dextrans based on molecular weight.

Kinetic studies and blocking of the classical pathway by EGTA indicated that when the alternative pathway is responsible for C activation in the presence of Ab, C3 localized at the edge of the capsule. Our data confirm prior reports that the C3 deposition kinetics are accelerated in the presence of mAbs to the capsule (Kozel *et al*., 1998). This observation supports the concept of a physical or steric block, by bound Ab, whereby C3 deposition by alternative C activation occurred only at the capsule edge

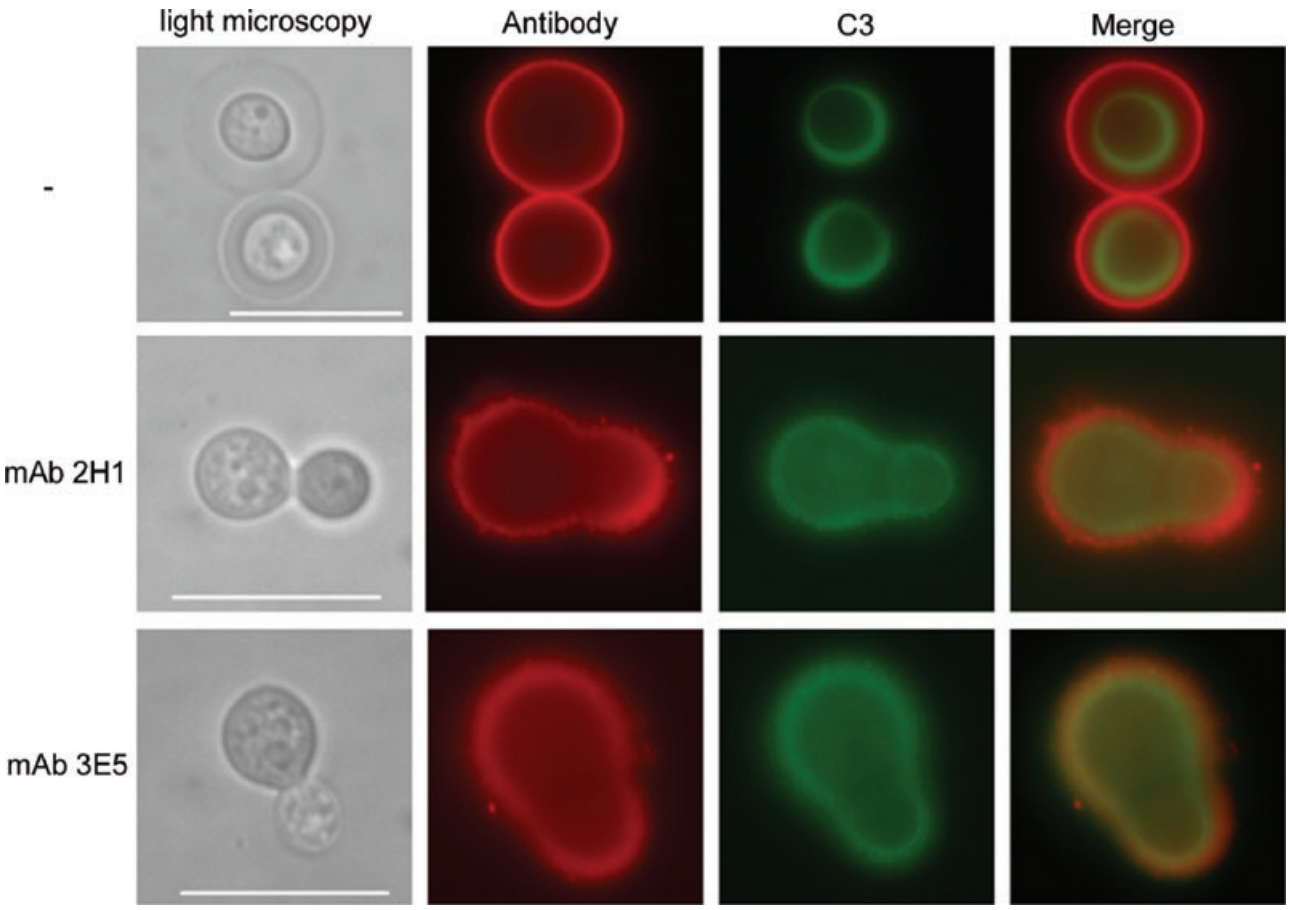

Fig. 7. Different IgG1 mAbs have different effects on C3 localization. Cells were treated exactly as described in the legend to Fig. 1, but mAbs 2H1 and 3E5 were used at a concentration of 100 μg ml^{-1}. For the negative control (top row) cells were incubated first with serum and the mAb 18B7 (10 μg ml^{-1}) was added to detect the capsule. The Ab (rhodamine) and C3 (fluorescein) bound to the capsule were detected with secondary conjugated Abs. Panel on right shows a composite generated by merging the two images. Scale bars in left panel denote 10 microns.

because C proteins cannot penetrate to the capsular interior in the presence of mAb 18B7. Consistent with this, we noted a correlation between the penetrations of dextrans of different molecular weight, and consequently, different Stokes radius, and the concentration of mAb 18B7. We observed significantly less penetration of dextran to Ab-coated cells, and this effect was visible with low molecular weight dextrans, such as 3 and 10 kDa. Considering that the molecular weight of C3b, the main C protein that binds to the cryptococcal capsule, is around 180 kDa, the dextran penetration data would suggest C3 is largely excluded from the capsular interior by mAb 18B7 binding. The fact that in cells non-coated with mAb, dextrans larger than 70 kDa are excluded, but C3 (which has a larger molecular weight) is not, indicates that C3 preferentially binds to the inner part of the capsule, and can penetrate, even though it is a large molecule. Our results in mAb-coated cells show that in these conditions the capsule is really sealed, because C3 cannot penetrate anymore. Hence, Ab binding can change the localization of C3 binding by steric hindrance whereby C3 penetration is blocked.

The Ab-mediated localization of C3 was not observed with all the mAbs tested. In the course of these experiments, we noticed that the binding pattern differed between Abs. For example, mAb 18B7 produced a clear annular immunofluorescence pattern when bound to cells with large capsule, while both mAbs 2H1 and 3E5 gave a more diffuse and punctuate pattern. Only Abs that produced both annular patterns and capsular reactions mediated a change in C3 localization, suggesting that an annular pattern was necessary to block penetration of C3 to the deeper regions of the capsule where it bound preferentially in the absence of Ab. However, an annular pattern and a capsular reaction are not sufficient conditions for this effect, because some Abs, such as mAb 12A1, produced annular fluorescence pattern and a capsular reaction but did not promote a change in C3 localization. The molecular basis for the punctate and annular fluorescence pattern is not understood, but it is believed to reflect

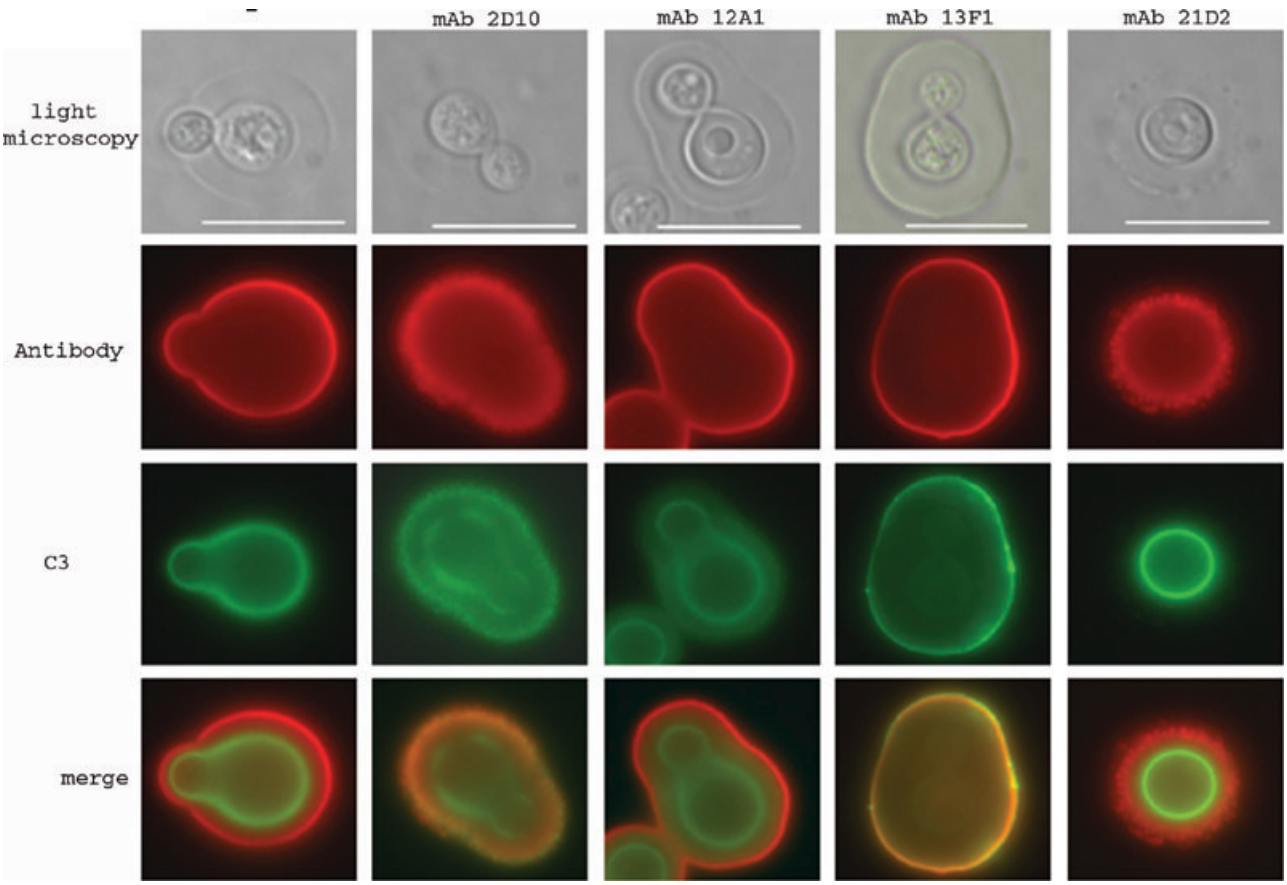

Fig. 8. Effect of IgM on C3 localization serotype A *C. neoformans* strain. The cells were treated as in Fig. 1, but different IgM mAbs to the cryptococcal capsule (2D10, 12A1, 13F1 and 21D2) were used at 100 μg ml^{-1}. The Ab (rhodamine) and C3 (fluorescein) bound to the capsule were detected with secondary conjugated Abs. Panel on bottom shows a composite generated by merging the two images. The negative control was treated with mAb 18B7 to detect the capsule after the serum incubation. Scale bars in upper row denote 10 microns.

differences in Ab epitope specificity (MacGill *et al*., 2000). Interestingly, some mAbs produced different fluorescence patterns in small and large capsule cells. Assuming that large and small cells produced the same type of polysaccharide this result would be consistent with a mechanism whereby the different patterns reflected the formation of different complexes, possibly as a result of conformational effects on the part of the polysaccharide capsule. In addition, it is conceivable that when using different Abs with different affinity, the amount of Ab bound to cell would vary, even when using the same concentration. That would produce different degrees of steric hindrance, and consequently, different effects on C3 localization.

We investigated whether the Ab-mediated localization of C3 correlated with a change in the mechanism of phagocytosis. The fact that both opsonins were located at the edge of the capsule raised the interesting question of how these two opsonins interacted in the polysaccharide capsule to induce phagocytosis. The Ab-mediated localization of C3 produces C3-mediated phagocytosis, in addition to other phagocytosis mechanisms known, such as IgG1–FcRI/II/III, GXM–CD14, GXM–TLR2,4 and GXM–CD18 interactions. In this regard mAb binding to the cryptococcal capsule can mediate phagocytosis by changing the structure of the capsule and facilitating the binding of GXM to CD18 (Dong and Murphy, 1997; Taborda and Casadevall, 2002; Netski and Kozel, 2002). Finally, not all the phagocytosis was mediated through the CR, because blocking CR3 and CR4 resulted in a moderate reduction in phagocytosis, suggesting a role of Abs as opsonin in these conditions (result not shown). We conclude that in the presence of opsonins, phagocytosis of *C. neoformans* is a complex process that involves multiple interactions (Fig. 9). When cells with large capsule are incubated in serum, no phagocytosis is obtained, as a consequence of C3 localization deep in the capsule. In the presence of mAb, there is phagocytosis through two different mechanisms, which are mAb–FcR and GXM–CD18. Finally, when the cells are first coated with Ab and then with C3, there is a third mechanism, which involves C3–CR3 and C3–CR4 interactions. Hence, in the presence of Ab and C3 there are at least three types of

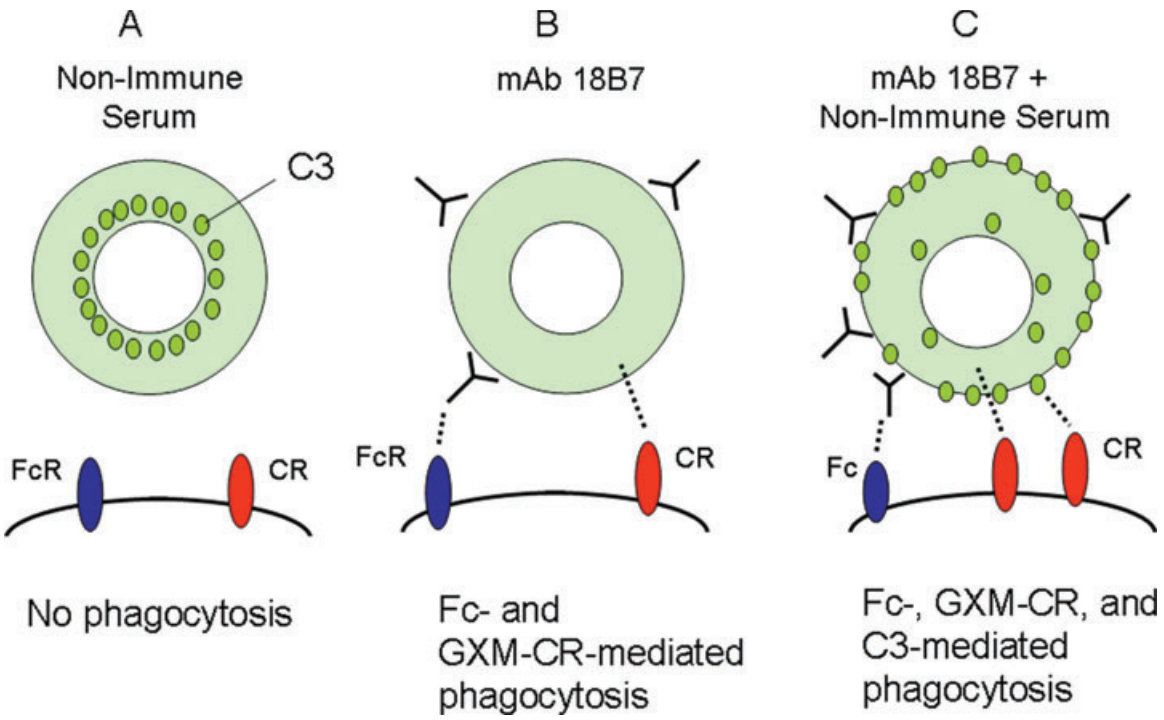

Fig. 9. Model of phagocytosis for *C. neoformans*.
A. In the presence of non-immune serum, C3 is the only opsonin. C3 deposition in the capsule is mediated by alternative C activation and results in binding deep in the capsule, where it cannot interact with CR3 or CR4, and consequently, there is no phagocytosis.
B. In the presence of mAb to GXM, there are two different mechanisms for phagocytosis, Ab–FcR and GXM–CR3/4 interaction, which is described in Taborda and Casadevall (2002). The GXM–CR3 and GXM–CR4 interaction presumably occurs as a consequence of structural changes in the polysaccharide capsule that allow the interaction between GXM and CD18.
C. In Ab and C3- coated cells, all three mechanisms for phagocytosis can occur.

opsonic interactions. In fact, the phagocytosis of *C. neoformans* is a much more complex process. GXM can interact with other receptors in the absence of opsonins (see review in Levitz, 2002; Vecchiarelli, 2005). In dendritic cells, the mannose receptor and FcRII are involved in the phagocytosis and presentation of GXM to the T-cells (Syme *et al.*, 2002). CD14 is required for phagocytosis of *C. neoformans* by human microglia (Lipovsky *et al.*, 1997), and moreover, it can bind GXM (Shoham *et al.*, 2001). Other receptors that could enhance phagocytosis of *C. neoformans* are the Toll-like receptors (see reviews in Levitz, 2004; Netea *et al.*, 2004; Roeder *et al.*, 2004). GXM binds TLR2 and TLR4 (Shoham *et al.*, 2001), and survival after *C. neoformans* infection is mainly dependent on $TLR2^{-/-}$ and $MyD88^{-/-}$ deficient mice are more susceptible to *C. neoformans* infection (Yauch *et al.*, 2004). These observations indicate that phagocytosis of *C. neoformans* is a complex process in which the pathogen is recognized by several receptors that in combination could contribute to the efficient ingestion of the pathogen.

The strong dose dependence of this effect presumably reflects a requirement for a large number of Ab molecules to seal the capsule to C3. *In vitro*, the change in C3 localization was observed after incubation of the yeast cells at relatively high Ab concentration. In addition, we have also observed that, although the Abs titers to GXM during pulmonary infection manifest great variations between mice, the effect can be observed with serum from immunized animals. Hence, the effect can occur with physiological concentrations of specific polyclonal Ab. As most people have Ab as a result of initial infection, the phenomenon described here may occur during subsequent re-infections, which are presumably common. Our results are also relevant in the case of passive Ab administration for the treatment of cryptococcosis, where relatively large doses of Ab are administered (Larsen *et al.*, 2005). The concentrations that we used *in vitro* are in the range achievable in passive Ab studies in mice (Mukherjee *et al.*, 1994). Furthermore, serum concentrations of mAb 18B7 in clinical trials are also expected to be in the range studied here, at least shortly after infusion (Larsen *et al.*, 2005). Hence, we consider the effects described here to be relevant for the conditions where passive Ab is used therapeutically or experimentally.

Our results are almost certainly relevant to the prozone-like effects observed in Ab protection studies against *C. neoformans* whereby protective mAbs lose efficacy and

can become disease-enhancing at high doses (Taborda and Casadevall, 2001; Taborda *et al*., 2003). We note that Ab-mediated effects on C3 localization were not observed at low concentrations of specific Ab but manifested themselves only at high Ab doses. The mechanism for that effect was attributed to interference with oxidative killing by the deposition of a proteinaceous layer in the capsule in the form of bound mAb and alterations in cytokine expression in conditions of high and low specific Ab (Taborda *et al*., 2003). The finding that high Ab concentrations seal the capsule and promote C3 deposition on the surface of the yeast cell can shield the microbe against oxidants by providing additional protein at the surface of the capsule. Furthermore, the shift in FcR- to C3-mediated uptake as Ab concentration increases implies qualitative differences in the interactions with phagocytic cells at high and low Ab concentrations which may affect intracellular survival of fungal cells and cytokine expression through different signalling pathways.

There are previous reports in the literature that indicate that Abs change C3 localization but we note that those findings involved differences in C3 deposition mediated by Abs that bound to different Ags. For example, in *S. pneumoniae,* Abs to the capsule or to the cell wall promoted C3 binding to the capsule or the cell wall respectively (Brown *et al*., 1983). Hence, in that system Ab-mediated differences in C3 localization were caused by Abs that bound to different antigens. In contrast, we describe how a single Ab alters C3 deposition on a microbial capsule depending on its specificity and concentration. In addition, this is the first demonstration whereby a change in C3 localization in a microbe is correlated with a change in the mode of phagocytosis. C activation can occur through the alternative pathway and the classical pathway, and the role of each one is very dependent on the organism. In *C. neoformans*, the capsule activates C through the alternative pathway, but occurs at a much slower rate than when activated by Abs through the classical pathway (Kozel, 1996). In *S. pneumoniae*, C can be activated by the alternative pathway by the cell wall (Hummell *et al*., 1981; 1985), although it has been shown that classical pathway and natural occurring Abs are important during innate immunity (Brown *et al*., 2002). In the case of other pathogenic fungi, such as *Candida albicans*, C activation occurs also through the alternative pathway, although the kinetics and C3 binding present some differences to the activation by *C. neoformans*. (Kozel *et al*., 1987).

In summary, our results indicate that Ab binding to a microbial capsule can result in a qualitative difference in C3 binding that affects the mechanism of phagocytosis. As C3 deposition in cells with large capsules occurs away from the capsule edge, specific Ab provides a mechanism for localizing C3 at the outer edge of the capsule and thus allowing C3–CR3 and C3–CR4 interactions. These results suggest a new mechanism of action of Ab whereby they activate C3-mediated phagocytosis by changing C3 localization, and therefore, allow the presence of multiple pathways of phagocytosis of the pathogen.

Experimental procedures

Yeast strains and growth conditions

Strain H99 [serotype A (Franzot *et al*., 1999)] was used in all experiments. We used cells in which the capsule size was induced because this allowed greater discrimination of C3 localization by virtue of a greater distance between the capsule edge and the cell wall. Capsule size was induced by placing the cells in Sabouraud (10%) diluted with 50 mM MOPS buffer pH 7.3 (Zaragoza and Casadevall, 2004b).

India Ink staining and microscopy

Capsule size was measured in India Ink solution using an Olympus AX70 microscope. Pictures were taken with a QImaging Retiga 1300 digital camera using the QCapture Suite V2.46 software (QImaging, Burnaby BC, Canada), and processed with Adobe Photoshop 7.0 for Windows (San Jose, CA).

Antibodies to GXM

The following Abs to GXM were used in this study: 18B7 (IgG1), 2H1 (IgG1), 3E5 (IgG1), 2D10 (IgM), 21D2 (IgM), 13F1 (IgM) and 12A1 (IgM). mAb 18B7 was purified by protein A affinity chromatography (Pierce, Rockford, IL) following the manufacturer's recommendations. For the other Abs, we used concentrated supernatants from the corresponding hybridoma cells. These mAbs are characterized based on their variable gene use and affinity for GXM (Mukherjee *et al*., 1993). Ab concentrations were determined by ELISA relative to isotype-matched standards of known concentration.

Antibody treatment, C deposition and immunofluorescence

Fluorescence was performed as in Zaragoza *et al*. (2003). Yeast cells were suspended in freshly obtained mouse serum and in some experiments, the cells were directly incubated in mouse serum containing mAb to the capsule. For blocking the classical C activation pathway, 10 mM EGTA was added. The cells were washed again, and bound mAb and C3 were detected by immunofluorescence. C3 was detected with a FITC conjugated goat Ab to mouse complement C3 (4 μg ml^{-1}, Cappel). Negative controls consisting of cells without Ab added were carried out in parallel to observe C3 localization in untreated cells. To detect the capsular edge, mAb 18B7 (10 μg ml^{-1}) was added after serum incubation with the GAM-C3-FITC Ab. To visualize Abs bound to the capsule, a goat anti-mouse IgG or IgM conjugated to TRITC (Southern Biotechnology Associates, Birmingham, AL, 10 μg ml^{-1}) was added. The cells were suspended in mounting medium (50% glycerol and 50 mM N-propyl gallate in PBS) and observed under a fluorescence microscope.

In some experiments, cells with induced capsule were suspended in 0.2 mg ml^{-1} fluorescent-labelled dextrans (Molecular

Probes, Eugene, OR) to measure capsule porosity as a function of molecular weight. We used dextrans of 3000, 10 000, 40 000 and 70 000 Da labelled with tetramethylrhodamine.

Mouse infection

C57BL/6J (Jackson Laboratories, MA, USA) were infected intratracheally with 10^6 yeast cells, and after 7 days of infection, blood was obtained from the retro-orbital cavity, and serum was obtained. Abs to GXM were measured by ELISA (Casadevall *et al*., 1992). For immunofluorescence, the serum was heat-inactivated, and 10^6 yeast cells were incubated in 50 μl of serum. C3 and Abs to GXM were detected by immunofluorescence as described above.

Phagocytosis experiments

Phagocytosis assays were performed as in Zaragoza *et al*. (2003), using the macrophage-like cell line J774.16. We used this cell line because it expresses both Fc and CR (Taborda and Casadevall, 2002) and has been extensively used in phagocytosis studies of *C. neoformans*. These cells were maintained in DME medium (7–13 passages) with 10% inactivated FCS, 1% nonessential amino acids and 10% NCTC, in the presence of 10% CO_2. Yeast cells washed with PBS and suspended in blocking solution (1% BSA/0.5% horse serum). Then, the cells were treated in different ways. In some experiments, cells were first incubated with mAb 18B7 (100 μg ml^{-1}, 1 h at 37°C), washed, and then placed in 100 μl of mouse serum. In other experiments, either the Ab or serum incubation was omitted or the cells were first incubated in mouse serum for 1 h at 37°C, washed, and then incubated with mAb 18B7 100 μg ml^{-1}. When indicated, the yeast cells were finally incubated with 100 μg ml^{-1} of goat $F(ab')_2$ fragment to mouse complement C3 (Cappel, ICN, Aurora, OH).

The phagocytosis was performed for 1 h at 37°C as described in Taborda and Casadevall (2002) and the phagocytic index was calculated as the number of cryptococcal cells phagocytosed per 100 macrophages counted.

Capture ELISA to detect C3 bound to Abs

Cells from H99 strain with induced capsule were incubated with mAb 18B7 (100 μg ml^{-1}) for 1 h at 37°C and after washing with PBS, they were incubated for 1 h in 100 μl of mouse serum. Then, after washing again, the cells were incubated overnight in 0.1 M HCl at 30°C without shaking. The cells were discarded by centrifugation, and the supernatants were neutralized with NaOH. Ab and C3 were detected by capture ELISA. Briefly, 96-well plates were coated with goat anti-mouse IgG1 (1 μg ml^{-1}) and the neutralized samples were added afterwards. Ab or C3 were detected using GAM-IgG1-HRP or GAM-C3-HRP (3 μg ml^{-1}, ICN/Cappel, Aurora, OH) conjugated Abs. To develop the plates, we used ABST tablets (Roche, Indianapolis, IN) in the buffer recommended by the manufacturers, and the absorbance was read at 405 nm.

Statistics

Kruskal–Wallis analysis was used to compare the phagocytosis index using Unistat 5.5 software for Excel (London, England). This test was used due to the lack of normality of our samples.

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