

Reduced Expression of the Functionally Active Complement Receptor for iC3b but Not for C3d on an Avirulent Mutant of *Candida albicans*

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Pseudohyphae of *Candida albicans* bear surface receptors for iC3b and C3d. In order to determine a possible role for these receptors in the pathogenesis of candidiasis, a spontaneous *C. albicans* mutant, m-10, which exhibits reduced ability to adhere in vitro to fibrin platelet clots and epithelial cells or to cause endocarditis in a rabbit model, and its parent wild-type (wt) strain were compared for receptor expression in rosetting assays with sheep erythrocytes carrying iC3b (EAC1423bi) or C3d (EAC1423d). An equally high attachment to wt and m-10 was seen with EAC1423d, whereas rosetting with EAC1423bi was reduced by 53% in m-10 compared with wt. In inhibition studies, rosetting of wt with EAC1423bi was markedly inhibited by culture filtrate, hyphal-cell extract, and DEAE-fractionated material prepared from wt (54, 87, and 70% decreases in rosetting, respectively), thus suggesting the presence of the soluble, functionally active iC3b receptor of *C. albicans* in each of these preparations. Minimal inhibition of iC3b rosetting, however, was seen with the identical materials from m-10 (21, 5, and 12%, respectively). All of the preparations from the two strains were equally effective in their inhibitory activities against rosetting of C3d. A human serum specimen obtained from a patient with chronic mucocutaneous candidiasis blocked iC3b rosetting of the wt strain almost completely. When used in an immunoblot, this serum recognized proteins of 68 to 71, 55, and 50 kilodaltons (kDa) in hyphal-cell extracts of the wt. With the same preparation of the avirulent mutant, only weak reactions with the 68- to 71-kDa and 55-kDa proteins occurred, while the 50-kDa protein was not detectable. Taken together, these results indicate that the expression of the functionally active iC3b receptor on *C. albicans* may be involved in the virulence of the organism, possibly by mediating adherence to mammalian cells.

Human peripheral blood cells bear several membrane receptors that bind cleavage products of the third component of human complement (C3) (1, 19). Recently it was found that *Candida albicans* is able to mimic the human complement receptors type 2 (CR2) and type 3 (CR3) by binding either C3d- and iC3b-coated sheep erythrocytes (3, 6, 7, 10) or radiolabeled fluid-phase iC3b (8). Except for *Candida stellatoidea*, the other less pathogenic *Candida* spp. lack this ability, thus implying a role for these binding proteins in the pathogenesis of candidiasis (10). The C3d-binding protein of *C. albicans* has been identified by C3d affinity chromatography as two proteins with molecular masses of 62 and 70 kilodaltons (kDa) under reducing gel conditions (3). More recently we used monoclonal antibodies which specifically block C3d binding to *C. albicans* to purify the C3d-binding structure from hyphal-cell extracts (HCEs) (16). By using the OKM-1 monoclonal antibody, which is directed against the alpha chain of the human CR3, Eigentler et al. (7) were able to inhibit the binding of iC3b-coated sheep erythrocytes (SRBC) to *C. albicans*. The same monoclonal antibody immunoprecipitated three proteins (130, 100, and 50 kDa) from *C. albicans* pseudohyphal extracts. Very recently, Hostetter and Kendick (12) used a different monoclonal antibody (BU-15) directed against the mammalian CR3, which reacted with a 185-kDa protein from *C. albicans* under nonreducing conditions and with three proteins with molecular masses of 70, 67, and 55 kDa under reducing conditions.

Previously we isolated a spontaneous avirulent mutant of *C. albicans* (11). This mutant, originally isolated for its resistance to the antibiotic cerulenin, has a reduced ability to adhere in vitro to fibrin platelet clots and epithelial cells, to cause endocarditis in a rabbit model (2), and to cause vaginitis in a murine model (15). The purpose of the present study was to determine whether altered virulence of the mutant strain 4918-10 (m-10) was accompanied by changes in iC3b and C3d receptor expression in comparison with the virulent *C. albicans* wild-type (wt) strain 4918.

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MATERIALS AND METHODS

Organisms and culture conditions. *C. albicans* 4918, a virulent clinical isolate (wt), and 4918-10 (m-10), a spontaneous, avirulent mutant of the wt, were used throughout these studies and have been described previously (4, 11, 18). Both strains were maintained on Sabouraud dextrose agar slants at 4°C and subcultured monthly. For rosetting assays, cells were first transferred to a synthetic medium as described by Lee et al. (14) and then grown to stationary yeast phase for 20 h at 24°C. Yeast cells were harvested, washed, and resuspended in the synthetic medium as described previously (14). The mycelial-growth form of *C. albicans* was induced at 37°C in the same medium. Cells were collected after 16 h, washed, and resuspended for rosetting assays.

Preparation of HCE and CF. For the preparation of HCE and culture filtrate (CF), *C. albicans* was grown as described above. Hyphae were harvested after 16 h by centrifugation

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at $8,000 \times g$, washed, suspended in sterile distilled water containing 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.), and homogenized with 0.45-mm-diameter glass beads in an MSK cell homogenizer (B. Braun Instruments, San Mateo, Calif.). The cell components released by this procedure were subsequently centrifuged at $10,000 \times g$ for 20 min, at $25,000 \times g$ for 20 min, and at $100,000 \times g$ for 60 min (18). After the last centrifugation, the clear supernatant was membrane filtered and lyophilized. To prepare the CF, the culture medium was separated from the hyphae by centrifugation as described above, filtered through a 0.45- μ m-pore-size membrane filter (Millipore Corp., Bedford, Mass.), concentrated 200 times, dialyzed extensively against phosphate-buffered saline, and finally lyophilized (5).

Rosetting assay. To quantitate the binding of the complement components iC3b and C3d to *C. albicans*, a rosetting assay with SRBC was performed as described previously (3). Samples of SRBC were sequentially incubated with a sensitizing antibody (hemolysin) and with human C1, C4, C2, and C3 to generate EAC1423b (all components were from Dia-medix Corp., Miami, Fla.). The intermediates EAC1423bi and EAC1423d were formed by incubating EAC1423b with human factors I (Cytotech, Inc., San Diego, Calif.) and H (generously provided by M. M. Frank, National Institutes of Health, Bethesda, Md.) or with trypsin (Worthington Diagnostics, Freehold, N.J.), respectively. For inhibition studies, $50 \mu\text{l}$ (7×10^7 cells) of complement-coated SRBC was incubated for 30 min at 37°C with $50 \mu\text{l}$ of a standardized *Candida* extract ($0.7 \mu\text{g}$ of protein per ml of the material to be tested as determined by the method of Lowry et al. [17]). Subsequently, $20 \mu\text{l}$ of *C. albicans* hyphae (approximately 4×10^6 cells) suspended in gelatin-Veronal-sucrose buffer was added and incubated for another 30 min at 37°C . Rosetting was determined and evaluated by light microscopy as described previously (3). To test the inhibitory activity of antisera, $50 \mu\text{l}$ of the desired antiserum dilution was incubated with *C. albicans* hyphae for 30 min at 37°C before the complement-coated SRBC were added.

DEAE-Trisacryl chromatography. HCEs from both *C. albicans* strains (the wt and m-10) were fractionated on DEAE-Trisacryl M (LKB Produkter AB, Bromma, Sweden) by a method previously reported by our laboratory with a stepwise NaCl gradient for protein elution (3). Eluted fractions were assayed for inhibition of rosetting as described above.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting were performed according to established procedures (13, 20). Electrophoresis was carried out in a minigel system (Bio-Rad Laboratories, Richmond, Calif.). The total amount of protein loaded per lane was $3 \mu\text{g}$ in each of the tested preparations (17). A human serum sample from a patient with chronic mucocutaneous candidiasis (CMC) was used as the primary antibody (diluted 1:400) for immunoblotting of HCE and DEAE fractions from the wt and m-10. The antiserum was a kind donation from H. Buckley, Philadelphia, Pa. Binding of the primary antibody to *C. albicans* antigens was detected with alkaline phosphatase-labeled protein A as described previously (3).

RESULTS

Interactions of *C. albicans* wt and m-10 strains with iC3b and C3d. The hyphal form of wt *C. albicans* bound both

complement intermediates, EAC1423bi and EAC1423d, to a very high extent. In comparison, hyphae of m-10 and of the wt formed rosettes with EAC1423d to an equal extent, but expression of the iC3b-binding protein was reduced by a margin of more than 50% in m-10 hyphae. With iC3b, the percent rosetting was 40 ± 3.9 with m-10 hyphae (mean \pm standard deviation) and 85 ± 3.6 with wt hyphae. With C3d, the percentages of rosetting were 91 ± 3.2 with m-10 hyphae and 92 ± 2.0 with wt hyphae. At least 150 hyphal cells were counted in each experiment; a minimum of four SRBC per hypha was required for a positive test. To further confirm these results, HCE and CF from both the wt and m-10 were prepared and tested for their abilities to inhibit iC3b and C3d binding to wt hyphae. These preparations were incubated with EAC1423bi and EAC1423d before the addition of wt hyphae. Both the HCE and the CF obtained from the wt strain markedly decreased rosetting with the two complement intermediates EAC1423bi and EAC1423d (Fig. 1). In a similar way, HCE and CF from strain m-10 were significant inhibitors of EAC1423d binding to wt *C. albicans*. However, EAC1423bi binding to wt hyphae could not be inhibited with HCE from m-10 and could be inhibited only slightly with CF from m-10 (Fig. 1).

HCE from both strains was chromatographed on DEAE-Trisacryl, and the eluted fractions were tested for their inhibitory activities on iC3b and C3d rosetting by wt hyphae. When HCE from the wt strain was separated, several protein peaks were typically obtained. Only one of the peaks (designated as P4 and eluted at 0.2 M NaCl), however, contained significant receptor activity for iC3b and C3d (Table 1). An almost identical elution profile was obtained when HCE from strain m-10 was separated by DEAE-Trisacryl. The corresponding peak P4, however, contained mostly C3d-blocking activity but only a low degree of iC3b-blocking activity (Table 1).

Immunoblotting. An immune serum specimen obtained from a patient with CMC was tested for its ability to block iC3b rosetting with wt *C. albicans*. This serum significantly blocked iC3b rosetting at the tested dilution range of 1:10 to 1:100 (Fig. 2). At the same dilutions, normal human serum had only slight blocking activity. The immune serum, which was a potent inhibitor of iC3b binding to wt *C. albicans*, was used for immunoblot analysis of HCE and DEAE fractions from the wt and m-10 (Fig. 3). With the wt HCE and the wt DEAE fraction P4, both of which contain significant iC3b receptor-blocking activity, the immune serum recognized a major protein complex with a molecular mass of 68 to 71 kDa, an additional protein with a molecular mass of 55 kDa, and a minor band with a molecular mass of 50 kDa (Fig. 3, lanes 1 and 3). In the identical preparations of the mutant strain m-10, however, there were only weak reactions with the 68- to 71-kDa complex and the 55-kDa protein. The 50-kDa band was not detectable in m-10 (Fig. 3, lanes 2 and 4).

DISCUSSION

The presence of a binding protein for iC3b and C3d on the surface of the pathogenic yeast *C. albicans* but not on the surfaces of virulent *Candida* spp. has raised the question of whether such a factor is involved in the virulence of the organism (3, 6, 10). In the present study, we investigated the binding of iC3b- and C3d-coated SRBC to the mycelial forms of a virulent *C. albicans* wt and its derived, avirulent mutant. This mutant strain is characterized by its reduced adherence to epithelial cells in vitro and by its diminished virulence in

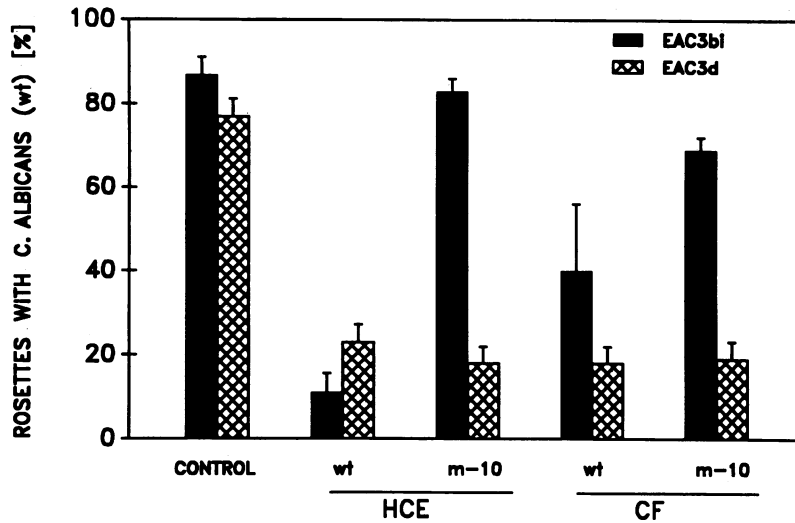


FIG. 1. Inhibition of C3d and iC3b binding to wt *C. albicans* by HCE and CF obtained from the wt and m-10 strains. Complement-coated SRBC were preincubated with the inhibitory fractions before the incubation with *C. albicans* wt hyphae. Buffer was used in control experiments to determine the normal level of rosette formation with EAC1423bi (EAC3bi) and EAC1423d (EAC3d). Error bars represent standard deviations.

a rabbit endocarditis model (2) and a murine vaginitis model (15). Because of the differences in adherence and virulence between these two *C. albicans* strains, we decided to compare their complement receptor activities.

The mutant strain m-10 formed rosettes with C3d-coated SRBC to the same high degree as the wt did, but the binding of EAC1423bi to m-10 was reduced by more than 50% compared with its binding to the wt (see Results), thus indicating a reduction in iC3b receptor expression on m-10. An alternative explanation would be the expression of a functionally inactive receptor protein on m-10. This reduction in iC3b rosetting with m-10 correlates well with the previously reported reductions in m-10 virulence in two different animal models (2, 15). Furthermore, a total cell extract and a CF, both of which, when isolated from the wt, were able to inhibit iC3b rosetting, lacked this activity when isolated from m-10 (Fig. 2). These results, taken together with the previously reported characteristics of the m-10 mutant (2, 4, 15), strongly indicate that the iC3b-binding protein on *C. albicans* represents a virulence factor of this opportunistic pathogen. It also can be suggested from the data presented here that the *C. albicans* iC3b receptor might be related to the adhesin on hyphal cells. Supporting evidence for this theory is derived from a recent report from another laboratory. Gustafson et al. (9) showed that certain monoclonal antibodies directed against the alpha subunit of the mammalian CR3 not only block iC3b binding to *C. albicans* but also are inhibitors of *C. albicans* adherence to human endothelial cells in vitro. These studies, however,

were done with *C. albicans* yeast cells, which have a six- to sevenfold lower iC3b receptor expression than *C. albicans* hyphae and pseudohyphae have (M. W. Ollert and R. A. Calderone, unpublished observation). Additional studies are therefore necessary to investigate the role of the iC3b receptor on *C. albicans* hyphae in the adherence of the organism to mammalian cells. These studies are under way in our laboratory.

The *C. albicans* iC3b receptor was partially cloned recently by Hostetter and Kendrick (12) and appears to be a

TABLE 1. Inhibition of EAC1423bi and EAC1423d rosetting with wt *C. albicans* by DEAE-separated fractions from wt and m-10

Inhibitory fraction or buffer	% Rosettes ^a (mean ± SD) with:	
	EAC1423bi	EAC1423d
wt P4	26 ± 3.1	13 ± 9.0
m-10 P4	88 ± 7.2	10 ± 6.1
Buffer	90 ± 4.2	93 ± 4.2

^a See Results for description of determination of rosetting.

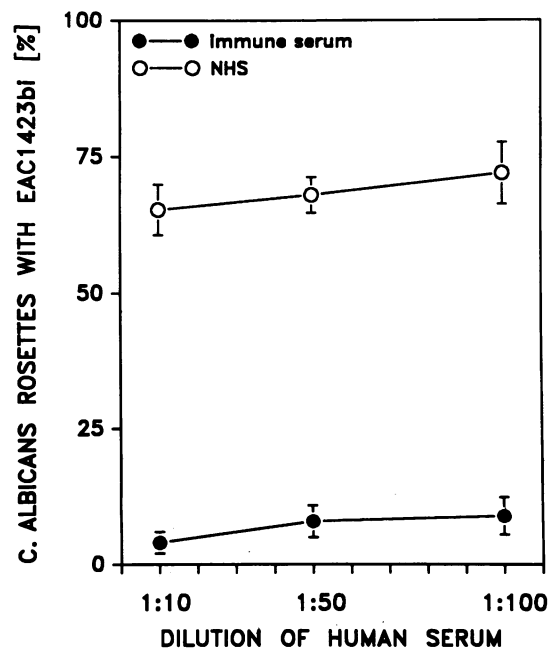


FIG. 2. Inhibition of EAC1423bi rosetting with wt *C. albicans* by an immune serum specimen obtained from a patient with CMC. Normal human serum (NHS) served as a control. Error bars represent standard deviations.

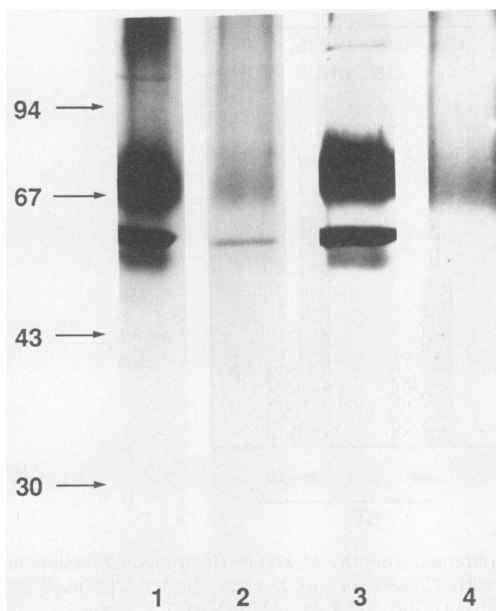


FIG. 3. Immunoblotting of HCE and DEAE fractions from *C. albicans* wt and m-10 strains with a CMC patient serum specimen (diluted 1:400). Lane 1, wt HCE; lane 2, m-10 HCE; lane 3, wt DEAE fraction P4; lane 4, m-10 DEAE fraction P4. Molecular masses are indicated in kilodaltons.

member of the integrin receptor superfamily, a fact that makes the protein an even more likely candidate to be an adhesin on *C. albicans*, as integrin receptors are known to be involved in adhesive mechanisms of mammalian cells (19). In the same study, by using a monoclonal antibody directed against the mammalian CR3, three proteins of 72, 68, and 55 kDa were identified under reducing electrophoresis conditions as subunits of the iC3b receptor on *C. albicans* (12). In our studies, we used a patient serum specimen which was a potent inhibitor of iC3b binding to *C. albicans* (Fig. 2) for immunoblot detection of the protein structures involved in this process. The proteins recognized by this procedure had molecular masses of 68 to 71, 55, and 50 kDa (Fig. 3). Except for the minor 50-kDa band, the molecular sizes of the detected proteins correlate well with the report by Hostetter and Kendrick (12). The same antiserum detected only weak bands of the corresponding proteins in m-10 (Fig. 3). Therefore, with the partial sequence information of the wt protein now available, it will be of great interest to determine the sequence of the mutant iC3b-binding protein from m-10 in order to map binding sites for iC3b and determine possible mammalian cell acceptor molecules involved in host-parasite interactions.

We have previously identified the C3d receptor of *C. albicans* as two proteins of 62 and 70 kDa under reducing conditions (3). These molecular masses are very similar to those of what we identify here as the putative iC3b receptor on *C. albicans* by Western blot (immunoblot) analysis (Fig. 3). Therefore, no final conclusion can be drawn from these experiments as to whether there are separate receptors for the two ligands iC3b and C3d or only a single receptor with separate binding sites for the two ligands. The fact that the m-10 strain has altered iC3b-binding properties but binds as many C3d-coated SRBC as the wt does would suggest that the binding sites for the two ligands are different. Additional evidence for this theory is derived from experiments with a

previously described monoclonal antibody (3) that effectively blocks C3d rosetting with *C. albicans*. This monoclonal antibody has almost no inhibitory effect on iC3b binding to *C. albicans* (M. W. Ollert and R. A. Calderone, unpublished observation).

In conclusion, the iC3b receptor on *C. albicans* appears to be associated with the virulence of the opportunistic fungus, possibly by mediating adherence to mammalian cells (9). It also seems to be a major antigen which evokes the humoral immune response, as a CMC patient serum specimen reacted very strongly to it in immunoblots. Future studies will be necessary to exactly define the role of the iC3b receptor in the pathogenesis of candidiasis.

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