A Role for Complement Receptor–like Molecules in Iron Acquisition by *Candida albicans*

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Summary

Candida albicans, an opportunistic fungal pathogen of humans, is dependent upon iron for growth. Consequently, human serum inhibits *C. albicans* growth due to the presence of high affinity iron-binding proteins that sequester serum iron, making it unavailable for use by the organism. We report that in the inhibitory environment of human serum, the growth of *C. albicans* can be restored by the addition of exogenous hemoglobin or heme, but not by protoporphyrin IX, the heme precursor that does not contain iron. We further report that *C. albicans* can utilize cell surface proteins that are homologues of the mammalian complement receptors (CR) to rosette complement-coated red blood cells (RBC) and obtain RBC-derived iron for growth. The ability of *Candida* to acquire RBC-derived iron under these conditions is dependent upon *Candida*-RBC rosetting mediated by CR-like molecules. Unopsonized RBC do not support *Candida* growth in serum, and restoration of *Candida* growth in serum by complement-opsonized RBC is inhibited by monoclonal antibodies to the human CR type 3 (CR3). In addition, activation of the human alternative pathway of complement by *Candida* leads to "bystander" deposition of C3 fragments on the surface of autologous, unopsonized RBC, generating the ligands necessary for *Candida*-RBC rosetting. These results suggest that *C. albicans* has evolved a unique strategy for acquiring iron from the host, which exploits the host complement system, and which may contribute to the pathogenic potential of the organism.

Candida albicans, a member of the normal human gastrointestinal flora, is a major cause of opportunistic fungal infection in immunocompromised hosts, especially those with hematologic malignancies (1, 2) and AIDS (3). The complex array of factors, both host related (4), and pathogen related (5), that are responsible for the virulence of *C. albicans* under these circumstances have been the subject of considerable study. Factors that have been implicated in the virulence of *C. albicans* include the ability of the organism to form germ tubes (6, 7), to produce toxins and hydrolytic enzymes (8, 9), and to adhere to and invade host tissues (10, 11).

C. albicans expresses cell surface proteins that bind C3 degradation fragments, and as such appear to be functional homologues of mammalian complement receptors (CR) (12–15). Expression of CR-like molecules by *C. albicans* was discovered when it was observed that *C. albicans* hyphae could rosette complement-coated sheep erythrocytes (12). The function of the *Candida* CR–like molecules is unknown, but CR expression appears to be associated with the more pathogenic species of *Candida* (12, 13, 16), thus implicating the receptors in virulence of the organism.

C. albicans is growth inhibited in human serum (17–19). Although several possibilities have been suggested to account for this inhibition (20–22), a number of reports have correlated inhibition with the presence of transferrin (18, 23, 24). Transferrin is a serum protein that binds iron with high affinity, restricting free iron to levels insufficient for microbial growth (25). Since iron is essential for a variety of cellular metabolic processes, pathogenic microorganisms must have mechanisms for obtaining iron that is sequestered by the host (25). The mechanism(s) by which *C. albicans* acquires iron for growth in the iron-restricted environment of the human host have not been extensively investigated.

Since hemoglobin is a rich iron source, the ability of *C. albicans* to rosette complement-coated RBC led us to investigate the possibility that *C. albicans* obtains iron from receptor-bound RBC. In the present study we demonstrate that *C. albicans* utilizes hemoglobin from RBC as an iron

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source. In addition, we show that *C. albicans* uses CR-like molecules to bind complement-coated RBC, allowing the fungus to obtain RBC-derived iron for growth. A mechanism by which this mode of iron acquisition may occur in vivo is suggested by the fact that activation of the alternative complement pathway by *Candida* cultured in fresh human serum leads to deposition on surrounding, autologous RBC of the C3-derived ligands necessary for *Candida*-RBC rosetting.

Materials and Methods

Iron Compounds. $Fe(NO_3)_3$ (no. F3002), bovine hemoglobin (no. H2625), bovine hemin chloride (no. H2250), protoporphyrin IX (no. P5889), human apotransferrin (no. T4515), and human holotransferrin (no. T3400) were obtained from Sigma Chemical Co. (St. Louis, MO). All compounds except hemin chloride were prepared as stock solutions in water that was deferrated by passage through a Chelex filter (Bio-Rad Laboratories, Richmond, CA). Hemin chloride was dissolved in 0.2 M NaOH prepared with Chelex-treated water.

Candida albicans. *C. albicans* strain B311 (32354; American Type Culture Collection, Rockville, MD) was passaged weekly on Sabourauds dextrose agar (SDA)[1] plates. Six clinical isolates of *C. albicans* were obtained from Temple University Hospital (Philadelphia, PA), and were maintained in the same manner as the American Type Culture Collection strain. A culture of yeasts was streaked onto a fresh SDA plate 24 h before experiments.

Assays for Serum-induced Growth Inhibition of C. albicans. Yeast phase organisms from 24-h SDA plate cultures were washed with sterile saline and resuspended in RPMI 1640 supplemented with glutamine, penicillin, and streptomycin (RPMI medium) plus the indicated concentrations of heat-inactivated (56°C, 45 min) pooled normal human serum (NHS). Yeasts were plated at 250/well in polystyrene microtiter dishes and incubated at 37°C with 5% CO_2. *C. albicans* cultured under the above conditions convert from the yeast to the hyphal form in the presence or absence of human serum (26, 27). The extent of hyphal growth after 18 h of culture was determined by [^{3}H]glucose uptake assay (26, 27) and by MTT assay (28).

[^{3}H]Glucose Uptake Assay. Assay for *Candida* hyphal growth by uptake of radiolabeled glucose has been described in detail elsewhere (26, 27). Briefly, after 18 h of growth under the described experimental conditions, medium was removed, wells were washed with sterile distilled water, and 25 μl of 2,5[^{3}H]glucose (Net-807, sp act 67 Ci/mMol; New England Nuclear, Boston, MA), diluted to 10 μCi/ml in sterile water, was added to each well. Plates were incubated for 30 min at 37°C, 25 μl bleach was added to wells to remove plastic-adherent hyphae, and radiolabeled *C. albicans* were harvested onto glass fiber filters using an automatic cell harvester. Radioactivity incorporated into *C. albicans* hyphae, which is proportional to the amount of hyphal growth (26, 27), was counted in a liquid scintillation counter.

MTT Assay. Quantitation of *Candida* growth by tetrazolium-based colorimetric assay was performed as described by Levitz and Diamond (28). Microtiter wells containing adherent *Candida* hyphae grown for 18 h under the described experimental conditions were washed twice with sterile water and 5 μg/well of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; M2128; Sigma Chemical Co.), diluted in RPMI 1640 without phenol red (Gibco Laboratories, Grand Island, NY) was added for 4 h at 37°C. Conversion of MTT to insoluble MTT-formazan by *Candida*-derived dehydrogenases was quantitated after solubilization of MTT-formazan with 10% SDS and warm acid alcohol. The change in OD at 570 nm was determined using a microELISA reader.

SRBC Opsonization. RBC obtained from fresh, heparinized sheep blood were washed three times with HBSS, adjusted to 10^9/ml in HBSS containing 1:150 anti-SRBC IgM (Diamedix Co., Miami, FL), and incubated for 30 min at 23°C with shaking. IgM-coated SRBC were washed twice with HBSS and incubated for 20 min at 37°C in HBSS + 10% C8-deficient serum (C8ds) for complement deposition. C8ds was obtained from a C8-deficient donor, as previously described (29). Cells were washed twice and incubated for an additional 15 min at 37°C in HBSS + 5% heat-inactivated C8ds to convert remaining C3b to iC3b. Previous studies have shown that using this protocol for opsonization, the predominant form of C3 on the RBC surface is iC3b (D. M. Mosser, unpublished data). Complement-coated SRBC were washed three times and adjusted to the desired concentration in RPMI medium for addition to *C. albicans* cultures. Unopsonized SRBC underwent the same incubations without the addition of antibody and C8ds.

Rosetting of SRBC to C. albicans. 10^5 *C. albicans* hyphal colonies grown in suspension for 18 h in RPMI medium + 0.25% NHS were washed and cultured with 10^8 unopsonized or complement-opsonized SRBC. After 1 h at 37°C, an aliquot of hyphae was taken and examined microscopically for *Candida*-SRBC rosetting. To assay for *C. albicans* growth in the presence of RBC, 250 yeast phase organisms were cultured in microtiter wells as described above, in RPMI medium supplemented with 0.25% heat-inactivated NHS. Medium was removed from wells and complement-opsonized or unopsonized SRBC diluted in RPMI medium plus 5% heat-inactivated C8ds were added to the wells at the indicated concentrations. In these assays, heat-inactivated C8ds is used as a source of transferrin to create an iron-restricted environment. After culture for an additional 18 h at 37°C, the extent of hyphal growth was determined by uptake of ^{3}H-glucose.

mAb LPM19c and 14B6E.2, mAbs specific for the human CR3 α chain (CD11b) (30) and R6.5, a mAb specific for ICAM-1, were generously provided by Dr. T. A. Springer (Harvard Medical School, Boston, MA). mAb to human iC3b neoantigen was obtained from Quidel (San Diego, CA).

Preparation of Radiolabeled Rat RBC (RRBC). ^{59}Fe-labeled RRBC were obtained from anemic Sprague-Dawley rats according to the method described by Pippard et al. (31). ^{59}Fe-labeled RRBC were opsonized using the method described for SRBC, except red cells were coated with anti-RRBC IgM, 1:1,500 dilution, (Accurate Chemical Co., Westbury, NY) before the deposition of complement.

Assay for Uptake of Iron from Radiolabeled RRBC by C. albicans Hyphae. 2×10^4 yeasts were cultured in polypropylene microfuge tubes at 37°C in RPMI medium plus 0.25% heat-inactivated NHS. After 18 h, hyphae were washed and incubated for an additional 2 h with 10^7 complement-opsonized or unopsonized ^{59}Fe-RRBC diluted in RPMI medium plus 2.5% heat-inactivated C8ds. Hyphae were washed with HBSS + 0.1% BSA, followed by three washes with water to lyse RRBC, and a final wash with HBSS-BSA to remove unincorporated radiolabel. The amount of ^{59}Fe taken up after 2 h by *C. albicans* hyphae was counted in a gamma counter.

"Bystander" Rosetting of Human RBC (HRBC) to C. albicans.

[1] *Abbreviations used in this paper:* C8ds, C8-deficient serum; HRBC, human red blood cells; NHS, normal human serum; RRBC, rat red blood cells; SDA, Sabourauds dextrose agar.

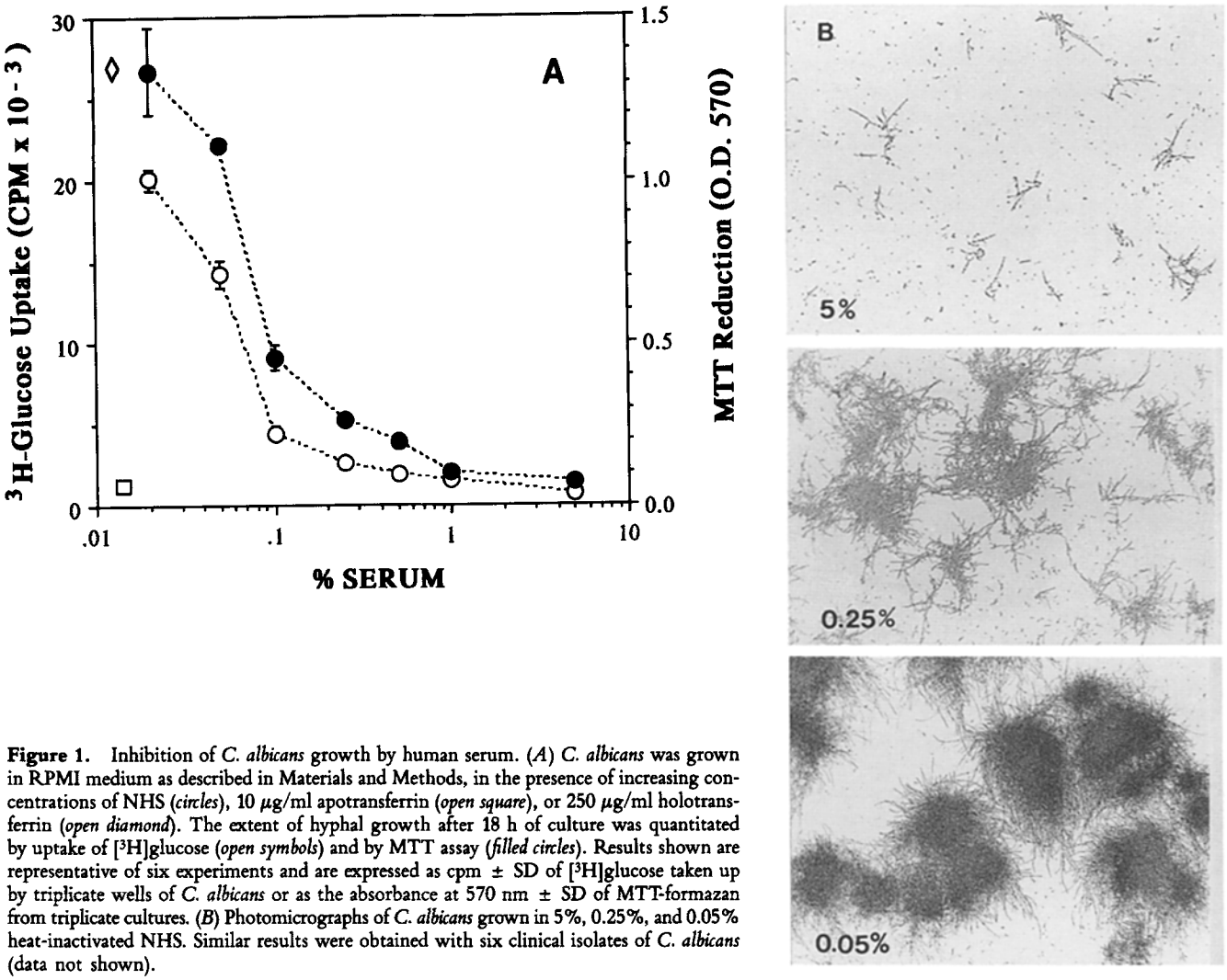


Figure 1. Inhibition of *C. albicans* growth by human serum. (*A*) *C. albicans* was grown in RPMI medium as described in Materials and Methods, in the presence of increasing concentrations of NHS (*circles*), 10 μg/ml apotransferrin (*open square*), or 250 μg/ml holotransferrin (*open diamond*). The extent of hyphal growth after 18 h of culture was quantitated by uptake of [^{3}H]glucose (*open symbols*) and by MTT assay (*filled circles*). Results shown are representative of six experiments and are expressed as cpm ± SD of [^{3}H]glucose taken up by triplicate wells of *C. albicans* or as the absorbance at 570 nm ± SD of MTT-formazan from triplicate cultures. (*B*) Photomicrographs of *C. albicans* grown in 5%, 0.25%, and 0.05% heat-inactivated NHS. Similar results were obtained with six clinical isolates of *C. albicans* (data not shown).

5×10^3 yeasts were cultured in individual wells of 96-well microtiter dishes at 37°C in RPMI medium plus 0.25% NHS. After 18 h, medium was removed and 5×10^6 HRBC diluted in 50 μl RPMI medium plus 10% autologous fresh serum, heat-inactivated serum, C3-depleted serum, or fresh serum treated with EDTA or EGTA/Mg^{2+} were added to the wells. After 1 h at 37°C, wells were washed gently three times to remove excess unbound HRBC and cultures were scored blindly for the presence of *C. albicans*–HRBC rosettes. For a quantitative assay of rosetting, the amount of hemoglobin associated with *C. albicans*–HRBC rosettes was measured. 5×10^5 *C. albicans* yeasts were grown in RPMI medium with 0.25% NHS for 18 h in 1.5-ml microcentrifuge tubes. Medium was removed and hyphae were resuspended in a 50-μl volume of serum plus 2.5×10^7 HRBC. After 1 h at 37°C, 200 μl medium was added and the tubes were incubated for an additional 1 h, during which time nonrosetted HRBC settled into a pellet below the hyphae. Hyphae were carefully removed to fresh tubes without disturbing the unbound RBC pellet, washed once, and resuspended in water to lyse bound RBC. The amount of hemoglobin released by bound RBC was measured by determining the absorbance at 420 nm.

Flow Cytometry. HRBC for flow cytometry analysis were obtained from rosetted and nonrosetted cultures containing *C. albicans* hyphae and fresh or heat-inactivated autologous serum, respectively. Wells were washed three times gently to remove excess unbound HRBC from the wells, followed by more vigorous washing to remove remaining rosetted and nonrosetted HRBC. Cells obtained in this manner were assayed for the presence of complement by incubating for 45 min on ice with a 1:15 dilution of monoclonal anti-iC3b followed by goat anti-mouse Ig-FITC (National Cancer Institute Repository, Bethesda, MD). *C. albicans* hyphae, which are firmly adherent to plastic, are not removed from the wells by this procedure. Fluorescence analysis was performed on a FACScan® analyzer (Becton Dickinson & Co.).

Results

Inhibition of C. albicans Growth in Human Serum and Restoration of Growth by Ferric Nitrate and Hemoglobin. Growth of *C. albicans* hyphae was dramatically inhibited in the presence of increasing concentrations of heat-inactivated NHS, as indicated by a dose-dependent decrease in the uptake of radiolabeled glucose into hyphae (Fig. 1 *A*). Serum-induced growth inhibition was also reflected by a dose-dependent decrease in the reduction of MTT to MTT-formazan by *C. al-*

bicans, as well as by colony size (Fig. 1, *A* and *B*). This inhibitory effect was reproduced by substituting 10 μg/ml purified human apotransferrin for serum in the growth medium (Fig. 1 *A*). The addition of up to 250 μg/ml iron-saturated holotransferrin, in contrast, did not inhibit *Candida* growth. Furthermore, inhibition of *Candida* growth in 5% serum could be completely reversed by the addition of increasing concentrations of ferric nitrate to the growth medium (Fig. 2), indicating that serum-induced growth inhibition is the result of iron restriction.

To determine if *Candida* could utilize hemoglobin as an iron source, medium containing 5% serum was supplemented with increasing concentrations of hemoglobin or heme, the iron-containing moiety of hemoglobin (Fig. 2). Both hemoglobin and heme restored *Candida* growth in serum. Protoporphyrin IX, the heme precursor that does not contain iron, did not, however, support *Candida* growth.

Taken together, these results indicate that growth inhibition of *C. albicans* hyphae in human serum is due to iron restriction, and that *C. albicans* grown under these conditions is unable to efficiently access iron bound by transferrin. Iron from hemoglobin, however, is accessible to *Candida* and restores hyphal growth in serum.

Complement-Opsonized SRBC Rosette C. albicans Hyphae and Restore C. albicans Growth in Serum. Since hemoglobin is sequestered in RBC, heme-derived iron is generally inaccessible to pathogenic microorganisms. We hypothesized that *C. albicans* has a mechanism for obtaining heme-derived iron from RBC, and that this mechanism involves binding of RBC to *Candida* via CR-like molecules. To test this, *C. albicans* hyphae were cultured for 18 h in the presence of SRBC opsonized with complement. Hyphae formed rosettes with opsonized SRBC (Fig. 3 *A*), but not unopsonized SRBC (Fig. 3 *B*), after 1 h in culture. If binding of RBC to complement receptors on *Candida* enables the organism to obtain RBC-derived iron, it would be expected that opsonized, but not unopsonized, SRBC would restore *Candida* growth in serum. As shown in Fig. 3 *C*, in the presence of serum, unopsonized SRBC have a minimal effect on *Candida* growth, but hyphal growth in serum is increased 15–50-fold when opsonized SRBC are added to the cultures. Rosetting of opsonized SRBC to hyphae, therefore, results in enhanced growth of *C. albicans* in human serum.

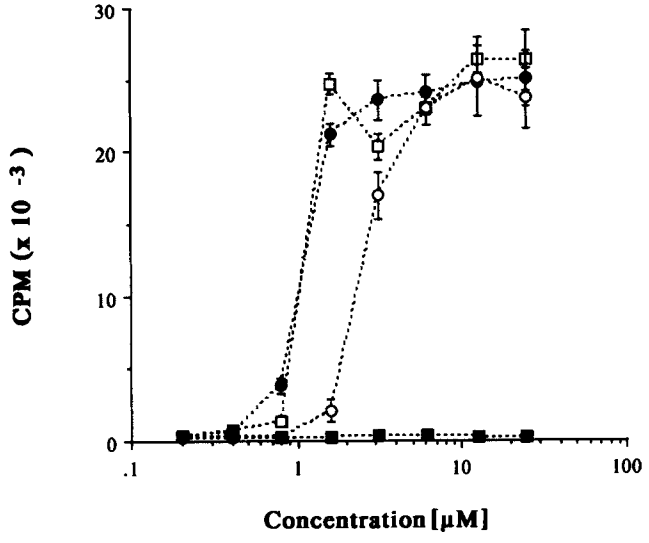
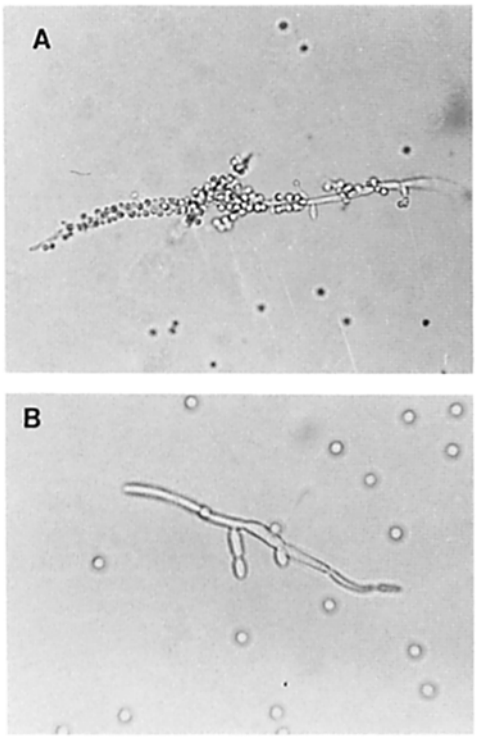


Figure 2. *C. albicans* can utilize heme-derived iron for growth in human serum. *C. albicans* was grown as described in Materials and Methods in RPMI medium supplemented with 5% NHS + equimolar concentrations of hemoglobin (*filled circles*), heme (*open circles*), protoporphyrin IX (PPIX) (*filled squares*), or $Fe(NO_3)_3$ (*open squares*). After 18 h at 37°C, the extent of *C. albicans* growth was quantitated by uptake of [^{3}H]glucose. Results shown are representative of five experiments and are expressed as cpm ± SD of radiolabeled glucose taken up by triplicate wells of *C. albicans*.

mAbs to Human CR3 Block Opsonized SRBC-induced Growth of C. albicans in Serum. Others have shown that certain mAbs to the human CR3 α chain (CD11b) react with *C. albicans* (13, 15, 32). When *C. albicans* hyphae were preincubated with two anti-human CR3 mAbs, LPM19c and 14B6E.2, rosetting to iC3b-coated SRBC was inhibited (data not shown), and the capacity of complement-coated SRBC to restore *Candida* growth in serum was dramatically decreased (Fig. 4). The two antibodies in combination almost completely reversed the opsonized SRBC-induced growth of *Candida* in serum. An isotype-matched control mAb, R6.5, which is specific for ICAM-1, had no effect on opsonized SRBC-induced *Candida* growth. *Candida* growth in iron-sufficient medium, in the absence of serum and SRBC, was not inhibited by mAbs to the CR3 (Fig. 4, legend). These results indicate that binding of RBC to *Candida* hyphae via surface CR-like molecules enhances growth of the organism under conditions of iron limitation.

Uptake of ^{59}Fe from Complement-opsonized, Radiolabeled RBC. To demonstrate directly that *C. albicans* is able to obtain iron from RBC bound to complement receptors, RRBC were radiolabeled in vivo with ^{59}Fe, and the uptake of radiolabeled iron by iron-deprived *Candida* was measured. As shown in Fig. 5, *Candida* grown in serum acquired significantly more radiolabeled iron from opsonized than from unopsonized RRBC. Moreover, uptake of iron from opsonized RRBC was inhibited by antibodies to the human CR3. We conclude, therefore, that *C. albicans* has a mechanism for obtaining heme-derived iron from RBC and that binding of RBC to CR-like molecules on *Candida* is a requisite step in this mechanism.

"Bystander" Deposition of C3 Fragments on RBC as a Result of Alternative Pathway Activation by C. albicans. For *Candida* to utilize the above mechanism for iron acquisition in the human host, the C3-derived ligands for *Candida* CR-like molecules must be present on HRBC. Others have shown that activation of complement can lead to deposition of C3 fragments not only on the surface of the complement activator itself, but also on host tissues in the vicinity of activation (33, 34). Indeed, complement activation by *C. albicans* was found to lead to deposition of complement components along the basement membranes in cutaneous lesions of patients with chronic mucocutaneous candidiasis (35). We hypothesized that in the presence of fresh human serum, complement would



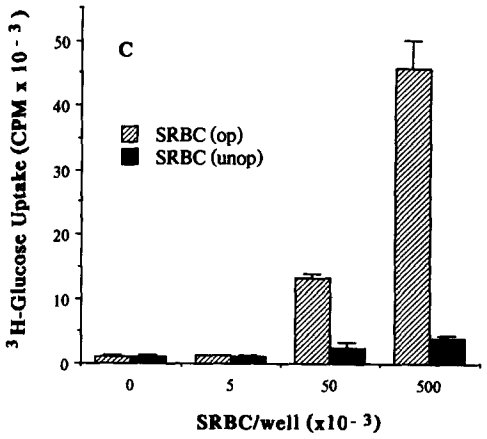


Figure 3. Rosetting of complement-coated SRBC to hyphae restores growth of *C. albicans* in human serum. *C. albicans* hyphae cultured for 1 h with complement-opsonized (*A*) or unopsonized (*B*) SRBC. (*C*) *C. albicans* hyphae were incubated for 18 h with increasing doses of SRBC (opsonized or unopsonized) in medium + 5% C8ds as described in Materials and Methods, and growth in triplicate wells was quantitated by uptake of [^{3}H]glucose. Results shown are representative of three experiments.

become deposited on autologous RBC by a "bystander" mechanism after activation of the alternative complement pathway (36) by *C. albicans*. To test this hypothesis, we cultured *Candida* hyphae with HRBC in the presence of fresh autologous serum or serum treated to inactivate complement. After 1 h at 37°C, the cultures were examined for *Candida*-RBC rosettes. In addition, rosetting was measured spectrophotometrically by the amount of hemoglobin released after water lysis of bound HRBC. Rosetting occurred in all wells in which the alternative complement pathway was intact, including wells that received fresh, autologous serum and serum treated with EGTA/Mg^{2+} (Table 1). Rosetting did not occur under conditions in which the alternative pathway was rendered nonfunctional, including serum that was exposed to heat (56°C, 45 min), treated with EDTA, or immunologically depleted of C3 (Table 1). Photomicrographs of *C. albicans* cultured with HRBC and fresh or heat-inactivated serum are shown in Fig. 6. These results indicate that in an autologous human system, activation of the alternative pathway of complement by *C. albicans* can lead to complement deposition on "bystander" RBC, and consequently to *Candida*-RBC rosetting.

To show directly that C3 degradation fragments may become deposited on HRBC as a result of alternative pathway activation by *Candida*, HRBC that were washed from rosetted and nonrosetted wells were analyzed by flow cytometry for the presence of surface C3 fragments using a mAb specific for human iC3b. A significant percentage of HRBC washed from rosetted wells (those that received fresh serum), but not nonrosetted wells (those that received heat-inactivated

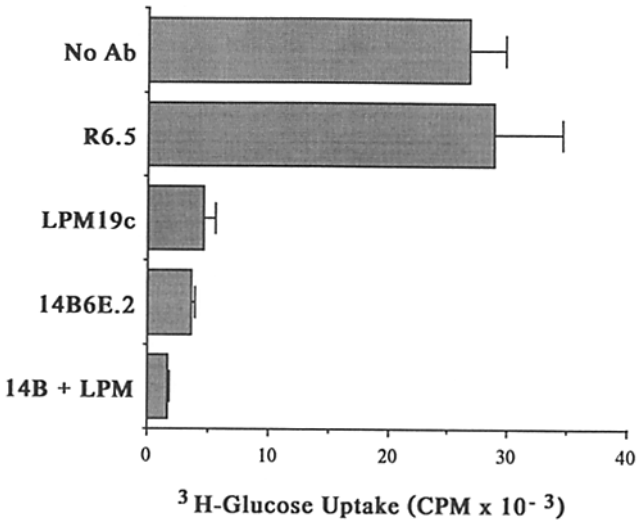


Figure 4. mAbs to human CR3 inhibit SRBC(op)-induced growth of *C. albicans* in human serum. Growth assay was performed as in Fig. 3, except hyphae were preincubated for 30 min at 37°C with 4 μg/well of anti-CR3 mAbs LPM19c and 14B6E.2, or control mAb R6.5 (anti-ICAM-1). 10^5/well opsonized SRBC were added for 1 h, wells were washed free of nonrosetted RBC, and cultures were incubated for 18 h in media containing 5% C8ds. Hyphal growth was quantitated by uptake of [^{3}H]glucose. Results shown are from one of two experiments and are expressed as cpm ± SD of radiolabeled glucose taken up by triplicate samples of *C. albicans*. In a separate experiment, *C. albicans* grown for 18 h in RPMI medium alone yielded 57,081 ± 2,219 cpm; *C albicans* grown in RPMI medium + 4 μg/well LPM19c yielded 53,276 ± 1,000 cpm; and *C. albicans* grown in RPMI medium + 4 μg/well 14B6E.2 yielded 70,482 ± 6,795 cpm.

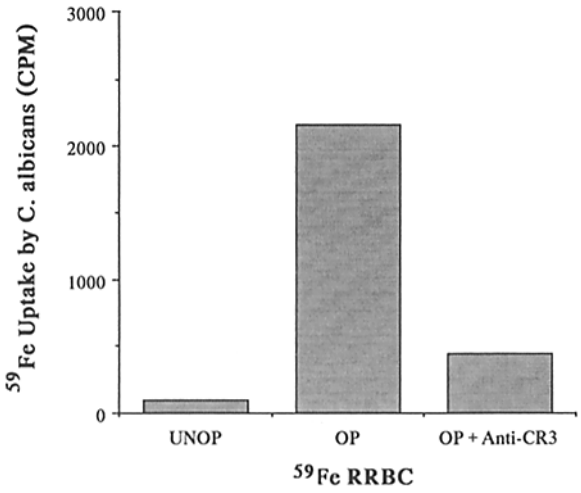


Figure 5. *C. albicans* hyphae take up radiolabeled iron from complement-coated RRBC. *C. albicans* were cultured with opsonized or unopsonized ^{59}Fe-labeled RRBC as described in Materials and Methods. In one sample, hyphae were preincubated for 30 min at 37°C with 4 μg/well anti-human CR3 mAb LPM19c before addition of opsonized RRBC. Results are expressed as cpm of ^{59}Fe taken up by *C. albicans*. Results shown are from one of three experiments. In three independent experiments, the ratio of cpm taken up by *C. albicans* from opsonized RRBC compared with unopsonized RRBC ranged from 3 to 10.

Table 1. *Rosetting as a Result of Alternative Pathway Activation by C. albicans*

Serum	Rosetting	OD_{420}
Fresh	+	1.39 ± 0.38
Heat inactivated	−	0.12 ± 0.01
C3 depleted	−	0.04 ± 0.03
Fresh + EDTA (10 mM)	−	0.02 ± 0.03
Fresh + EGTA/Mg^{2+} (10 mM/10 mM)	+	1.59 ± 0.27

C. albicans hyphae were cultured with HRBC and 10% fresh autologous serum, heat-inactivated (56°C, 45 min) serum, serum depleted immunologically of C3, or serum chelated with EDTA, or EGTA/Mg^{2+}. After 1 h at 37°C, cultures were scored blindly for *Candida*-HRBC rosettes (+/−). In addition, rosetting is expressed as the amount of hemoglobin associated with HRBC bound to *Candida*, and was determined by measuring the absorbance of hemoglobin at 420 nm as described in Materials and Methods. Results are representative of three experiments.

serum), were positive for bound human iC3b (Fig. 7). These results indicate that activation of the human alternative pathway by *Candida* in the presence of HRBC leads to deposition of the ligands for *Candida* CR-like molecules on the surface of host RBC by a "bystander" mechanism, resulting in *Candida*-RBC rosetting.

Discussion

Molecules that mimic mammalian complement-binding proteins are expressed by various pathogenic microorganisms. Expression of CR-1-like molecules by HSV (37, 38) and EBV (39), and of a decay-accelerating factor–like protein by *Trypanosoma cruzi* (40), have been previously reported. In each case it has been speculated that these molecules facilitate survival in the host, possibly by interfering with host complement–mediated lysis (38–41). Expression of complement-binding molecules on *C. albicans* has also been demonstrated (12, 13). Since these proteins represent potential virulence factors of the organism (12, 13, 16), it is of interest to determine their function(s).

The human host presents potential pathogens with a highly iron-restricted environment. As a result of their need for iron, pathogenic microorganisms have adapted a variety of mechanisms for obtaining host-derived iron (25, 42–44). In fact, the possession of an efficient mechanism for obtaining host-derived iron is a factor that has been shown in some systems to distinguish virulent from avirulent bacterial strains (25). To access iron from the host, many bacteria and fungi produce siderophores, low molecular weight molecules with high iron-binding affinities. These molecules are able to compete successfully with host iron-binding proteins for elemental iron (25). In addition, some bacteria obtain iron from transferrin by directly binding transferrin-iron complexes (43), while others have the ability to utilize heme-containing compounds (44–46).

Numerous studies have demonstrated serum-induced growth inhibition of *C. albicans* (17–22). In these studies, inhibition was attributed to a variety of factors (20–22), however, a number of reports have related serum-induced growth inhibition to the presence of transferrin (18, 23, 24). In the latter studies it was demonstrated that growth inhibition could be reversed by the addition of exogenous iron. These studies indicated that iron is necessary for the growth of *C. albicans*. The mechanism(s) by which *C. albicans* acquires iron for survival in the iron-limiting environment of the human host are largely unknown.

In the present report we propose a novel mechanism by which *C. albicans* may obtain iron for growth in the human host, which involves the use of CR-like molecules. Specifically, we have shown that expression by *C. albicans* of a molecule with iC3b-binding activity enables the organism to bind host RBC and obtain heme-derived iron for growth. Evidence to support this model is as follows: (*a*) *C. albicans* hyphae rosette complement-opsonized, but not unopsonized RBC; (*b*) in the presence of inhibitory concentrations of serum, growth of *C. albicans* is increased up to 50-fold as a result of *Candida*-RBC rosetting. Unopsonized, non-rosetted RBC have little effect on *Candida* growth in serum; (*c*) rosetting of *Candida* to complement-opsonized RBC is inhibited by monoclonal antibodies to the human CR3. Inhibition of rosetting correlates with inhibition of opsonized SRBC-induced *Candida* growth; (*e*) rosetting of hyphae to opsonized, radiolabeled RBC results in the uptake of radiolabeled iron by *Can-*

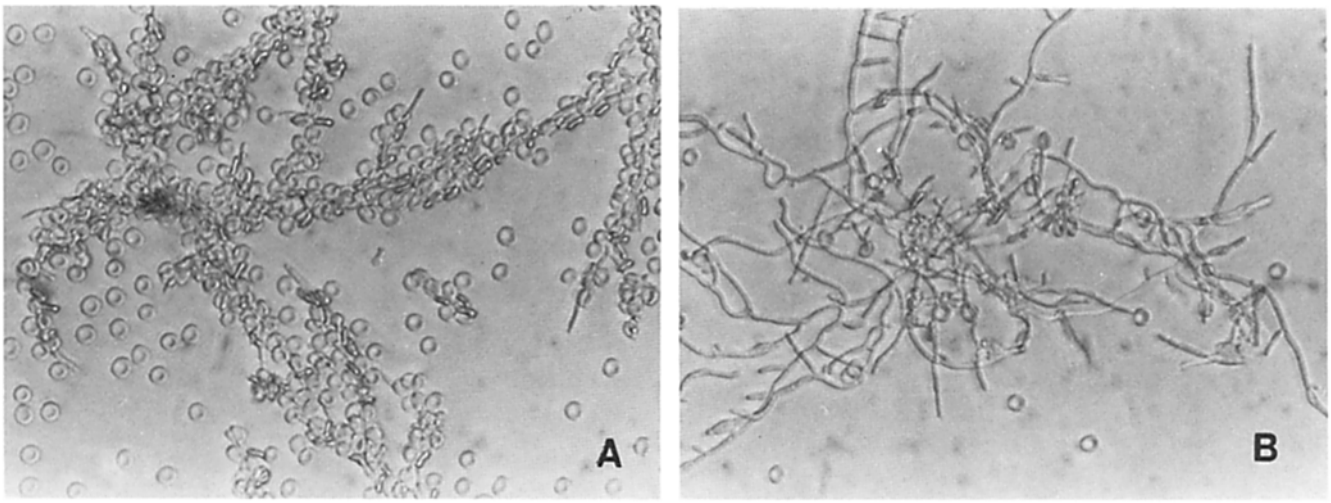


Figure 6. Activation of the alternative pathway of complement by *C. albicans* results in "bystander" deposition of complement on autologous RBC. Photomicrographs of *Candida* hyphal colonies that were cultured for 1 h as described in Materials and Methods with HRBC + 10% fresh autologous serum (*A*) or heat-inactivated autologous serum (*B*).

dida. Radiolabeled iron uptake is inhibited by antibodies to the human CR3.

That unopsonized RBC do not restore *Candida* growth in the manner observed with opsonized RBC indicates that close contact between *Candida* and RBC, mediated by a specific receptor-ligand interaction, is a necessary prerequisite for uptake of RBC-derived iron by *Candida*. The importance of this interaction for uptake of RBC-derived iron is further substantiated by the blocking of this process by mAbs to the human CR3 (Figs. 4 and 5). The events that occur subsequent to *Candida*-RBC binding and before iron uptake are presently unknown, and are currently under investigation. Preliminary data, however, suggest the involvement of a *Candida*-derived hemolysin in this process (D. M. Mosser, unpublished data).

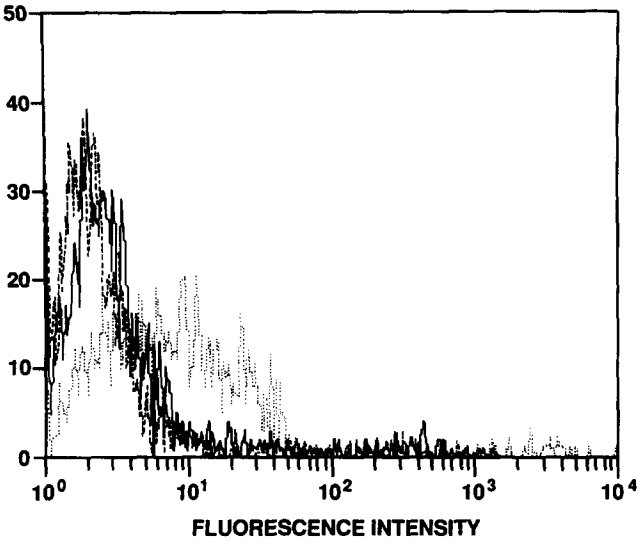


Figure 7. iC3b is present on HRBC cultured with *C. albicans* and fresh autologous serum. HRBC obtained as described in Materials and Methods from rosetted and nonrosetted *Candida* cultures were stained with anti-human iC3b mAb followed by goat anti-mouse Ig FITC-conjugated antibody and analyzed by flow microfluorimetry for iC3b expression. The dashed line represents the goat anti-mouse Ig FITC control, the solid line represents heat-inactivated serum, and the dotted line represents fresh serum. Results shown are representative of four experiments.

The production of siderophores by *C. albicans* has been reported (42, 47), but the conditions under which *Candida* utilizes these molecules have not been described in detail. It is clear that in our system, however, *Candida*-derived siderophores do not function efficiently (Fig. 1). *C. albicans*, in addition to being a potential pathogen, is a commensal organism in the human gut, where an abundance of nutrients, including elemental iron, are available as a result of digestive processes. It is possible that under circumstances such as these, in which iron is not bound by transferrin, that *Candida*-derived siderophores operate to scavenge iron. Acquisition of iron from RBC by the mechanism that we describe may be important in disseminated candidiasis, at sites of invasive infection such as kidney and liver, where elemental iron is not readily available and where there is an abundance of RBC. In addition, *C. albicans* may utilize this mechanism in mucocutaneous lesions in which the organism has penetrated the superficial layers of the skin resulting in an inflammatory response (35, 48). It is likely that the ability to utilize more than one mechanism for iron acquisition, depending upon the local environment, would contribute to the pathogenic versatility of the organism.

The method of iron acquisition that we propose would be dependent upon the presence of the CR3 ligand, iC3b, on the surface of host RBC. It is a well described phenomenon that complement activation can lead not only to deposition of complement component C3b on the surface of the complement activator, but also in the fluid phase (49) and on

the surface of surrounding host tissues (33, 34). In lesions of patients with chronic mucocutaneous candidiasis (35), as well as in an experimental animal model (48), C3 fragments were detected on the surface of host tissues as well as on the surface of *C. albicans* as a result of complement activation. Similarly, we have shown that activation of the alternative complement pathway by *C. albicans* can result in deposition of C3 fragments on the surface of surrounding autologous RBC (Figs. 6 and 7). Once deposited by this "bystander" effect, C3b would become degraded by serum factors H and I to its inactive form, iC3b, the CR3 ligand. Moreover, this process would be facilitated by the presence of CR1 on HRBC (50). CR1 has factor I cofactor activity for the conversion of C3b to iC3b, and further to C3dg (50), a fragment that has also been shown to bind *Candida* CR-like molecules (12–14).

Others have proposed that the *Candida* CR3, like the mammalian CR3, which is a member of the integrin family of proteins (51), functions as an adhesion molecule, promoting the adherence of *Candida* to host tissues (16, 52). The host tissue ligand for this interaction, however, has not been determined. In light of the data presented here, the possibility that C3 degradation fragments may also serve as adherence ligands for *Candida* CR-like molecules deserves consideration.

In summary, we propose a novel mechanism of iron acquisition by *C. albicans*, which involves the use of molecules that mimic host complement receptors. In addition, we show that *C. albicans* can utilize hemoglobin from RBC as an iron source. To our knowledge, this is the first report of the utilization of a heme-containing compound as an iron source by a pathogenic fungus. We are currently investigating whether *C. albicans* acquires RBC-derived iron by a hemoglobin receptor, similar to certain bacteria (T. L. Stull, unpublished results).

The predisposition of certain immunocompromised patients to infection with *C. albicans* is clearly a result of host factors such as leukocyte deficiencies and prolonged exposure to chemotherapeutic agents (4). However, the ability of *C. albicans* to persist in mucocutaneous and disseminated lesions may be facilitated by the ability of the organism to exploit the host complement system to acquire and utilize host RBC–derived iron. The use of CR-like molecules for iron acquisition by *C. albicans* may therefore be an important virulence factor of the organism.

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