The effects of monoclonal antibodies against iC3b receptors in mice with experimentally induced disseminated candidiasis

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SUMMARY

CR3 (iC3b receptor), composed of CD11b/CD18, is a β2 integrin. A protein that shares antigenic and structural homology with the α-chain of CD11b/CD18 has been isolated from the surface of Candida albicans. This molecule is thought to be essential in the pathogenesis of disseminated candidiasis. To evaluate the effects of anti-iC3b receptor antibodies on adhesion between human dermal microvascular endothelial cells (HDMEC) and C. albicans, and in treatment of candidal infection, a binding assay of C. albicans to cultured HDMEC was performed in vitro. An anti-iC3b receptor-specific monoclonal antibody was administered to mice infected with C. albicans. The mice were monitored for mortality and renal involvement by culture and histopathological findings. Flow cytometric analysis demonstrated surface expression of iC3b receptor on C. albicans. The adherence of C. albicans to HDMEC was significantly decreased by treatment with anti-iC3b receptor antibodies. Anti-iC3b receptor antibodies significantly increased the survival time and rate while lowering the renal fungal burden. The iC3b receptors are involved in the adherence of C. albicans to vascular endothelial cells and are likely to be involved in the pathogenesis of disseminated candidiasis. The increased survival in mice infected with C. albicans after treatment with anti-iC3b receptor antibodies indicates that this modality may be beneficial for future development of a new therapy for candidiasis.

INTRODUCTION

Adherence of a pathogen to host tissue is essential in the pathogenesis of infectious disease and presumably takes place in all forms of candidiasis. Studies concerning the modification or prevention of infection in relation to adhesion mechanisms have been performed in animal and human models.1,2 The virulence of a species of Candida can be correlated with its ability to adhere to various cell types, with the most virulent C. albicans being most adherent to epithelial cells. In animal models of invasive disease, the pathogenicities of candidal strains in vivo correlate directly with yeast adhesion in vitro.3 Endothelial attachment and subsequent penetration are requisite stages in the induction of haematogenously disseminated candidiasis.

Receptors [complement receptor (CR)1, CR2 and CR3] for C3 degradation fragments (C3b, iC3b, C3dg) are present on many human cells operative in the immune and inflammatory host defence process and may be critical in the attachment of invading micro-organisms.3 C3-binding structures are not specific for mammalian cells in general but can also be found in micro-organisms pathogenic for humans. CR3 is present on the surface of mammalian neutrophils, monocytes and phagocytes and has been known as CD11b/CD18, Mol, Mac-1, or iC3b receptor. It is composed of an α-chain of 165000 MW and a 95000 MW β-chain common to all leucocytes. There are iC3b receptors on the surface of C. albicans which share homology with the α-chain of the neutrophilic receptors for iC3b.4-6 Western blotting and immunoprecipitation have confirmed the similar relative molecular weights of CR3 on the human neutrophil and the iC3b receptors isolated from C. albicans.6,7

Since in disseminated candidiasis the fungus is delivered to target organs through the vascular system, targets for adherence of blood-borne fungi ultimately include the endothelial cell. Experiments on adhesion of C. albicans to cultured human umbilical vein endothelial cells (HUVEC) demonstrate that increasing glucose concentrations increase adhesion and monoclonal antibodies (mAb) recognizing epitopes on neutrophil CR3 inhibit glucose-enhanced adhesion of C. albicans to HUVEC.8 However, the exact role of iC3b receptors in haematogenously disseminated candidiasis in humans has not been elucidated.
We assessed the role of iC3b receptors on *C. albicans* in the adhesion of yeast to cultured human dermal microvascular endothelial cells (HDMEC), and the inhibition of this adhesion by mAb against iC3b receptors. In addition, we observed the effects of intravenous injection of anti-iC3b receptor mAb with respect to mortality and long-term infection in mice with experimentally induced disseminated candidiasis.

**MATERIALS AND METHODS**

**Organisms**

One randomly selected pathogenic strain of *C. albicans* (American Type Culture Collection, Rockville, MD; ATCC 44808) recovered from human blood was used for these experiments. Yeast cells were grown to log-phase in Sabouraud's broth, as determined by colony counts and spectrophotometric measurement of OD at 420 nm, and were then frozen at −70 °C until use. On the evening before the experiment, aliquots of frozen yeasts were inoculated into RPMI-1640 media (Gibco Laboratories, Grand Island, NY). After attaining log-phase growth, the yeasts were pelleted, washed three times with phosphate-buffered saline (PBS) and diluted spectrophotometrically to an OD of 0.5 at 420 nm.

**Animals**

BALB/c mice (Daehan Laboratory Animal Research Centre, Seoul, Korea) weighing around 25 g were used for all experiments.

**Monoclonal antibodies**

Four mAb directed against epitopes on a subunit of CR3 were Mol (mouse IgM, Coulter Immunology, Hialeah, FL), 44 (mouse IgG1, R&D Systems, Minneapolis, MN), Leu 5 (mouse IgG2a, Becton Dickinson, San Jose, CA), and M1/70. M1/70 (rat IgG2b) was purchased as a hybridoma (ATCC TIB 128) and was purified from supernatant by affinity chromatography on protein G-Sepharose (Hitrap Protein G column, Pharmacia Biotech, Uppsala, Sweden). The mAb ECCD-2 (anti-E-cadherin, rat IgG2a) was obtained from M. Takeichi (Kyoto University, Kyoto, Japan). ECCD-2 was purified from hybridoma supernatants by protein G-Sepharose affinity chromatography. MEM 48 (mouse IgG1, R&D Systems, Minneapolis, MN) is a mAb directed against the common β-chain of CR3, leucocyte function associated antigen-1 (LFA-1).

**Flow cytometric analysis**

Fifty million colony-forming units (CFU)/ml of *C. albicans* were washed in PBS with 0.5% bovine serum albumin (BSA), counted and aliquotted for staining. Primary antibody at the appropriate dilution was added to each tube. Either an isotype control mAb or PBS without antibody was used as a first-step control with identical results. The cells were incubated for 30 min on ice, washed, and second-step antibody, diluted 1:20 in PBS with 0.5% BSA, was added. After another 30 min of incubation, the cells were washed and resuspended in 0.5 ml PBS with 0.5% BSA. Propidium iodide was added immediately before flow cytometric analysis to identify dead cells. Fluorescence was examined by a Becton Dickinson FACStar flow cytometer (Becton Dickinson, Mountain View, CA) and expressed as log mean channel fluorescence.

**Isolation and culture of HDMEC**

HDMEC were isolated from human neonatal foreskins by trypsinization and Percoll gradient centrifugation as described previously. Briefly, foreskins were cut into small pieces, treated with 0.3% trypsin (Sigma Chemical Co., St Louis, MD), and 1% ethylenediamine tetraacetic acid (EDTA; Sigma) and individual segments were compressed with a scalpel blade to express microvascular fragments. The microvascular segments were layered onto a 35% Percoll (Pharmacia AB, Sweden) gradient in Hank's balanced salt solution (HBSS) and spun at 400 g for 15 min at room temperature. The fraction with a density less than 1.048 g/ml was applied to gelatin (Sigma) - precoated tissue-culture dishes and cultured in endothelial basal media (Clonetics Corp., San Diego, CA) with epidermal growth factor 1 ng/ml (Clonetics), hydrocortisone acetate 1 μg/ml (Sigma), dibutylryl cyclic AMP 5 × 10−5 M (Sigma), penicillin 100 U/ml, streptomycin 100 μg/ml, amphotericin B 250 μg/ml (Sigma), and 30% human serum (Irvine Scientific, Santa Ana, CA). The resulting cell cultures were free of contaminating fibroblasts as assessed by expression of CD31 (Fig. 1) and uptake of acetylated low-density lipoproteins. Experiments were conducted with endothelial cells at passages 2–6.

**Candida albicans–HDMEC adherence assay**

HDMEC were plated in gelatin-coated 96-well flat-bottomed culture plates. An isolate of *C. albicans* was grown in the RPMI medium, labelled with [35S]methionine, washed extensively in PBS and resuspended in HBSS with calcium and magnesium, to which was added 1% BSA (BSA-HBSS*+*). Yeast cells were then held at 4 °C to prevent germination before incubation with HDMEC (5% germ tubes present for *C. albicans*). Then 4 × 10^4 CFU were added to each well of confluent HDMEC monolayer, and incubated for 1 hr at 37 °C. For quantification of non-specific bindings, a 100-fold excess of unlabelled yeast cells were added to labelled yeast cells. The supernatant was removed from each well and the monolayers washed twice with BSA-HBSS*++* to remove non-adherent yeasts. These were counted in a scintillation counter (Beckman Instruments, Inc., Palo Ato, CA). Adherent yeast cells and HDMEC monolayers were removed with 1% Triton-X (Sigma), then solubilized in 0.5 M NaOH and counted in a

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**Figure 1.** Flow-cytometric analysis showing the expression of CD31 on HDMEC.
β-scintillation counter. The percentage binding of *C. albicans* was calculated as follows:

\[
\frac{C.\ p.m. \ (\text{adherent yeasts} - \text{non-specific binding})}{C.\ p.m. \ (\text{total yeasts added} - \text{non-specific binding})} \times 100.
\]

**Inhibition of adhesion with mAb**

*C. albicans* was grown in RPMI medium and was then preincubated with 100 μl of mAb to α-subunit of CR3 for 45 min. 100 μl of 20 μg/ml of mAb were used. After preincubation with antibodies, the adhesion assay was performed as described above in the continuous presence of antibodies. All the individual experiments were performed three times independently.

**Experimental infection**

Mice were divided into three experimental treatment groups for the assessment of anti-iC3b receptor antibody on the resulting prevention of systemic candidiasis. The three groups were: Group I, controls, received the vehicle for the mAb (0.5% human serum albumin) and was inoculated with one dose of 10^6 CFU of *C. albicans* grown in RPMI-1640 per mouse into the lateral tail veins of a group of 45 normal mice; Group II, mAb M1/70, beginning before infection received mAb M1/70 2 mg/kg infused intravenously once per day for 5 days (day 0 to day 5). Group III, isotype control, beginning before infection received mAb ECCD-2 2 mg/kg infused intravenously once per day for 7 days (day 0 to day 5); The fourth group received only equivalent amounts of mAb M1/70 at the same time. All animals were monitored for 30 days, and mortality was recorded. Two groups of 20 mice each were studied, an only *C. albicans*-inoculated group and a group treated anti-M1/70 mAb and inoculated with *C. albicans* as previously described. On days 0, 2, 4, and 6, five mice per experimental group were selected at random and killed by cervical dislocation. The kidneys of each of these animals were removed aseptically, transferred into sterile polyethylene bags, and homogenized in 5 ml of sterile saline. Samples were removed from each homogenate, serially diluted in 0.9% NaCl, plated onto Sabouraud dextrose agar plates, and incubated at 25°C for 16 hr. The colonies were then enumerated, and CFU were calculated per gram of each organ. All experiments were performed in triplicate. To document the administration of M1/70 mAb sufficient to produce antibody excess, samples of serum were analysed for the presence of residual M1/70 antibody. Blood was collected from the orbital, venous plexus. This was assessed by indirect immunofluorescence analysis in which *C. albicans* were incubated in serum for 30 min at 25°C, and then, after washing, incubated in fluorescein isothiocyanate (FITC)-conjugated goat anti-rat immunoglobulin for an additional 30 min at 25°C. Antibody binding to *C. albicans* was quantified by flow cytometry. Formalin-fixed portions of kidneys obtained at 2, 4 and 6 days were stained with periodic acid-Schiff reagent (PAS) to search for fungi in kidneys.

**Statistical analysis**

All data are expressed as mean ± standard error. All differences in survival were analysed by the Wilcoxon rank sum test and Kruskal–Wallis test. Product limit method was used to analyse survival data and repeated ANOVA were applied to compare the mean CFU per mean kidney weight. Statistical significance was defined as a *P*-value of <0.05.

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**RESULTS**

**Expression of iC3b receptor on *C. albicans***

The iC3b receptor expression was observed by immunofluorescence flow cytometry from *C. albicans* pretreated with anti-iC3b receptor antibody purified from culture supernatant of ATCC M1/70 antibody by affinity chromatography (Fig. 2).

**Effect of anti-iC3b receptor antibody on adhesion of *C. albicans* to HDMEC**

Preliminary studies were done to compare the rate of adhesion between HDMEC and *C. albicans* according to the number of *C. albicans* and elapsed time. The binding of 5 × 10^5 CFU, 1 × 10^6 CFU and 2 × 10^6 CFU of *C. albicans* to HDMEC were 25±1 ± 3.8%, 29±9 ± 2.2% and 28±3 ± 1.5%, respectively. The adherence increased significantly after 1 hr of culture. Further studies were carried out under culture conditions with 10^6 CFU of *C. albicans* being cultured for 1 hr.

The adhesion of *C. albicans* without any prior treatment to HDMEC was 24±0 ± 3.6%. Adherence was 25±4 ± 5.4%, 27±9 ± 1.8%, 26±3 ± 0.3% and 16±5 ± 2.2% when *C. albicans* was treated before inoculation with rat IgG2b, mAb ECCD-2, anti-CD18, and M1/70 mAb, respectively (Fig. 3).

**Effect of anti-iC3b receptor antibody on mortality in mice with experimentally induced disseminated candidiasis**

Preliminary studies were performed to determine the mortality rates of BALB/c mice according to the number of *C. albicans*. All mice injected with 5 × 10^6 CFU of *C. albicans* cultured in RPMI-1640 media expired within 1 day. At 1 × 10^6 CFU and 5 × 10^5 CFU, the average survival durations were 2±7 ± 0.6 days and 4±3 ± 0.6 days, respectively. Inoculation was done with 1 × 10^6 CFU of *C. albicans* to determine mortality and effects of the anti-iC3b receptor antibodies. Group I was inoculated with only *C. albicans*. Group II was pretreated with...
The mAb ECCD-2 with experimentally induced at M1/70 with inoculated C1997 mice in both all 45 mice cytometry initial albicans collected from the surface of C. albicans with inoculation concentrations of 20 mg/ml at 37°C for 45 min, and incubated with HDMEC. The results are shown in Table 3. Inoculation group mAb-treated C. albicans+control mAb§ anti-iC3bM1/70 only§ Group I was inoculated with only C. albicans.

<table>
<thead>
<tr>
<th>Inoculation group</th>
<th>No. of animals</th>
<th>Survival duration (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans only*</td>
<td>45</td>
<td>2.7±0.7</td>
</tr>
<tr>
<td>C. albicans + mAb M1/70†</td>
<td>45</td>
<td>5.3±1.8</td>
</tr>
<tr>
<td>C. albicans + control mAb‡</td>
<td>45</td>
<td>3.4±0.9</td>
</tr>
<tr>
<td>mAb M1/70 only§</td>
<td>45</td>
<td>&gt;30</td>
</tr>
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*Group I was inoculated with only C. albicans. †Group II was inoculated with C. albicans and intravenously injected with anti-iC3b receptor antibody M1/70 at 2 mg/kg of concentration. ‡Group III was intravenously injected with mAb ECCD-2 at 2 mg/kg of concentration and inoculated with C. albicans. §Group IV was injected with only anti-iC3b receptor antibody M1/70.

days after inoculation. All mice in Group II expired between 2 and 10 days after inoculation. However, the mortality rate in Group II at 4 days after inoculation was 31.1%, which was significantly lower than the mortality rates at this time in Groups I and III (P=0.0001, Fig. 4). The average survival durations for Groups I, II and III were 2.7±0.7 days, 5.3±1.8 days, and 3.4±0.9 days, respectively, (Table 1). These differences were significant. White blood cell counts and morphology of mouse neutrophils were not different between the irrelevant mAb-treated group and the M1/70-treated group.

**Effect of anti-iC3b receptor antibody on renal invasion of C. albicans in mice with experimentally induced disseminated candidiasis**

To evaluate the effects of anti-iC3b receptor antibody in disseminated candidiasis, mice were injected intravenously with 5×10^6 CFU of C. albicans cultured in RPMI-1640. The mice were killed at random and the kidneys were removed, homogenized and cultured on a Sabouraud dextrose agar plate.

**Figure 4. Effect of anti-iC3b receptor antibody on mortality of mice with experimentally induced disseminated candidiasis.** Group I was inoculated with only C. albicans (○). Group II was inoculated with C. albicans and intravenously injected with anti-iC3b receptor antibody M1/70 at 2 mg/kg (▲). Group III was intravenously injected with mAb ECCD-2 at 2 mg/kg and inoculated with C. albicans (■). Group IV was injected with only anti-iC3b receptor antibody M1/70 (▼). The mortality and survival duration of the four groups were compared.

**Figure 5. Effect of anti-iC3b receptor antibody on renal invasion of mice with experimentally induced disseminated candidiasis.** Group I was inoculated with only C. albicans. Group II was inoculated with C. albicans and intravenously injected with anti-iC3b receptor antibody M1/70 at 2 mg/kg. Renal specimens were harvested every 2 days for 6 days to evaluate the invasion of kidneys. A quantitative culture assay was performed three times for each mouse. The average CFU of C. albicans/g of kidney were compared.
Figure 6. (a) Histopathological findings of kidney of mice with experimentally induced disseminated candidiasis and treated with anti-iC3b receptor antibody (PAS stain × 40). (b) Higher magnification (×400). Group I was inoculated with only *C. albicans*. Group II was inoculated with *C. albicans* and intravenously injected with anti-iC3b receptor antibody M1/70 at 2 mg/kg. Renal specimens were harvested every 2 days for 6 days to evaluate the invasion of kidneys. Specimens were paraffin-embedded and stained with periodic Schiff reagent. This staining revealed many fungal spores and hyphae in the glomerulus at the second day of infection in Group I, whereas Group II showed only few fungal spores and hyphae. G-I-2, Group I-day 2; G-I-4, Group I-day 4; G-I-6, Group I-day 6; G-II-2, Group II-day 2; G-II-4, Group II-day 4; G-II-6, Group II-day 6.
On the days 2, 4 and 6 of infection, Group I had 3.0 ± 0.2 × 10^6 CFU, 3.3 ± 1.8 × 10^6 CFU, 3.2 ± 0.1 × 10^7 CFU of C. albicans, respectively, in 1 g of kidney. By comparison, Group II had 1.6 ± 1.3 × 10^6 CFU, 2.8 ± 1.2 × 10^5 CFU, 1.9 ± 1.2 × 10^4 CFU of C. albicans, respectively, in 1 g of kidney. These differences in renal involvement were statistically significant (P=0-0001, Fig. 5).

Periodic acid-Schiff staining of the extracted renal specimens revealed many fungal spores and hyphae in the renal medulla on day 2 of infection in Group I. Group II showed only a few fungal spores and hyphae on day 2 of infection. Group I showed heavy infiltration of inflammatory cells at the sixth day of infection (Fig. 6).

**DISCUSSION**

The importance of the adherence of Candida in the pathogenesis of disseminated disease is increasingly recognized. There have been reports that pathogenicity of candidal strains in vivo correlate directly with yeast adhesion in vitro. However, studies on fungus-to-host cell adhesion mechanisms have been relatively sparse. It is possible to categorize these adhesion mechanisms by considering the components of each ligand-receptor. The first category includes protein–protein interactions such as the iC3b receptor on C. albicans. The second category includes interactions in which a surface Candida protein with lectin activity recognizes sugar residues associated with the host-cell plasma membrane. The third category involves a carbohydrate ligand, mannan, on the Candida cell surface that is recognized by an unknown host-cell receptor. Complement fragments adhere more readily to Candida than to endothelial cells in vitro. CR3 on neutrophils is involved in recognition of iC3b on the surface of micro-organisms and subsequent phagocytosis. The iC3b receptor on the surface of C. albicans has recently been isolated and characterized. The iC3b receptor in Candida shares antigenic and structural homology with CR3 on neutrophils.

As we, and others, have observed the expression of iC3b receptor is usually below 50% depending on the type of mAb used, condition of culture medium, species or strains of Candida used, etc. The low expression in our study was due to the RPMI medium with low glucose concentration. When the glucose concentration was increased, the expression of iC3b receptor was up-regulated (unpublished results). However, the difference in the expression could be attributed to the type of mAb used.

The relationship between hyperglycaemia and candidal infection has been well documented. However, the exact mechanism by which sensitivity to candidal infection is increased is unknown. There have been reports that expression of iC3b receptor on Candida and adhesion to cultured HUVEC correlate with glucose concentrations. Moreover, adhesion to cultured HUVEC is suppressed by 50–80% with mAb directed against the α-chain of the iC3b receptor.

As there are marked differences between the endothelial cells of various vessels, and as C. albicans infects the skin and mucous membranes easily, we performed adhesion experiments using cultured HDMEC isolated from skin. Our results show that adherence of C. albicans correlates with reaction time and number of organisms. But there was a marked decrease when anti-iC3b receptor mAb were added. These findings are similar to previous studies using HUVEC.

Intercellular adhesion molecule-1 (ICAM-1), which acts as a ligand for neutrophil CR3, has no role in the adhesion of iC3b receptor on C. albicans. Recently, the host ligand for the iC3b receptor on C. albicans has been characterized as iC3b. C3 is synthesized from endothelial cells and it is plausible to assume that C3 is degraded by proteolytic enzymes in the serum or cell with the resultant iC3b attaching to the endothelial surface and acting as a ligand for the iC3b receptor.

In the present study, binding of C. albicans to HDMEC was partially inhibited by mAb M1/70. This would be explained by the finding of 50 000, 100 000, 130 000 and 165 000 MW proteins in extracts of C. albicans reacting with anti-iC3b receptor antibodies in immunoblotting and immunoprecipitation studies. Hence, the combination of this variability of the iC3b receptor epitopes and specificity of each mAb might have resulted in the partial inhibition as seen in our study.

We confirmed that the iC3b receptor on C. albicans is essential for adhesion to cultured HDMEC. Thus we assumed that prevention of the yeast-to-host cell adhesion could block the dissemination of Candida to surrounding tissue. Haematogenously disseminated candidiasis was induced in mice by intravenous injection with C. albicans. The mortality rate of the group injected with anti-iC3b receptor mAb was significantly lower than that of the normal control. The 50% survival rate increased significantly. The amount of yeast and the extent of invasion of renal tissue was significantly less. The affinity constant for the iC3b receptor is 2.1 ± 0.5 × 10^6 L/M for human neutrophils and virtually identical to that of C. albicans. However, Candida possess 2 × 10^6 iC3b receptors per yeast which is nearly 100 times higher than neutrophils. The increased survival rate in mice treated with anti-iC3b receptor antibodies indicates that this modality might be beneficial for future development of a new therapy for disseminated candidiasis.

The iC3b receptor on Candida is homologous in structure to human CR3, however, it is not the same molecule. There is no doubt that antibodies directly against the Candida iC3b receptor would be likely to bind better to Candida and to have better inhibitory effects. However, at this time, we could not obtain the purified candidal iC3b receptor and its mAb. It is desirable that similar experiments using the above antibody be carried out as a next step to evaluate the clinical usefulness of anti-iC3b receptor antibody in the treatment of haematogenously disseminated candidiasis.

The anti-iC3b receptor mAb may have deleterious effects on host neutrophils and aggravate the course of disease, but our in vivo results indicate that they have a therapeutic effect in haematogenously disseminated candidiasis without significant alterations in host neutrophils. Attachment to the iC3b receptor on C. albicans blocks adhesion to endothelium and subsequent systemic invasion. Adherence to the endothelium is correlated with virulence. However, it is too early to discuss the interactions between intravenously injected C. albicans and the anti-iC3b receptor mAb based solely on the current experiment. We plan to use radioisotope-labelled anti-iC3b antibodies for future studies and to evaluate the safety profiles of anti-iC3b mAb in humans.
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