MYCOLOGY

GM-CSF-modulated phagocytosis of *Trichosporon beigelii* by human neutrophils

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*Trichosporon beigelii* has emerged as a lethal opportunistic pathogen in granulocytopenic and corticosteroid-treated patients. Little is known of the host defence mechanisms against this yeast. The interaction between human neutrophils and serum-opsonised *T. beigelii* and the effect of GM-CSF on binding and ingestion of the yeast by neutrophils were investigated by a microscopic analysis of neutrophil monolayers stained with FITC-Concanavalin A. Positive staining with FITC-Concanavalin A distinguished between intracellular and extracellular yeast cells. Binding of *T. beigelii* to neutrophils was an energy- and complement-dependent process involving movement of actin in the neutrophil cytoskeleton. The mean percentage binding of *T. beigelii* was 37.5% and the mean binding index (BI) was 1.30 whereas the mean percentage ingestion was 3.5% and the mean phagocytic index (PI) was 1.34. GM-CSF increased percentage ingestion of *T. beigelii* from 2.8% to 30.5% and the PI was increased from 1.3 to 1.86. The percentage binding was 36.8% and the mean BI was 1.3 in control experiments compared with 49.3% and 1.6, respectively, in the presence of GM-CSF. In conclusion, GM-CSF significantly increased percentage ingestion of opsonised *T. beigelii* by neutrophils, but its effect on percentage binding of the yeast was not statistically significant.

Introduction

During the past two decades *Trichosporon beigelii* has emerged as an infrequent but usually lethal opportunistic pathogen in granulocytopenic and corticosteroid-treated patients [1–9]. It is widely distributed in the soil and may be present on the skin and in the mouth as part of the normal flora. *T. beigelii* causes a wide spectrum of infection [10]. It may cause superficial disease of hair shafts (white piedra), cutaneous infection, onychomycosis and mucosal infection. Deep *T. beigelii* infection is a potentially life-threatening localised visceral or disseminated mycosis, usually in immunocompromised patients. Clinical symptoms include fever, fungaemia, funguria, pulmonary infiltrates and cutaneous lesions with invasion of the kidneys, lungs, skin and other tissues. *T. beigelii* may also cause summer-type pneumonitis hypersensitivity in Japan [11].

Granulocytopenia is the most common apparent risk factor among reported cases of disseminated *T. beigelii* infection. Moreover, resistance to amphotericin B treatment has been reported in these granulocytopenic patients. Although the host defence mechanisms against the organism are not well understood, neutrophils are thought to be important in defence against fungal infections. Neutrophils act by undergoing a sequence of events: chemotaxis, adhesion, ingestion and killing. It has been found that phagocytosis of *T. beigelii* was significantly less than that of *Candida* species but was similar to that of *Cryptococcus neoformans* [12]. The mechanism of inhibition of phagocytosis of *T. beigelii* is not well understood. There is evidence that *T. beigelii* produces a heat-stable antigen which shares antigenic determinants with the capsular antigen of *Cr. neoformans* [13] which may abrogate binding of *T. beigelii* to the neutrophil surface.

Interest in the effects of cytokines on the fungicidal properties of neutrophils and their potential use in immunotherapy of fungal diseases is increasing [14, 15]. GM-CSF is a 22-kDa glycoprotein cytokine which may modulate the behaviour of mature human neutrophils in various ways. However, the way in which GM-CSF actually enhances the neutrophil function is not well understood. Recent reports have demonstrated that GM-CSF can enhance phagocytosis and intracellular killing of several fungi [16]. Evidence is provided for GM-CSF-mediated enhancement of phagocytosis of *T. beigelii*. 

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Preparation of *T. beigelii*

A recent isolate of *T. beigelii* was subcultured on glucose peptone-chloramphenicol (GPC) agar at 28°C; the cultures used were 3–4 days old. *T. beigelii* was emulsified in 10 ml of distilled water and vortex mixed to give a single cell suspension. Dilutions were made in Hanks's Balanced Salts Solution (HBSS) to give a suspension containing $1 \times 10^5$ *T. beigelii* blastoconidia ml. A fresh suspension was prepared for each experiment. *T. asahii* and *C. albicans* were used for comparison.

Opsonin

Unpurified isologous serum complement as a component of peripheral blood was used as an opsonin when required. The complement was stored at -70°C but pre-equilibrated to 37°C before use.

Cytokine

The specific activity of rHu GM-CSF (Leucomax; Sandoz Oncology) was $1.67 \times 10^7$ U/mg. It was then diluted in HBSS to obtain the desired concentration.

Preparation of neutrophil monolayer

Neutrophil monolayers were prepared by placing drops of blood from fingertip puncture (a single donor throughout) on untreated, dust-free coverslips (16 mm diameter). The coverslips were incubated for 30 min at 37°C in a humidified chamber. They were then immersed in saline 0.83% w/v at 37°C and agitated to remove clots and adherent red blood cells. The coverslips were then washed again in fresh, warm saline, resulting in a monolayer of neutrophils. Viability of the phagocyte monolayer was assessed after the final washing step by trypan blue (0.16% w/v) dye exclusion and in all cases was found to be >80%.

Assessment of phagocytosis

*T. beigelii* (1 ml, $1 \times 10^5$ blastoconidia/ml) was added to neutrophil monolayers held in individual wells of plastic tissue-culture repti dishes (Sterilin). Normal human serum (NHS, 100 μl) was added to each well before incubation at 37°C for 30 min. The monolayers were then removed from the wells and washed in saline 0.85% w/v. A drop of FITC-Concanavalin A 0.025% w/v, which binds only to extra-cellular organisms, was added to each coverslip. The coverslip was then inverted (monolayer facing downwards) over glass cavity slides. Monolayers were viewed microscopically by bright-field illumination and fluorescence by epifluorescence illumination with blue light excitation at 1000× magnification. Ten random fields of views were examined – the only constraint being that at least two neutrophils were present in each field. The total number of neutrophils/field was counted and the number of *T. beigelii* adhered or ingested by each neutrophil was noted.

From these observations the following parameters were calculated:

$$
\text{Binding (\%)} = \frac{\text{Number of neutrophils with one or more bound blastoconidia}}{\text{Total neutrophil number}}
$$

$$
\text{Binding index (BI)} = \frac{\text{Number of bound blastoconidia}}{\text{Neutrophils containing one or more blastoconidia}}
$$

$$
\text{Ingestion (\%)} = \frac{\text{Number of neutrophils with one or more ingested blastoconidia}}{\text{Total neutrophil number}}
$$

$$
\text{Phagocytic index (PI)} = \frac{\text{Number of ingested blastoconidia}}{\text{Neutrophils containing one or more blastoconidia}}
$$

Cytochalasin D treatment

Some populations of complement receptors (CR1 and CR3) have been shown to be linked to the cytoskeleton of neutrophils and macrophages. This study investigated whether inhibiting actin filaments would affect binding of *T. beigelii* to neutrophils. A cytochalasin D stock solution was prepared by dissolving 1 mg of cytochalasin D (Sigma) in 1 ml of dimethyl sulphoxide (DMSO) and stored at 4°C. Where indicated, 100 μl of stock solution were added to each monolayer to achieve a final concentration of 10 μg/ml in HBSS. Controls consisted of 100 μl of HBSS and 100 μl of DMSO (to give a 1% DMSO concentration, as in test wells). Neutrophil monolayers were incubated in the presence of cytochalasin D 10 μg/ml solution in HBSS for 30 min at 37°C. The solution was aspirated, 1 ml of HBSS was added to wash the neutrophils and the HBSS removed. The binding assay was then performed as described.

Simultaneous addition of GM-CSF to neutrophils and *T. beigelii*

Neutrophil monolayers were incubated with $10^5$ blastoconidia of *T. beigelii* in HBSS plus rHuGM-CSF 40 U/ml, serum complement 100 μl, or saline 100 μl and incubated for 30 min. The experiments were performed to see if GM-CSF would promote neutrophil ingestion of *T. beigelii* blastoconidia in the presence or absence of opsonin.

Statistical analysis

All statistical analyses were by Student's *t* test. As duplicate experiments were carried out on different days and at different times of the day, the test results were compared with the control results determined at the same time. The pooled variance and statistical
Results

Phagocytosis of blastoconidia

Binding and ingestion were assessed by incubating neutrophil monolayers together with $10^5$ blastoconidia/ml and serum opsonin for 30 min. The mean percentage binding of *T. beigelii* was 37.47(SEM 5.62)% and the mean BI was 1.30 (SEM 0.05). The mean percentage phagocytosis for *C. albicans* was 56.53 (SEM 6.70)% compared with 4.75(SEM 0.78)% for *T. beigelii* and the mean PI for *C. albicans* was 1.32 (SEM 0.09) compared with 1.34(SEM 0.05)% for *T. beigelii* (Table 1). The percentage binding for *T. asahii* was 70.75(SEM 3.25)% and percentage ingestion was 5.05(SEM 2.55)%.

Requirement of normal human serum for binding of *T. beigelii*

In the absence of normal human serum, no binding or ingestion took place at the blastoconidia concentration of $10^5$/ml.

Kinetics of *T. beigelii* binding to neutrophils

During an incubation period of 2 h, the percentage binding of blastoconidia (opsonised) to neutrophils increased from 10% to >90% while the BI increased from 1.27 to 1.93 (Table 2). The percentage ingestion increased from 8.35 to 14.60% while the PI did not increase significantly. In the absence of opsonin, the percentage binding and PI of the blastoconidia were zero and did not change with increasing incubation time.

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Percentage binding (SEM)</th>
<th>PI (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em></td>
<td>56.53 (6.70)</td>
<td>1.32 (0.09)</td>
</tr>
<tr>
<td><em>T. beigelii</em></td>
<td>4.75 (0.78)</td>
<td>1.34 (0.05)</td>
</tr>
<tr>
<td><em>T. asahii</em></td>
<td>5.05 (2.55)</td>
<td>-</td>
</tr>
</tbody>
</table>

Data points represent a minimum of eight neutrophil monolayers.

Effect of temperature on binding of blastoconidia

At 4°C, the percentage binding of blastoconidia was 1.54(SEM 0.25)%, the BI was 0.7 (SEM 0.2) and the percentage ingestion and PI were both zero.

Effect of cytochalasin D on the binding of blastoconidia

In the presence of cytochalasin D, the percentage binding of opsonised blastoconidia was reduced to zero (Table 3). The percentage ingestion of opsonised blastoconidia was also zero.

Effect of GM-CSF on phagocytosis of blastoconidia

In the presence of GM-CSF, the percentage binding was 49.30(SEM 6.23)% compared with 36.76(SEM 6.25)% of the control (HBSS) (Table 4). The BI was 1.62 (SEM 0.11) compared with 1.32 (SEM 0.05) of the control. The percentage ingestion was increased from 2.83(SEM 1.66)% to 30.50 (SEM 7.55)% in the presence of GM-CSF and the PI was increased from 0.26 (SEM 0.15) to 0.86 (SEM 0.20).

Opsonin-dependent action of GM-CSF on binding and ingestion of blastoconidia

In the absence of opsonin, both the percentage binding and percentage ingestion of blastoconidia in the presence of GM-CSF were reduced to a very low level, indicating that the action of GM-CSF on binding and ingestion of blastoconidia was opsonin-dependent (Table 5).

Effect of depleting complement on binding and ingestion of blastoconidia

When the serum was depleted of complement (by heating at 56°C), both percentage binding and percentage ingestion were reduced to zero (Table 6).

Discussion

Although disseminated trichosporon infections are uncommon they have a poor prognosis in immunocompromised patients. Little is known regarding the

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Percentage binding</th>
<th>Percentage ingestion</th>
<th>BI</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum + cytochalasin D</td>
<td>0</td>
<td>0</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Serum + HBSS</td>
<td>36.84</td>
<td>18.42</td>
<td>1.79</td>
<td>0.92</td>
</tr>
<tr>
<td>Serum + DMSO</td>
<td>22.2</td>
<td>18.42</td>
<td>1.79</td>
<td>0.92</td>
</tr>
</tbody>
</table>

Data points represent a minimum of eight neutrophil monolayers.
Table 4. Effect of GM-CSF (100 μl, 40 U/ml) on binding and ingestion of opsonised blastoconidia of T. beigelii

<table>
<thead>
<tr>
<th>Blastoconidia treatment</th>
<th>Percentage binding (SEM)</th>
<th>Percentage ingestion (SEM)</th>
<th>BI (SEM)</th>
<th>PI (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF + serum</td>
<td>49.30 (6.23)</td>
<td>30.5 (7.57)</td>
<td>1.62 (0.11)</td>
<td>0.86 (0.20)</td>
</tr>
<tr>
<td>Serum + HBSS</td>
<td>36.76 (6.25)</td>
<td>2.83 (1.66)</td>
<td>1.32 (0.05)</td>
<td>0.26 (0.15)</td>
</tr>
</tbody>
</table>

Data represent a minimum of eight neutrophil monolayers.

Table 5. Effect of GM-CSF (100 μl, 40 U/ml) on binding and ingestion of blastoconidia of T. beigelii

<table>
<thead>
<tr>
<th>Blastoconidia treatment</th>
<th>Percentage binding (SEM)</th>
<th>Percentage ingestion (SEM)</th>
<th>BI (SEM)</th>
<th>PI (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM-CSF + serum</td>
<td>50.70 (11.82)</td>
<td>39.34 (16.76)</td>
<td>1.65 (0.12)</td>
<td>1.21 (0.05)</td>
</tr>
<tr>
<td>GM-CSF + HBSS</td>
<td>4.78 (6.73)</td>
<td>3.98 (5.61)</td>
<td>0.84 (0.85)</td>
<td>0.58 (0.85)</td>
</tr>
</tbody>
</table>

Data represent a minimum of eight neutrophil monolayers.

Table 6. Effect of depleting complement on binding and ingestion of blastoconidia

<table>
<thead>
<tr>
<th>Blastoconidia treatment</th>
<th>Percentage binding</th>
<th>Percentage ingestion</th>
<th>BI</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS + serum (37°C)</td>
<td>25.69</td>
<td>0</td>
<td>1.38</td>
<td>...</td>
</tr>
<tr>
<td>GM-CSF + serum (37°C)</td>
<td>46.00</td>
<td>11.4</td>
<td>1.29</td>
<td>1.38</td>
</tr>
<tr>
<td>HBSS + serum (56°C)</td>
<td>0</td>
<td>0</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>GM-CSF + serum (56°C)</td>
<td>0</td>
<td>0</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

pathogenicity of T. beigelii, but granulocytopenia is the most apparent risk factor for disseminated trichosporon infection. Furthermore, the fungicidal activity of both neutrophils and monocytes against T. beigelii has been shown to be impaired compared with that of C. albicans.

The results of the present study demonstrate that phagocytosis of T. beigelii is much lower than that of C. albicans; even in the presence of opsonin, the organisms were minimally phagocytosed. However, the PIs were similar to those observed with C. albicans, suggesting that there may be a subpopulation of neutrophils capable of phagocytosing these organisms.

The importance of opsonin was shown by zero binding and ingestion of the blastoconidia in the absence of opsonin. This was confirmed by depleting complement by heating serum at 56°C, after which no binding and ingestion occurred. The study also demonstrated that binding and ingestion of T. beigelii to neutrophils was an energy-dependent, receptor-mediated process which required re-arrangement of actin in the cytoskeleton of neutrophils. It was assumed that the complement factors C3b and iC3b were the important opsonins of the blastoconidia, because of their relatively high level in serum. However, it is likely that mannose, fibrinogen and lectins may also act as putative opsonins of T. beigelii. It is known that complement receptors CR1 and CR3 for C3b and iC3b, respectively, can be upregulated [17], and the increased number of receptors may result in increased association of complement components to their receptors and hence result in increased binding and ingestion. Alternatively, the complement receptors that normally exist in inactive forms are activated by a number of factors which results in increased binding of opsonised blastoconidia. In this study, the effect of GM-CSF on percentage binding of opsonised T. beigelii was shown to be statistically insignificant compared to that of controls. However, the percentage ingestion of T. beigelii was significantly increased in the presence of GM-CSF. Under some conditions, C3b-coated particles are ingested only if the phagocyte receives a second membrane signal. CR3 mediates the binding of C3bi-coated particles. As with CR1, a second signal facilitates microbial ingestion. It is possible that the percentage binding by opsonised T. beigelii was already sufficiently high and so was not altered by addition of GM-CSF. However, the increase in percentage ingestion by GM-CSF could be explained by activation of the signal which facilitates ingestion.

A previous report showed that the recombinant human cytokines, M-CSF, GM-CSF and IFN-γ, enhanced the fungicidal activity of elutriated human monocytes against T. beigelii [18]. This work also suggested that the response of cytokine-activated monocytes to T. beigelii could be predicted because of its relationship to other basidio-mycetous yeasts, such as C. neoformans, where monocytes and monocyte-derived macrophages have a well characterised role in host defence. However, this study showed that pre-treatment of
neutrophils with IFN-γ at concentrations of 10–1000 units/μl or with G-CSF at concentrations of 100–10,000 units/μl did not enhance neutrophil fungicidal activity against Trichosporon isolates.

Failure of phagocytes to interact with *T. beigelii* may be related to a surface component of the yeast that restricts phagocyte-*T. beigelii* interaction. One possible component is the extractable heat-stable antigen that shares antigenic determinants with the capsular polysaccharide component glucuronoxylomannan (GXM) of *C. neoformans* [19]. The possibility that this GXM-like antigen is a virulence factor of *T. beigelii* is further supported by evidence that there is direct correlation between antigen production and resistance to killing by polymorphonuclear leukocytes [19].

The recovery of neutrophil count appears to be the most important factor influencing the outcome of *T. beigelii* infection [20]. The results of the present study suggest that ingestion of *Trichosporon* can be enhanced by GM-CSF – it is not known whether the fungicidal activity of neutrophils is enhanced by cytokines.

**References**