Immunoglobulins G could prevent adherence of *Candida albicans* to polystyrene and extracellular matrix components

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Immunocompromised patients are at high risk of developing *Candida* infections. Although cell-mediated immunity is generally believed to play the main role in defence against fungi, antibodies could also be effective in immune defence by different mechanisms of action. The adherence capacity of four strains of *Candida albicans* to polystyrene and to some extracellular matrix components was investigated after incubation of the yeasts with non-specific and specific anti-*C. albicans* IgG. Experiments were carried out using a colorimetric method based upon the reduction of XTT tetrazolium (2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide) by mitochondrially active blastospores in the presence of menadione. Incubation of the yeasts with IgG, specific or not, caused a decrease in the capacity for adherence to the surfaces studied. There was no significant effect of the specificity of the tested antibodies on the reduction of adherence capacity. In conclusion, total IgG could play a role in blocking the binding of *C. albicans* to host and medical device surfaces. These results suggest that regular survey of levels of total IgG in patients suffering from severe hypogammaglobulinaemia could be of interest for the prevention of systemic candidiasis.

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INTRODUCTION

Candida albicans, a saprophyte of the human digestive tract, is frequently responsible for systemic infections in immunocompromised patients, even if other species of *Candida* are reported with increased frequency (Vincent *et al.*, 1998). According to a wide-ranging American study, the rate of invasive fungal infections among hospital patients approximately doubled between 1980 and 1990, and the incidence of nosocomial candidaemia alone increased fivefold (Beck-Sagué & Jarvis, 1993).

C. albicans possesses virulence factors that are required for the establishment of candidiasis, involved in processes such as adhesion, phenotypic switching and morphogenesis (Calderone & Fonzi, 2001). Adhesion of the organism to mucosal epithelium is a prerequisite for colonization and is therefore regarded as the initial step in the process leading to infection. Furthermore, adhesion to endothelium and extracellular matrix (ECM) components are required for dissemination of *C. albicans* (Klotz, 1992). A number of ECM proteins bind to the yeast, including fibronectin (Klotz *et al.*, 1994), laminin (Sakata *et al.*, 1999), vitronectin and type I and IV collagens (Klotz *et al.*, 1993). Moreover, *C. albicans* is

also able to adhere to the surface of intravascular catheters, usually colonized by intra- or extraluminal migration of *Candida* spp. from the skin surface (Flanagan & Barnes, 1998). Candidaemia occurs commonly in the presence of a colonized intravascular catheter (Rex, 1996).

Nevertheless, the ability of this yeast to cause human infectious disease relates more to the immunological status of the host than to obvious virulence factors produced by the fungus. *C. albicans* is an opportunist yeast that becomes a pathogen in hosts whose local or systemic immune functions are impaired, for whatever reason (Matthews & Burnie, 1996). Neutropenia represents a crucial risk factor, because neutrophils are indispensable for antifungal immunity (van Spruiel *et al.*, 2001). Derangements in antibody immunity often accompany defective cellular immunity. The role of antibody immunity in fungal infections remains a controversial subject. Many *in vivo* or *in vitro* studies have provided evidence for or against the importance of antibody immunity to *C. albicans* (Casadevall, 1995).

Some authors have reported that antibody immunity against *C. albicans* may participate in host defence by preventing attachment (Han & Cutler, 1995). Most *in vitro* studies have

Abbreviation: ECM, extracellular matrix.

concerned the inhibition of attachment by antibodies that recognize cell-wall proteins (Masuoka *et al.*, 1999; San Millan *et al.*, 1996).

We study here the adherence of *C. albicans* to polystyrene and to ECM components after incubation of the yeast with IgG directed against cytoplasmic antigens of *C. albicans*, compared with its adherence after contact of the yeast with non-specific IgG.

METHODS

Polyclonal antiserum. *C. albicans* strain 2091, obtained from the Pasteur Institute (Paris, France), was grown for 48 h at 37 °C on Sabouraud's dextrose agar slants (Sanofi Diagnostics Pasteur). The cells were harvested in Tris-buffered saline (TBS: 140 mM NaCl, 10 mM Tris/HCl, pH 7.2), washed three times at 4 °C by centrifugation (600 g, 10 min) and suspended to a final concentration of 10^9 cells ml⁻¹ in the same buffer. Cells were disrupted for 10 min in an MSK cell homogenizer (B. Braun) with glass beads (0.45–0.55 mm) with cooling under CO₂. Disrupted yeast cells were centrifuged at 100 000 g for 30 min at 4 °C. The supernatant fluid represented the cytoplasmic extract and was used for immunization of rabbits.

Antiserum directed against the *C. albicans* cytoplasmic extract was obtained after six subcutaneous injections, at 15 day intervals, of this extract at a concentration of 0.5 mg ml⁻¹, emulsified (v/v) with QuilA-purified saponin (Superfos Piosector), into three New Zealand white rabbits (Hy/Cr, Charles River Laboratories). After immunization, the three sera were pooled and the specificity of the resulting antiserum was verified by immunoblotting and indirect immunofluorescence assay.

IgG purification. Purification of the rabbit IgG before and after immunization was achieved with HiTrap Protein A columns (Pharmacia). The column was pre-equilibrated with 100 mM Tris/HCl, pH 7.5. The pooled antiserum sample was diluted 1:1 with this buffer and filtered (0.22 µm) prior to its application to the Protein A column, so that the optimal ionic strength and pH for binding were maintained. The diluted serum was then applied to the column and was allowed to flow completely into the gel. The bound IgG was eluted with 100 mM glycine buffer, pH 2.7. Elution of bound proteins was monitored by measuring the A₂₈₀. Pooled IgG obtained from the rabbits before and after immunization was tested by an indirect immunofluorescence test with blastospores of *C. albicans* in order to confirm the effect of immunization and to determine the titre of the specific antibodies.

Organisms and growth conditions. *C. albicans* strains 1066, 2091 and NIH 311 (Pasteur Institute) and a clinical isolate (165-CA) obtained from blood-stream culture of a patient were used for this study. Identification of isolate 165-CA was performed by conventional physiological and morphological studies such as the germ-tube test in serum, agglutination (Bichro-Latex; Fumouze), metabolic properties (API 20C, API ID 32C; bioMérieux) and growth on Staib agar as described previously (Al Mosaid *et al.*, 2001).

Yeasts were first grown for 48 h at 28 °C on Sabouraud's agar slants (Sanofi Diagnostics Pasteur) to obtain a fresh culture of synchronous stationary-phase yeast. A loopful of this culture was transferred to 25 ml yeast nitrogen base (Difco) supplemented with 300 mM galactose (Sigma), which promotes adherence of *C. albicans* yeasts to cellular and plastic surfaces, and incubated without shaking for 36 h at 37 °C, as described previously (Imbert *et al.*, 2002).

Incubation of blastospores with IgG. Prior to use for adherence experiments, blastospores were harvested and washed twice in 0.1 M PBS (pH 7.2) (bioMérieux). Cell counts were determined and the

suspension was adjusted to 10^7 blastospores ml⁻¹ and then incubated for 1 h at 37 °C in PBS with the purified specific or non-specific IgG at different concentrations (800, 300, 100 and 50 µg ml⁻¹).

Protein concentration was determined as described by Bradford (1976) using BSA (Sigma) as a standard. A control consisted of incubating the yeasts in PBS alone.

Adherence of *C. albicans* to polystyrene. Adherence experiments were performed in untreated 96-well tissue-culture plates as described previously (Imbert *et al.*, 2002). XTT tetrazolium (2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide) was used to assess the adherence of *C. albicans* blastospores to wells of tissue-culture plates: the principle was based upon the reduction of XTT tetrazolium to XTT formazan by mitochondrially active *C. albicans* blastospores in the presence of an electron-coupling agent, menadione. Briefly, *C. albicans* blastospores were added, after pre-incubation with or without IgG at various concentrations, in culture plates as inocula of 10^7 cells ml⁻¹ in 150 µl PBS alone for control or containing the IgG at the same concentrations. They were then allowed to adhere to polystyrene for 2 h at 37 °C; half of the wells were then washed twice with PBS to remove non-adherent yeasts. Thereafter, 300 µg XTT ml⁻¹ and 0.13 mM menadione (both from Sigma) were added to all wells. Plates were incubated for 3 h at 37 °C without shaking and then gently agitated and XTT formazan was measured by monitoring the A₄₉₂ in an LP400 micro-plate reader (Sanofi Diagnostics Pasteur) in washed and unwashed wells. The adherence capacity of each isolate was calculated as the mean absorbance in washed wells divided by the absorbance in unwashed wells.

Background formazan absorbance was determined with plates that contained PBS only or PBS, XTT and menadione; these values did not exceed 0.005 and were therefore not significant. All experiments were performed at least twice in six samples.

Adherence of *C. albicans* to ECM proteins. ECM gel and fibronectin (both from Sigma) were coated onto wells of 96-well tissue-culture plates (polystyrene; Evergreen Scientific) according to the manufacturer's instructions. ECM and fibronectin were used at a concentration of 10 µg ml⁻¹. Aliquots of 250 µl of each were coated onto wells overnight at 4 °C. ECM gel was composed primarily of laminin, collagen type IV, heparan sulfate proteoglycan and entactin. Adherence of *C. albicans* to ECM proteins was determined in the same way as adherence to polystyrene, after the same steps of incubation, with or without the IgG at various concentrations.

Statistical analyses. Analysis of variance and Scheffé's test were conducted to determine differences among the test groups ($P < 0.05$).

RESULTS

Verification of the immunization of rabbits

Pooled sera from rabbits before and after immunization were tested by an immunofluorescence assay using *C. albicans* blastoconidia. The pooled serum obtained before immunization was negative, whereas the titre of serum obtained after immunization was 1:1280.

Influence of IgG on adherence of *C. albicans* to different surfaces

Incubation of blastospores with IgG directed against cytoplasmic extract of *C. albicans* at different concentrations

Table 1. Effect of IgG on the capacity of four strains of *C. albicans* to adhere to polystyrene, fibronectin and ECM proteins

Adherence was determined as described in Methods. Non-specific (NS) and specific (S) IgG were used at the concentrations indicated. Control incubations contained PBS alone.

Strain	Control	800 µg ml ⁻¹		300 µg ml ⁻¹		100 µg ml ⁻¹		50 µg ml ⁻¹	
		NS	S	NS	S	NS	S	NS	S
Polystyrene									
1066	48.7 ± 5.1	25.2 ± 4.3 ^{a*}	24.0 ± 0.7 ^a	25.3 ± 0.4 ^a	24.2 ± 1.1 ^a	25.2 ± 0.3 ^a	25.6 ± 0.3 ^a	30.4 ± 3.0 ^a	28.2 ± 0.2 ^a
2091	47.2 ± 1.3	17.3 ± 3.0 ^a	20.3 ± 3.0 ^a	18.4 ± 0.9 ^a	19.6 ± 1.0 ^a	16.8 ± 2.1 ^a	17.4 ± 0.9 ^a	30.7 ± 2.3 ^a	28.2 ± 3.2 ^a
NIH 311	71.4 ± 2.5	29.3 ± 6.1 ^a	27.2 ± 2.5 ^a	31.6 ± 0.5 ^a	28.2 ± 3.2 ^a	58.2 ± 7.0 ^a	58.4 ± 4.2 ^a	57.5 ± 0.9 ^a	61.0 ± 4.3 ^a
165-CA	37.7 ± 1.2	30.2 ± 3.6 ^a	23.3 ± 2.5 ^{ab}	21.2 ± 1.4 ^a	25.3 ± 4.0 ^a	29.4 ± 3.4 ^a	32.2 ± 3.4 ^a	38.1 ± 3.0 ^a	35.5 ± 3.7 ^a
Fibronectin									
1066	67.2 ± 9.2	25.7 ± 4.2 ^a	23.9 ± 2.1 ^a	27.2 ± 3.5 ^a	23.2 ± 4.3 ^a	37.2 ± 3.2 ^a	36.4 ± 2.7 ^a	49.7 ± 2.1 ^a	45.3 ± 4.3 ^a
2091	25.3 ± 2.7	16.4 ± 2.9 ^a	17.3 ± 2.7 ^a	18.2 ± 1.2 ^a	17.7 ± 2.7 ^a	20.1 ± 1.2 ^a	19.2 ± 1.5 ^a	22.3 ± 1.3	21.5 ± 2.3
NIH 311	51.4 ± 4.2	33.9 ± 3.8 ^a	25.9 ± 2.9 ^{ab}	37.2 ± 2.5 ^a	35.3 ± 1.7 ^a	42.3 ± 1.8	41.8 ± 1.5 ^a	51.2 ± 1.7	49.3 ± 2.1
165-CA	55.7 ± 7.8	22.2 ± 3.5 ^a	25.3 ± 2.7 ^a	27.2 ± 3.5 ^a	28.3 ± 2.1 ^a	34.1 ± 1.7 ^a	35.3 ± 2.5 ^a	52.3 ± 4.2	51.1 ± 3.7
ECM proteins									
1066	51.2 ± 5.2	23.2 ± 7.3 ^a	20.5 ± 4.2 ^a	20.9 ± 3.2 ^a	21.5 ± 2.7 ^a	25.0 ± 3.1 ^a	27.0 ± 4.1 ^a	38.0 ± 1.2 ^a	39.0 ± 0.5 ^a
2091	25.2 ± 1.3	20.0 ± 2.7 ^a	18.2 ± 3.2 ^a	21.5 ± 0.2 ^a	21.2 ± 0.3 ^a	21.1 ± 0.7 ^a	20.8 ± 1.0 ^a	21.9 ± 1.1 ^a	20.0 ± 0.4 ^a
NIH 311	58.4 ± 3.7	32.7 ± 4.9 ^a	34.3 ± 7.5 ^a	31.2 ± 3.2 ^a	34.4 ± 0.7 ^a	37.5 ± 1.1 ^a	38.2 ± 3.2 ^a	57.1 ± 0.8	57.0 ± 0.7
165-CA	21.0 ± 1.8	21.7 ± 3.9	22.6 ± 4.8	22.1 ± 1.5	20.4 ± 1.8	19.5 ± 1.2	20.2 ± 1.3	23.2 ± 1.0	21.3 ± 1.5

*Significant differences ($P \leq 0.001$) are indicated in comparison with: *a*, control; *b*, non-specific IgG.

induced a significant decrease in the adherence of the four tested strains to polystyrene, fibronectin and ECM proteins ($P < 0.001$) when compared with the adherence capacity of these strains incubated with PBS only. Only low concentrations of IgG were unable to decrease adherence, in the case of strains 2091 and 165-CA to fibronectin and of strain NIH 311 to fibronectin and ECM gel. Moreover, for strain 165-CA, there was no effect of the presence of IgG (specific or not) on adherence to ECM protein. We found the same results after incubation of the yeasts with non-specific IgG from rabbits before immunization, in comparison with the same controls (Table 1).

Influence of specific IgG on the capacity of *C. albicans* to adhere to different surfaces

Regardless of the surface studied and the conditions used, we found only two significant cases where the specificity of the IgG for *C. albicans* antigens influenced (at the highest concentration) the capacity of adherence of the yeast: adherence of strain 165-CA to polystyrene and of strain NIH 311 to fibronectin (Table 1). In all the other cases, there was no significant difference between the adherence capacities of the strains incubated with specific or non-specific IgG, regardless of concentration.

DISCUSSION

In high-risk patients suffering from disseminated candidiasis, predisposition to infection has many components, including both immunodeficiency (humoral and cellular), related to the pathology itself, and the results of immunosuppression, related to treatment (Tsiodras *et al.*, 2000). Delayed immune reconstitution represents an increased risk of infectious complications (LaRocco & Burgert, 1997). The incidence of invasive fungal infections is particularly high in bone-marrow transplant recipients. The conditional regimen used to prepare the host is a major determinant of host tissue injury and may lead to mucositis diarrhoea, facilitating transmucosal arrival of blood-stream infections (van Burik & Weisdorf, 1999). Invasive monitoring with intravascular catheters predisposes to colonization and infection with *Candida* spp. (Flanagan & Barnes, 1998). In intensive-care units, patients are also immunosuppressed following major surgery, trauma, burns or corticosteroid administration. In these patients, this is often associated with a decrease in intestinal mucosal barrier function (Flanagan & Barnes, 1998).

Adhesion events to endothelium and ECM components are required for dissemination of *C. albicans*. This process could begin by yeasts gaining access to the blood stream through gastrointestinal perforation, by seeding from the biofilm of a medical device or by inoculation consecutive to a trauma (Glee *et al.*, 2001). For these reasons, adherence to implanted devices and to ECM are of great importance for development of the disease. The adhesins of *C. albicans* are diverse, reflecting the ability of the organism to colonize and invade a variety of host cells and tissues (Bailey *et al.*, 1995).

It has been reported in some *in vitro* studies that antibody immunity may contribute to host defence by direct candidacidal activity, by providing opsonins for more efficient phagocytosis, by binding to immunomodulating polysaccharides, by neutralizing extracellular proteases and by inhibiting the yeast-to-mycelium transition, but also by preventing attachment (Casadevall, 1995).

Administration of immune serum has been protective in some animal studies, but not in others. Polyclonal preparations are complex mixtures of antibodies, differing in isotype and specificity. This may explain why antibody protection has been observed only in some studies (Casadevall, 1995). Specific antibodies to mannoproteins and hsp90 have been shown to be protective against murine candidiasis (Matthews & Burnie, 1996; Cassone *et al.*, 1995). Specific antibody induction has been investigated as an immunotherapeutic preventative measure (Han & Cutler, 1995). Recently, oral administration of bovine anti-*Candida* antibodies has been used for passive immunization in allogeneic bone-marrow transplant recipients (Tollemar *et al.*, 1999).

The ability of a protein to block adherence may have potential in ameliorating or eliminating disease associated with this organism (Klotz & Smith, 1995), even if blocking of invasion of tissue by *C. albicans* in order to reduce infection has had modest success in several animal models dealing with different forms of candidiasis (Pendrak & Klotz, 1995).

In this study, we have investigated the effect of specific and non-specific IgG at different concentrations on the capacity of *C. albicans* to adhere to polystyrene and ECM components. IgG does adsorb spontaneously to polymer surfaces both *in vitro* and *in vivo* (Tang *et al.*, 1993; Inoue *et al.*, 1997). It has also been demonstrated that a biochemical interaction occurs between fibronectin and the Fc portion of IgG (Rostagno *et al.*, 2002). Antibodies used in this study were directed against a cytoplasmic extract of *C. albicans*. Nevertheless, these specific IgG were able to recognize proteins localized in the fungus cell wall, as proved by the immunofluorescence assay. We have shown that the presence of IgG, specific or not, at concentrations close to the *in vivo* situation, reduced the capacity of *C. albicans* to adhere to polystyrene and ECM components. More interestingly, this study highlights the fact that the hypogammaglobulinaemia found in immunocompromised patients (Hammarström *et al.*, 1994, 2000) could play a role in the dissemination of *Candida* infections, not only because of the decrease in specific antibodies, but also because total IgG could present a barrier to the interaction between pathogens and host components or medical device surfaces. This could have clinical implications: survey of the total level of immunoglobulins and maintenance of a sufficient amount of these immune proteins until the recovery of the immune system could have potential in the prevention of systemic candidiasis.

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