Effect of iron depletion on cell-wall antigens of *Candida albicans*

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Summary. Cell walls were isolated from stationary-phase cultures of *Candida albicans* grown at 25°C or 37°C, in iron-depleted and iron-sufficient conditions. Proteins solubilised from cell-wall fractions were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Approximately 40 protein bands were detected by Coomassie blue staining in all wall extracts, regardless of temperature or other growth condition. Sera from patients with oral or systemic candidosis, from whom the isolates were obtained, and pooled normal human serum were examined for the presence of IgG and IgM antibodies to cell-wall proteins by Western blotting. Patient sera recognised more antigens than pooled normal human serum. In particular, an antigen of 44 kda was detected by IgG antibodies in the sera of patients and two antigens of 41 and 14 kda were detected by their IgM antibodies when the sera were used as probes against walls from iron-depleted cells, but not from iron-sufficient cells, grown at 25°C. Two antigens of 45 and 40 kda were detected by IgM antibodies in the sera of patients tested against walls from iron-depleted but not from iron-sufficient cells grown at 37°C. IgG antibodies did not distinguish between these wall preparations from cells grown at 37°C. These results suggest that the specific cell-wall proteins induced during growth in iron-depleted conditions, as well as other proteins, were immunogenic and were recognised by the patients’ antibodies.

Introduction

The fungal cell wall plays an important role in interactions with host defences during infection. Studies of antigenic variability in *Candida albicans* have shown that surface antigen expression varies among *Candida* strains (Hasenclever and Mitchell, 1961; Poulain et al., 1982, 1985) and during growth of the yeast phase in vitro (Ballou, 1976; Manning and Mitchell, 1980; Brawner and Cutler, 1984, 1986; Chaffin et al., 1988; Sundstrom et al., 1988) and in vivo (Hopwood et al., 1986). Poulain et al. (1983) used polyclonal sera to show that a single strain of *C. albicans* may undergo antigenic variation during infection. This dynamic expression of surface antigens may be influenced by nutritional and environmental factors and may shed some light on the role of antibody in candida infections (Mouraud and Friedman, 1968) and other microbial infections (Brown and Williams, 1985).

*C. albicans* is an opportunistic, dimorphic yeast. As a commensal the organism is believed to exist primarily in the yeast phase (Simonetti and Stripoli, 1973) while the mycelial phase is expressed during invasive colonisation of the host (Saltarelli et al., 1975; Odds, 1979). It is well known that the withholding of iron constitutes an important component of mammalian host defences against infection (Weinberg, 1971, 1984; Finkelstein et al., 1983; Griffiths, 1983). Iron is relatively unavailable for microbial growth in vivo, and is mainly located intracellularly in haemoglobin and ferritin. Extracellularly, the iron-binding glycoproteins, transferrin (Aisen and Listowsky, 1980) and lactoferrin (Querinjean et al., 1971), ensure that the level of free ionic iron is too low for microbial growth (Bullen, 1981; Griffiths, 1983; Weinberg, 1984). For growth and survival in vivo, *C. albicans* must have a mechanism for the acquisition of iron from its host. To adapt to an iron-restricted environment, pathogenic micro-organisms may synthesise low molecular weight iron-chelating compounds called siderophores and induce envelope receptors for the uptake of iron-siderophore complexes (Neilands, 1981, 1982). These high-affinity iron transport systems can remove iron from the host iron-binding proteins and enable the pathogen to multiply in the

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iron-restricted environment of the host. Recent studies have provided direct evidence that pathogenic bacteria express iron-regulated outer-membrane proteins when grown in vivo (Griffiths et al., 1983; Brown et al., 1984; Brown and Williams, 1985; Shand et al., 1985). Such findings stress the importance of studying the physiology and antigenicity of cells grown in conditions of iron-deprivation.

The ability of C. albicans to sequester iron in vivo and the effects that an iron-restricted environment may have on its surface structure and properties is not known. With the increasing importance of C. albicans as a serious pathogen (Chandler, 1985; Sobel, 1985) a better understanding of the mechanisms of pathogenicity is needed to improve diagnostic, therapeutic and prophylactic measures. We have investigated the effect of iron-depletion on cell-wall proteins of clinical isolates of C. albicans by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. In addition, specific cell-wall proteins induced in iron-depleted conditions from a number of Candida strains, were probed with sera from normal individuals and from patients with superficial and systemic candidosis.

Materials and methods

C. albicans strains

C. albicans strain AU1, isolated from a patient with a superficial candida infection, and strain AU2, isolated from a patient with proven systemic candidosis, were kindly provided by Mr D. Sinfield, Department of Pathology, Hackney Hospital, London. Strain OMC3, isolated from a patient with oral candidosis, was kindly donated by Mr J. Hamburger, Oral Medicine Clinic, General Hospital, Birmingham. All strains were identified by Dr C. Fraser, Candida Typing Unit, Mycological Reference Laboratory, Colindale, London.

Removal of iron from medium

Asparagine (Sigma) 1% w/v and glucose (BDH) 1·25% w/v were dissolved in double distilled water to 1 L and the solution pumped through a column of Chelex-100 ion-exchange resin (Bio-Rad, Watford, Herts) at a rate of 2·45 ml/min for 36 h (Kadurugamuwa et al., 1987). To the iron-depleted eluate was added 0·045 M (NH₄)₂SO₄, 0·085 M NaCl, 1·4 mM KH₂PO₄, 0·8 mM MgSO₄·7H₂O (Analytical Reagents, BDH) and 0·67 mM L-methionine (Sigma), pH 4·5. After autoclaving at 115°C for 20 min, the medium was supplemented with 0·4 mM Biotin (BDH) from a stock solution sterilised by filtration through a 0·22-µm membrane filter (Millipore). Iron-depleted chemically defined medium (Fe-CDM) contained 0·35 ± 0·15 µM iron as determined by atomic absorption spectrophotometry (Perkin-Elmer, Beaconsfield, Bucks). Iron-sufficient chemically defined medium (Fe+CDM) was prepared by the addition of 100 µM FeSO₄·7H₂O to Fe-CDM. Glassware was treated as described previously (Kadurugamuwa et al., 1987).

Culture conditions

All strains were maintained on Sabouraud Dextrose Agar (Oxoid) slopes. Cells washed from a slope were inoculated into 125-ml Erlenmeyer flasks containing 25 ml of Fe-CDM. After shaking at 150 rpm for 48 h at 25°C, a portion of culture was transferred to similar flasks containing fresh Fe-CDM. Cultures serially transferred from this second culture were used for experimental purposes as follows: stationary phase cells were harvested by centrifugation at 2500 g for 5 min, washed twice and resuspended in double distilled water; the cells were re-inoculated into fresh prewarmed Fe-CDM at a concentration of 2 x 10⁸ cells/ml. Yeast-phase organisms were obtained by shaking flasks at 150 rpm at 25°C or 37°C.

Preparation of cell walls

Stationary phase yeast cells were harvested by centrifugation, washed in cold double distilled water and resuspended in 1 mM phenylmethylsulphonyl fluoride (PMSF). An equal volume of glass beads (0·45 mm; Sigma) was added and the suspension disrupted by blending on a vortex mixer for 3 min with intervals on ice. At least 95% cell breakage was observed by light microscopy. Walls were sedimented by centrifugation and washed several times in 1 mM PMSF (Chaffin and Stocco, 1983).

Serum samples

A single serum sample was collected by venepuncture from the patients from whom C. albicans strains AU2 and OMC3 were obtained. Normal human serum was collected from seven healthy donors and pooled.

Electrophoresis

Cell-wall proteins were added to equal volumes of sample buffer, denatured at 100°C and separated by SDS-PAGE on 12% gels in a discontinuous buffer system as described by Lugtenberg et al. (1975). Gels were either stained with Coomassie blue or used for Western blotting.

Western blotting

The proteins separated by SDS-PAGE were transferred on to nitrocellulose sheets as described by Towbin et al. (1979). Blotting was performed in a transblotting
chamber (Bio-Rad) for 1 h at a constant voltage of 90 V followed by 16 h at 50 V in ice-cold buffer (pH 8-3) containing 25 mM Tris, 192 mM glycine and methanol 20% v/v. After transfer, the nitrocellulose sheets were cut vertically into strips and washed with gentle shaking for 1 h at 37°C in a solution containing Tween-20 0.3% v/v, NaCl 0.9% w/v and 0.01 M Tris-HCl (pH 7-4) (TTBS) to block unbound sites on the nitrocellulose. The strips were rinsed six times in 0.01 M Tris-HCl buffer (pH 7-4) containing NaCl 0.9% w/v (TBS), then incubated with gentle shaking at 37°C for 4 h in either patient serum or pooled normal serum at a dilution of 1 in 25 in TTBS. The blots were rinsed six times in TBS and incubated with gentle shaking at 37°C for 1 h in alkaline phosphatase-conjugated rabbit anti-human IgG or IgM (DAKO Ltd, High Wycombe, Bucks) at a dilution of 1 in 1000 in TTBS. The strips were rinsed six times in TBS and incubated with gentle shaking at 37°C for 4 h in a solution containing H2O2 0.01% v/v and 4-chloro-1-naphthol (Sigma) 0.01% w/v in 0.01 M Tris-HCl buffer (pH 9-5). After 15 min the reaction was stopped by immersing the strips in a solution containing 0.02 M Tris and EDTA 0.2% W/V (pH 8-0).

For detection of glycosylated components with concanavalin A, the washed blots were gently shaken at 37°C for 2 h in the concanavalin A-peroxidase conjugate (Sigma) in TTBS 0.5 µg/ml. These blots were visualised after rinsing in TBS by adding a freshly prepared solution containing dioxygenate (Bio-Rad) 5-33 mg/ml and 4,6-diamino-2-phenylindole (Bio-Rad) 0.165 mg/ml in alkaline phosphatase buffer containing 0.1 M Tris, NaCl 0.58% w/v and MgCl 0.1% w/v (pH 9-5). After 15 min the reaction was stopped by rinsing with water.

Results

SDS-PAGE

SDS-PAGE of cell walls from each of the three isolates of C. albicans grown in the presence and absence of iron revealed similar antigenic profiles, indicating no interstrain variation. Therefore, strain AU2 was chosen as representative of these isolates. Fig. 1 shows the protein profiles of walls of strain AU2 grown in Fe-CDM and Fe+CDM, stained with Coomassie blue. All the profiles were similar. Approximately 40 bands were observed in each profile. Qualitative differences were not associated with temperature or other growth conditions for these stained preparations.

Recognition of wall antigens in cells grown at 25°C

Western blots of electrophoretically separated proteins from each of the three isolates grown in the presence or absence of iron revealed similar antigenic profiles when treated with serum from patients with oral or systemic candidosis or pooled normal human serum. Therefore, strain AU2 was chosen as representative of these isolates. Figs. 2 and 3 show Western blots of wall proteins from strain AU2 detected with serum from the patient with systemic candidosis and normal human serum. The IgG response was marked by a dense, diffuse staining to the upper portion of each blot (fig. 2). All blots showed similar antigenic profiles. This patient serum revealed two antigens of 44 and 35 kda (track 2), whereas normal serum revealed only the antigen of 35 kda (track 4), in walls of yeasts grown in Fe-CDM. Neither antigen was detected in walls of yeasts grown in Fe+CDM.

IgM antibodies reacted strongly with an antigen of 47 kda in each blot (fig. 3). Serum from the patient with systemic candidosis revealed three antigens of 45, 41 and 14 kda (track 2), whereas normal serum revealed only an antigen of 14 kda (track 4) in walls of yeasts grown in Fe-CDM. IgM antibodies did not reveal any of these antigens in walls of yeasts grown in Fe+CDM.
Recognition of wall antigens in cells grown at 37°C

The IgG response (fig. 4) was again marked by a dense, diffuse, but less intense staining to the upper portion of each blot. IgG of serum from the patient with systemic candidosis strongly revealed an antigen of 47 kda in walls of yeasts grown in the presence and absence of iron (fig. 4; tracks 1 and 2), otherwise the antigenic profiles were similar. Although normal serum did not recognise this antigen in walls of yeasts grown in Fe⁺ CDM, this antigen and three others of 45, 40 and 35 kda were clearly detected in walls of yeasts grown in Fe⁻ CDM (fig. 4; tracks 3 and 4).

IgM antibodies in the serum of the patient with systemic candidosis reacted strongly with an antigen of 40 kda, and revealed an antigen of 55 kda (fig. 5; track 2) in walls of yeasts grown in the absence, but not in the presence of iron (track 1). Pooled normal human serum revealed an antigen of 43 kda in walls of yeasts grown in the absence, but not in the presence of iron.

Lectin blotting with concanavalin A

Lectin blotting revealed different antigenic patterns in the walls of yeasts grown in the presence and absence of iron, and also at different growth temperatures (fig. 6). Concanavalin A-peroxidase reacted strongly with the antigen of 47 kda in all wall extracts except those obtained from cells grown in Fe⁺ CDM at 37°C (track 3). The lectin also gave strong reactions with the antigens of 41, 35 and 30 kda (track 2) in walls of yeasts grown in Fe⁻ CDM at 25°C, and with an antigen of 19 kda (track 4) in walls of yeasts grown in Fe⁻ CDM at 37°C. Weaker reactions were obtained with other antigens.
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Fig. 4. Western blots of cell walls from cells grown at 37°C as described in fig. 1 detected with IgG. Tracks 1 and 2, incubated with patients’ serum; tracks 3 and 4, incubated with pooled normal human serum.

Fig. 5. Western blots of cell walls from cells grown at 37°C as described in fig. 1 detected with IgM. Tracks 1 and 2, incubated with patients’ serum; tracks 3 and 4, incubated with pooled normal human serum.

Discussion

Factors such as growth rate, specific nutrient depletion and age of culture influence the surface composition and properties of bacteria (Brown and Williams, 1985). The role of iron in bacterial infections has received increasing attention (Field et al., 1986; Griffiths, 1987; Hall et al., 1987). Fungal pathogens in host tissue encounter the same iron-restricted environment as bacteria, and iron is an essential nutrient for these organisms too; growth of C. albicans is inhibited in vitro in the presence of iron-binding proteins or serum, and this inhibition can be reversed by the addition of iron (Caroline et al., 1964; Esterley et al., 1967; Kirkpatrick et al., 1971; Elin and Wolff, 1973). The mechanism by which C. albicans acquires iron when growing in vivo is not known. C. albicans produces siderophores of the hydroxamate-type (Holzberg and Artis, 1983), and certain clinical isolates may produce a phenolate-type siderophore (Ismail et al., 1985). The chemical structures of these chelators and their role, if any, during infection are not known. Similarly, there is no information on the effect of iron-restriction on the biological properties or cell envelope of C. albicans.

In the present study, the different growth conditions used had little effect on the expression of protein antigens visualised with Coomassie blue, but differences were revealed by Western blotting. By this technique walls of yeasts grown in the presence and absence of iron were shown to contain a range of proteins recognised as antigens by serum from patients with oral or systemic candidosis. IgG antibodies of the patients’ serum reacted strongly with an antigen of 47 kda in walls of yeasts grown
in both media at 25°C and at 37°C. This has been described as an immunodominant antigen of *C. albicans* (Matthews et al., 1984). Immunodominant antigens with similar molecular weights (Greenfield and Jones, 1981; Strockbine et al., 1984a, b; Au-Young et al., 1985; Bruneau and Guinet, 1987) may be identical or closely related, since apparent differences in molecular weight may arise through differences in methods of antigen preparation and antibody detection. This antigen appears to be characteristic of systemic candidosis because antibodies to it are rare in sera from uninfected individuals and infrequent in superficially-infected patients (Jones, 1980; Matthews and Burnie, 1988). Matthews et al. (1987) found that 40% of patients who produced antibody during systemic candidosis responded to an antigen of 60 kDa and 92% responded to an antigen of 47 kDa. The major antibody response to the latter antigen was predominantly of the IgM class. We found that IgG antibodies in patients' serum reacted strongly to this antigen in walls from cells grown at 25°C or at 37°C but IgM reacted strongly only when cells were grown at 25°C.

Expression of surface antigens varies among *C. albicans* strains (Hasenclever and Mitchell, 1961; Poulain et al., 1983; Brawner and Cutler, 1986; Chaffin et al., 1988; Sundstrom et al., 1988). They are expressed dynamically as a function of morphological state, growth phase and nutritional factors (Ballou, 1976; Manning and Mitchell, 1980; Brawner and Cutler, 1984, 1986; Hopwood et al., 1986). Brawner and Cutler (1987) characterised two antigens which reacted specifically with monoclonal antibodies and found them to be substituted mannans. These workers compared the intracellular and cell-surface expression of the same two antigenic determinants during growth of *C. albicans* in vitro and in infected mice by electronmicroscopy. Differences between in-vitro and in-vivo antigen expression were noted during early stages of growth. Antigen expression in vitro was confined to the surface layers and to the innermost wall layer of mother cells and hyphae, whereas antigen expression in vivo was concentrated in deeper cell-wall layers on mother cells and germ tubes.

The nutritional state of micro-organisms in vivo is difficult to assess, and may vary from one body site to another, but one may speculate about the nature of the growth-limiting nutrient for various localised sites (Brown, 1977; Brown and Williams, 1985). Hence, it is well established that in many infections the micro-organism is growing in an iron-restricted environment. We have shown that IgG and IgM antibodies from patients' sera and normal human serum revealed marked differences in their recognition of surface antigens in walls from cells grown in iron-rich and iron-depleted media. New proteins expressed in walls from the clinical isolate grown in iron-depleted conditions (but not expressed after growth in iron-rich medium) were immunogenic and were detected by antibodies in the patient's own serum. These new proteins may represent envelope receptors and enzymes involved in the uptake and transport of iron from siderophores. Because the cell surface of *C. albicans* consists mainly of glycoproteins, we examined the surface proteins for the presence of mannose residues by probing with the lectin, concanavalin A-peroxidase (Reeke et al., 1974). The lectin reacted strongly with specific antigens, many of which were recognised by patients' serum and normal human serum, indicating that these antigens, including those induced in conditions of

Fig. 6. Western blots of cell walls detected with concanavalin A-peroxidase. See fig. 1 for growth conditions of samples.
iron-depletion, contained mannose. The significance of these findings in infection remains to be determined. Further studies to characterise these new surface antigens may contribute to a better understanding of the mechanisms involved in the pathogenicity of *C. albicans*.

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### REFERENCES


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