Characterisation and cellular localisation of the immunodominant 47-Kda antigen of *Candida albicans*

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Summary. The 47-Kda component of *Candida albicans* is an immunodominant antigen in the serology of systemic candidosis. Immuno-electronmicroscopy with an affinity-purified antibody to the 47-Kda antigen showed that it was present in the cytoplasm and cell wall of both yeast and mycelial cells. It was found in discrete areas on the inner and outer borders of the cell wall and was mainly located within the wall rather than exposed on the outer surface. Sometimes it appeared to be in channels across the cell wall. In the cytoplasm, it was usually near the cytoplasmic membrane and occasionally appeared in vesicular areas. It was not detected in the nucleus or mitochondria. The 47-Kda antigen did not bind to Concanavalin A, and antigenicity was lost after protease digestion. Peptide mapping suggested that the antigen was highly conserved between different strains of *C. albicans*.

Introduction

The incidence of systemic candidosis is increasing and the mortality rate remains high at over 70% (Meunier-Carpentier *et al.*, 1981). The diagnosis is difficult to make clinically and blood cultures are negative in up to 56% of necropsy-proven cases (de Repentigny and Reiss, 1984). There is, therefore, much interest in developing a reliable serodiagnostic test. Several reverse passive latex agglutination tests (RPLA) have recently been developed, one of which is available commercially (Ramco Candtec). Some of these tests have used latex particles sensitised with rabbit antiserum raised against heat-killed *Candida albicans* blastospores but appear to detect a heat-labile antigen circulating in the serum (Gentry *et al.*, 1983; Bailey *et al.*, 1985). This antigen has been regarded as "cytoplasmic", as distinct from the better characterised heat-stable cell-wall mannoprotein detected by other latex tests (Kahn and Jones, 1986).

In an attempt to identify immunodominant antigens we assayed serial sera from patients with systemic candidosis against immunoblots of *C. albicans* (Matthews *et al.*, 1984; Matthews *et al.*, 1987). We found that 92% of patients with candidal antibodies produced a response to an antigen of 47 Kda. Ability to produce and maintain a good antibody response to this antigen was associated with recovery from candidosis whereas fatal cases

produced little or no antibody, or initially had antibody which subsequently fell in titre as their condition deteriorated. We next isolated the 47-Kda antigen from the sera of patients with systemic *C. albicans* infections and showed that the amount of antigen present correlated with the antigen titre obtained with our RPLA test (Burnie *et al.*, 1985a), in which particles are sensitised with rabbit antiserum against a pressate of *C. albicans* (Burnie, 1985). This would be consistent with the 47 Kda antigen being a major immunodominant component of the many antigens measured by such RPLA.

Greenfield and Jones (1981) described a major cytoplasmic antigen of 54·3 Kda (range 48·9–59·7 Kda) which was released from *C. albicans* during the course of invasive infections and elicited a specific antibody response in man and in experimental animals. Jones (1980) suggested, on the basis of the high titres of antibodies to the fraction containing this antigen in hyperimmunised mice, that antibody to this antigen was responsible for resistance to *C. albicans* infections. An immunodominant antigen of similar molecular weight (44–52 Kda) has been identified by Strockbine *et al.* (1984a) in an extract of *C. albicans*, grown in the mycelial phase and passed through a Concanavalin A sepharose affinity column to remove mannan. This antigen was recognised by antibodies in the sera of seven out of ten patients with disseminated candidosis. Au-Young *et al.* (1985) examined sera from 15 patients with systemic candidosis by

immunoblot analysis against a French-press extract of *C. albicans* and tested for anti-mannan antibody by an enzyme-linked immunosorbent assay (ELISA). They again described an immunodominant cytoplasmic antigen of similar molecular weight (45 Kda) and found that measurement of antibody to this antigen was more specific to systemic candidosis than anti-mannan antibody. Neale *et al.* (1987) identified the 47-Kda antigen in circulating immune complexes in the serum of a patient with *C. albicans* osteomyelitis.

It seems likely that these immunodominant cytoplasmic antigens are equivalent or closely related, the difference in molecular weight being due to the different methods of antigen preparation. Very little is known of the nature of this antigen and its role, if any, in the pathogenesis of candidal infections. In this paper we report the location of the 47-Kda antigen in *C. albicans* yeast and mycelial cells. We have also determined the extent to which it is conserved between different strains of *C. albicans* by peptide mapping.

Materials and methods

Affinity-purified antibody

A pressate of *C. albicans* NCPF strain 3153, serotype A was prepared in an Xpress (LKB, Bromma, Sweden) at a pressure of 200 mPa at -20°C ; 25 mg of this pressate was mixed with 0.2 ml of Freund's complete adjuvant and injected subcutaneously into three New Zealand White rabbits. A further injection was given 14 days later and serum was collected after 28 days. This serum contained high levels of candidal antibodies as determined by assay against an immunoblot of the *C. albicans* pressate, as previously described (Burnie *et al.*, 1985b). It was fractionated to produce monospecific antibody to the 47-Kda antigen by a modification of the method of Olmsted (1981). The candidal pressate was boiled for 5 min with cracking buffer sodium dodecyl sulphate 2.6%, 2-mercaptoethanol 1.3%, glycerol 6%, bromophenol blue 0.2%, 0.05 M Tris hydrochloride, pH 6.8). It was then loaded on to a 10% polyacrylamide gel. Electrophoresis and immunoblotting on to nitrocellulose membrane were performed as previously described (Matthews *et al.*, 1984). The horizontal band containing the 47-Kda antigen was excised. Free protein binding sites were blocked with non-fat dry milk 5% w/v in phosphate-buffered saline (PBS) pH 7.4. The strip was washed in PBS and then incubated for 2 h with the rabbit antiserum against *C. albicans* pressate. Then the strip was washed and the bound antibody eluted with glycine buffer (0.2 M glycine, 1 mM EGTA, pH 2.7). This was neutralised by adding an equal volume of 10 mM Tris base. The specificity of the affinity-purified anti-47-Kda antigen

probe was confirmed by immunoblotting against the pressate of *C. albicans*. The same procedure was applied to a rabbit hyperimmune antiserum to *Aspergillus* to obtain affinity-purified antibody against a 40-Kda antigen of *A. fumigatus*. Briefly, a rabbit was immunised by repeated subcutaneous injections of a pressate of *A. fumigatus* (25 mg) mixed with 0.2 ml of Freund's complete adjuvant and successful immunisation was determined by assay against an immunoblot of *A. fumigatus*. Electrophoresis and immunoblotting of *A. fumigatus* were performed as previously described (Matthews *et al.*, 1985). The horizontal band containing the 40-Kda antigen was excised and monospecific antibody prepared against it as described above.

Growth conditions for *C. albicans*

To obtain the yeast phase, *C. albicans* NCPF 3153 was inoculated into 10 ml of glucose broth 2% and grown overnight at 37°C with aeration. The mycelial phase was grown in 10 ml of Eagle's minimal essential medium supplemented with 1 ml fetal calf serum. Under these conditions about 70% of cells were in the mycelial phase.

Immuno-electronmicroscopy

Cell suspensions ($c. 2 \times 10^6$ cells/ml) were washed three times with PBS, pH 7.4, and then fixed in suspension in PLP fixative (McLean and Nakane, 1974) containing paraformaldehyde 4% for 20 min at room temperature. After washing three times with PBS the cells were quenched in 0.5 M ammonium chloride for 4 h, then washed in PBS and pelleted into 2% low gelling temperature agar. They were dehydrated to 70% ethanol and then embedded in L R White resin and polymerised at 50°C for 24 h (Newman *et al.*, 1982). Sections 70 nm thick were cut and mounted on formvar coated copper grids. Immuno-labelling was performed with the monospecific antibody to the 47-Kda antigen and binding sites were visualised with 5-nm gold particle goat anti-rabbit IgG probe. Sections were then stained with saturated aqueous uranyl acetate acid and Reynold's lead citrate and examined in a JEOL 100SX transmission electron-microscope. Control sections were incubated with pre-immune rabbit serum or affinity purified antibody against the 40-Kda antigen of *A. fumigatus*. Sections were also treated with the 5-nm gold probe without prior incubation with the affinity purified candidal antibody.

Protease digestion

The pressate of *C. albicans* NCPF 3153, protein concentration 1 mg/ml, was incubated at 37°C overnight with (i) pronase (0.1 mg/ml; Sigma) in 10 mM Tris chloride pH 7.5—1 mM EDTA (TE); (ii) trypsin (0.1 mg/ml; Sigma), in PBS, pH 7.5; (iii) TE without enzyme; (iv) PBS without enzyme. Each preparation was then separated on a SDS polyacrylamide gel, immunoblotted

and probed with the affinity purified antibody against the 47-Kda antigen.

Sodium periodate

The pressate of *C. albicans* NCPF 3153, protein concentration 1 mg/ml was incubated in the dark at 20°C overnight with (i) 0.1 M sodium periodate in 0.1 M sodium acetate buffer, pH 5 (ii), 0.1 M sodium acetate buffer, pH 5, without sodium periodate. Excess periodate was neutralised by adding an equal molarity of ethylene glycol before gel electrophoresis, immunoblotting and probing with the affinity-purified antibody against the 47-Kda antigen.

Concanavalin A

After electrophoresis of the pressate and transfer on to nitrocellulose membrane, it was blocked with bovine serum albumin 3% in Tris-buffered saline, pH 7.5, at 4°C overnight. It was then washed in Tris-buffered saline and incubated for 2 h at room temperature with 100 µl of Concanavalin A/horseradish peroxidase in Tris-buffered saline. After washing five times in Tris-buffered saline this was stained with 30 µl of H₂O₂ and 35 mg of 4-chloronaphthol in methanol: Tris-buffered saline (1:6).

Peptide mapping

Pressates were prepared from the yeast and mycelial phases of *C. albicans* NCPF 3153 and from the strain of *C. albicans* responsible for the outbreak of systemic candidosis on the intensive care unit at The London Hospital (Burnie *et al.*, 1985c). Pressates were prepared from the yeast phase of two other strains of *C. albicans*, chosen because they were among the commonest types identified by immunoblot fingerprinting (Lee *et al.*, 1986). These four strains represented 71% (158 of 190 isolates) of isolates typed by immunoblot fingerprinting.

Each pressate was run on an SDS 10% polyacrylamide gel and then the 47-Kda band was visualised by staining with a silver stain kit (Amersham International, Amersham, UK). The bands were cut from the gel, equilibrated with stacking buffer (0.125 M Tris HCl pH 6.8, SDS 0.1% and 1 mM EDTA) and set into the sample wells of a second SDS gel as described by Cheung *et al.* (1987). The second gel consisted of a 15% acrylamide resolving gel and a stacking gel of 6% acrylamide with 1 mM EDTA. Each gel slice was overlaid with 5 µg of chymotrypsin (type 1S; Sigma) in sample buffer (0.125 M Tris, pH 6.8, SDS 0.1%, glycerol 10%, bromophenol blue 0.002%). Electrophoresis was performed in the normal manner except that the current was turned off for 30 min when the bromophenol blue dye neared the bottom of the stacking gel. After blotting onto nitrocellulose membrane, the peptide digests were probed with hyperimmune rabbit antiserum to *C. albicans* diluted 1 in 25 in 3% bovine serum albumin buffered saline as previously described (Matthews *et al.*, 1987).

Results

Specificity of antibody

The monospecificity of the affinity-purified antibody against the 47-Kda antigen of *C. albicans* was confirmed by immunoblotting. At a dilution of 1 in 400 in bovine serum albumin 3% in buffered saline, the affinity-purified antibody gave a single prominent band at 47-Kda and very little reactivity with any of the other candidal antigens (fig. 1).

Immuno-electronmicroscopy

The antibody probe against the 47-Kda antigen of *C. albicans* produced the pattern of labelling illustrated in figs. 2–5. Reactivity was specific to this affinity-purified antibody and not present with any of the controls. The 47-Kda antigen was detected in both the yeast and the mycelial phase of *C. albicans*. It was located in the cytoplasm and the cell wall but not in the nucleus (fig. 2). In the cytoplasm it was usually located near the cytoplasmic membrane and adjacent to areas on the inner and outer borders of the cell wall showing strong concentrations of the antigen. Occasionally it localised in vesicular areas in the cytoplasm which appeared to be associated with endoplasmic reticulum (fig. 3). It was not uniformly distributed in the cell wall but located in discrete areas on both

Fig. 1. Immunoblot of *C. albicans* probed with (A) pre-immune rabbit serum, (B) affinity-purified antibody to the 47-Kda antigen.

Fig. 2. *C. albicans* showing labelling of 47-Kda antigen in the cytoplasm (large arrows) and cell wall (small arrows). The nucleus (n) and mitochondria (m) however are free of label; $\times 110\ 000$.

Fig. 4. Labelling of 47-Kda antigen on the outer aspect of the cell wall showing antigen distributed at intervals mainly within the cell wall. The antigen also appears in channels through the cell wall (arrows); $\times 110\ 000$.

Fig. 3. Vesicle (v) within the cytoplasm showing labelling of the 47-Kda antigen. Structures resembling endoplasmic reticulum can also be seen (arrows); $\times 110\ 000$.

Fig. 5. Mycelium showing 47-Kda antigen along the outer and inner borders of the cell wall and also on a developing septum (arrow); $\times 70\ 000$.

sides of the wall. In places, the antigen appeared to be in a channel through the cell wall (fig. 4). The antigen was found on developing septa of dividing cells (fig. 5).

Protease digestion

Incubation of the pressate of *C. albicans* with pronase or trypsin, followed by electrophoresis and immunoblotting, led to complete loss of reactivity with antibody to the 47-Kda antigen. There was no loss of reactivity following incubation with buffer alone.

Sodium periodate and Concanavalin A

Antigenicity was lost after incubation with sodium acetate, pH 5, alone; therefore, it is not possible to comment on the effect of periodate

oxidation. The 47-Kda antigen did not bind to Concanavalin A. Multiple bands of higher molecular weight did bind to Concanavalin A.

Peptide mapping

The chymotrypsin digests of the 47-Kda bands isolated from four different strains of *C. albicans* were indistinguishable. Apart from some undigested 47-Kda antigen, two further bands of lower molecular weight are prominent (fig. 6). The same digest pattern was obtained whether the 47-Kda antigen was prepared from the yeast or mycelial phase.

Discussion

Each group working on the antigen has described it as a major immunodominant component of the

Fig. 6. Peptide mapping of the 47-Kda antigen prepared from the yeast (A) and mycelial (B) phases of *C. albicans* NCPF 3153 serotype A, and from the yeast phases of an outbreak strain of *C. albicans* (C) and *C. albicans* NCPF 3156, serotype B (D).

cytoplasm and identified it in soluble extracts prepared after mechanical disruption of *C. albicans* in the yeast phase (Greenfield and Jones, 1981; Matthews *et al.*, 1984; Au-Young *et al.*, 1985) or the mycelial phase (Strockbine *et al.*, 1984a). It has also been isolated from the serum of systemically infected patients (Matthews *et al.*, 1987; Neale *et al.*, 1987). Recently we have used an antibody probe against the 47-Kda antigen to develop a serodiagnostic test for systemic candidosis (Matthews and Burnie, in press).

Immuno-electronmicroscopy with a monospecific affinity-purified antibody to the 47-Kda antigen confirmed the presence of the antigen in both yeast and mycelial cells. It was present in the cytoplasm and concentrated in discrete areas on both the inner and outer borders of the cell wall. Sometimes it appeared to be in channels across the cell wall. The antigen near the outer surface of the cell wall seemed to be mainly located within the cell wall rather than exposed on the cell-wall outer surface (figs. 2-5). There is evidence to suggest that the antigen is not easily accessible to antibody. Jones (1980) found that when antiserum raised against cytoplasmic extract was cross-absorbed with whole yeast or mycelial cells there was little or no reduction in antibody to the ammonium sulphate-soluble fraction which contained the immunodominant antigen. Perhaps the 47-Kda antigen present on the

cell wall is masked by other cell surface components so that it is not readily accessible to antibody.

Unfortunately the PLP fixative and short fixation time, chosen because it had less effect on the antigenicity of the 47-Kda antigen than glutaraldehyde, did not preserve all organelles sufficiently well for definite identification. Nevertheless, in some sites in the cytoplasm, the 47-Kda antigen appeared to be associated with membranous vesicles which may have been golgi apparatus (fig. 3). Secreted proteins are synthesised on ribosomes in the cytoplasm, become bound to the endoplasmic reticulum and are then transported via vesicles through the golgi stack before appearing on the cell surface. If the 47-Kda antigen is such a protein, determining its amino-acid sequence may identify a sorting signal, a sequence of amino acids which specifies the endoplasmic reticulum as its first destination.

Mannan is a major component of the candidal cell wall, but the 47-Kda antigen is not a mannoprotein because it does not bind to Concanavalin A. The same observation was made for the immunodominant antigens described by Strockbine *et al.* (1984a) and Au-Young *et al.* (1985). Each group of investigators has independently concluded that the immunodominant antigen is a protein on the basis of limited proteolysis (Strockbine *et al.*, 1984b), its ability to stain with Coomassie blue and failure to stain with periodic acid/Schiff reagent (Au-Young *et al.*, 1985), and amino acid analysis (Greenfield and Jones, 1981; Burnie *et al.*, 1985a). Greenfield and Jones (1981) found that the antigen was 5% carbohydrate. Therefore, the possibility exists that the antigenic determinants are carbohydrates attached to a protein. Jones (1980) found that antigenicity was lost following treatment with either periodate (which oxidises carbohydrates) or pronase. He suggested that the antigenic determinants contained carbohydrates and their expression required the native tertiary structure of the protein portion of the antigen. However, the immunodominant antigens described by Au-Young *et al.* (1985), Strockbine *et al.* (1984a) and by ourselves, retained their antigenicity after denaturing by boiling in 2-mercaptoethanol and SDS before electrophoresis and even after limited proteolysis. This is consistent with the antigenic determinants being defined by the primary structure of the protein but does not exclude the possibility of carbohydrate determinants. Like Jones (1980), we found that proteinases caused loss of antigenicity but we were unable to determine the effect of periodate because the presence of sodium acetate buffer, pH 5, alone removed antigenicity.

If probes to the 47-Kda antigen are to be used to develop tests for the serological diagnosis of systemic candidosis it is important to determine whether this antigen is conserved between different strains of *C. albicans*. Limited proteolysis with chymotrypsin was employed to determine the relatedness of the 47-Kda antigen isolated from four different strains and from the yeast and mycelial phases of two strains. The results indicate that the proteins had a similar primary sequence since they gave a series of polypeptides with the same molecular weights when digested with chymotrypsin. Strockbine *et al.* (1984b) applied limited proteolysis to three candidal proteins immunoprecipitated by monoclonal antibodies to the immunodominant antigen and demonstrated a significant

degree of relatedness in the primary structure of this antigen with two other proteins.

In conclusion, immunogold labelling suggests that the 47-Kda antigen of *C. albicans* is produced in the cytoplasm and is present within the cell wall of both yeast and mycelial cells. Its preponderance and conservation in structure between different strains explains its predominance in the sera of patients with invasive candidosis (Matthews *et al.*, 1987; Neale *et al.*, 1987) and suggests that assays detecting the 47-Kda antigen would form more sensitive and specific serodiagnostic tests. Its role in the disease process remains unclear.

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