Use of immunoblotting to identify antigenic differences between the yeast and mycelial phases of *Candida albicans*

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SUMMARY Western blotting was applied to the analysis of *Candida albicans* in the yeast and mycelial phases in an attempt to recognise mycelial specific antigens which might be of serodiagnostic value. The antisera were prepared in rabbits by immunising them with pressates of *C albicans* type A NCTC 3153 in the yeast phase or the mycelial phase. These were blotted against *C albicans* in the yeast and mycelial phases and the yeast phase of *C parapsilosis*, *C krusei*, *C tropicalis*, and *Torulopsis glabrata*. Cross reactivity was greatest against *C parapsilosis*. One yeast specific mannoprotein was identified with a molecular weight of 49 000. No mycelial specific antigens could be identified.

The profusion of published work on the serodiagnosis of systemic candidosis is a reflection of the difficulty in defining an antigen-antibody system which characterises the disease. Part of the difficulty lies in the fact that *Candida albicans* can exist in a yeast or a mycelial phase in vivo.¹ Most of the antigens used in serological tests are derived from the yeast phase, whereas the mycelial form is more typical of deep seated infections.¹ Thus the identification of mycelial specific antigens might form the basis of a serological test which could reliably differentiate between invasive disease and colonisation. Syverson *et al*,² using crossed immunoelectrophoresis, showed six antigens which were unique to each phase. Manning *et al*,³ using two dimensional electrophoresis, failed to show an antigen unique to the mycelial form.

By combining immunoblotting with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE), it is possible to characterise the specific antibody response to a particular antigen in a complex mixture of antigens. The limits of antigen detection are as low as 100 µg of protein.⁴ In the present study antisera to both the yeast and mycelial phases of *C albicans* were used to detect antigen differences between them. Using an enzyme substrate system the colour produced was proportional

to the amount of antibody bound. The serological response to any particular protein could therefore be semiquantified using a Chromoscan 3 densitometer.

Candidal antigens prepared by fragmentation at -70°C were used both to immunise the rabbits and to prepare the gels. Similar antigens have been used to investigate skin reactivity⁵ and as the basis of an enzyme linked immunoassay for the detection of *C albicans* cytoplasmic antigen.⁶ The antisera were also immunoblotted against other candidal species which commonly cause disease. This gives some idea of the immunological cross reactivity between species, which is reflected in the ability of serological tests designed to diagnose candidosis to detect these other species.

Material and methods

PREPARATION OF PRESSATES

The following yeasts were cultured: *C albicans* type A (NCTC 3153), *C parapsilosis* (NCPF 3104), *C krusei* (NCPF 3101), *C tropicalis* (NCPF 3111), and *Torulopsis glabrata* (NCPF 3240). Each was grown on neutral glucose peptone agar (2% glucose) at 26°C overnight and then harvested in distilled water and washed three times.

The mycelial phase of *C albicans* was grown in a defined minimal medium supplemented with amino

acids as described by Lee *et al.*⁷ Cultures were shaken in a gyratory incubator at about 200 rpm at 37°C for 12 h. Cells grown under these conditions were in the mycelial phase, although it is not possible completely to eliminate the yeast form.⁷

Cells were fragmented in an Xpress (LKB, Bromma, Sweden) at a pressure of 200 mPa. The press was frozen at -70°C overnight and then loaded with yeast and placed within plastic bags in a 70% methanol bath containing cardice for 30 min. The plastic bags prevented methanol from penetrating into the chamber containing the yeast.

The press was taken from the bath, the plastic bags were removed, and 200 mPa pressure was exerted. The process was repeated three times to obtain 90% or more disruption of cells. The disintegrated cells were removed from the press and centrifuged at 38 000 *g* for 1½ h at 4°C. The supernatant was filtered through a 0.22 µm Millipore membrane (Millex) to ensure sterility. Protein concentration was estimated as described by Lowry *et al.*⁸ The supernatants, described as pressates, were stored at -20°C before use.

PREPARATION OF MANNAN DEplete EXTRACTS
Concanavalin A Sepharose 4B columns (Pharmacia) were used to extract the mannan containing proteins from the pressates.⁹ By measuring the protein concentration of the pressate before running it through the column and afterwards, the proportion of yeast and mycelial phase proteins containing mannan could be estimated. The column was eluted with 0.2 M α-methylmannoside in 0.1 M sodium phosphate

1 M sodium chloride buffer according to the technique of Kennedy and Rosevear.¹⁰

RABBIT IMMUNISATION

New Zealand white rabbits were injected intravenously at weekly intervals with 25 mg of pressate. They were bled before the first injection to ensure that they were antibody free. Serum obtained after the fourth injection at 21 days was used in subsequent experiments. The titre of each antiserum was 1/10 000 by whole cell agglutination and strongly positive at 1/8 using countercurrent immunoelectrophoresis.¹¹

SDS-PAGE

Pressates and the mannan deplete extract were solubilised in 2.6% SDS and 1.3% 2-mercaptoethanol at 100°C for 5 min. Electrophoresis of these preparations was carried out using 10% polyacrylamide gels in a discontinuous buffer system.¹² Fifty micrograms of protein was applied to each well. Molecular weight standards were: myosin, 200 000; β-galactosidase, 116 250; phosphorylase B, 92 500; bovine serum albumin, 66 200; ovalbumin, 45 000; carbonic anhydrase, 31 000; soybean trypsin inhibitor, 21 500; and lysozyme, 14 400.

WESTERN BLOTS

Proteins were transferred⁴ on to nitrocellulose paper (Bio-Rad Laboratories, Richmond, California) in buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol (pH 8.3). Transfer was performed at 25°C with a current of 350 mA for 1½ h in a Bio-

Table 1 Densitometry of rabbit antiyeast and antimycelial antibody immunoblotted against the yeast phase antigen

Band number	Molecular weight (kilodaltons)	Antiyeast antibody	Antimycelial antibody
1	116	197 (10)	159 (5)
2	100	112 (30)	12 (20)
3	92	178 (20)	
4	84	190 (5)	
5	80	202 (10)	128 (15)
6	74	157 (20)	108 (30)
7	69	237 (13)	230 (5)
8	66	151 (17)	176 (15)
9	63	100 (10)	138 (5)
10	59	200 (5)	152 (15)
11	57	235 (3)	107 (20)
12	56	100 (15)	146 (30)
13	55	163 (20)	179 (5)
14	51	196 (5)	104 (5)
15	50	198 (3)	
16	49	184 (10)	
17	47	118 (5)	127 (20)
18	45	123 (20)	95 (10)
19	41	170 (13)	103 (20)
20	34	222 (30)	200 (30)
21	33	217 (10)	235 (5)

Values (in mm) given as mean (SD) of five immunoblots.

Table 2 Densitometry of rabbit antiyeast and antimycelial antibody immunoblotted against the mycelial phase antigen

Band number	Molecular weight (kilodaltons)	Antiyeast antibody	Antimycelial antibody
1	116	105 (5)	161 (20)
2	100	99 (5)	113 (10)
3	92	117 (7)	
4	84	133 (5)	115 (10)
5	80		112 (5)
6	74		
7	69	202 (5)	203 (17)
8	66	90 (20)	166 (20)
9	63	127 (5)	128 (5)
10	59	101 (15)	168 (10)
11	57	97 (30)	138 (20)
12	56		172 (15)
13	55	105 (10)	176 (7)
14	51		124 (5)
15	50	108 (5)	
16	49		
17	47		124 (30)
18	45		120 (15)
19	41	87 (17)	126 (30)
20	34	96 (20)	166 (13)
21	33	199 (10)	230 (30)

Values (in mm) given as mean (SD) of five immunoblots.

Table 3 Immunoblotting of rabbit antiyeast and antimycelial sera against mannan deplete *C albicans* yeast phase extract

Band number	Mannan deplete extract	
	Antiyeast	Antimycelial
1	+	+
2	+	+
3		
4		
5	+	+
6	+	+
7	+	+
8	+	+
9		
10	+	+
11	+	+
12	+	+
13	+	+
14	+	+
15		
16	Tr*	
17	±†	±
18	±	±
19	±	±
20		
21	+	+

*Trace consistently present in the five immunoblots examined.

†Not consistently present in the five immunoblots examined.

Rad Trans-Blot cell. Free protein sites were saturated by incubation in 3% bovine serum albumin (Sigma) in buffered saline (0.9% NaCl and 10 mM Tris, pH 7.4) at 4°C overnight. The nitrocellulose was then incubated at 25°C for 2 h with antiyeast or antimycelial rabbit antiserum, diluted 1/100 in 3% bovine serum albumin and 0.05% Tween 20 (Bio-Rad) in buffered saline. After washing five times over 30 min in 0.9% saline and 0.05% Tween 20, the nitrocellulose was incubated for 1 h at 25°C with alkaline phosphatase goat antirabbit IgG (Sigma). The conjugate was diluted 1/1000 immediately before use in 3% bovine serum albumin in buffered saline. After washing, as above, the nitrocellulose was incubated for 15 min at 25°C in a freshly prepared and filtered mixture of equal volumes of naphthol AS-MX phosphate (Sigma; 0.4 mg/ml in distilled water) and fast red TR salt (Sigma; 6 mg/ml in 0.2 M Tris, pH 8.2) as described by O'Connor and Ashmann.¹³ Antigen-antibody binding was semi-quantified a densitometer (Chromoscan 3, Joyce Loeb).

Fig. 1 Immunoblot of rabbit antiyeast serum against the following: (A) *C albicans* yeast phase, (B) *C albicans* mycelial phase, (C) *C parapsilosis*, (D) *C krusei*, (E) *T glabrata*, (F) *C tropicalis*, (G) mannan deplete *C albicans* (yeast phase).

Fig. 2 Immunoblot of rabbit antimycelial serum against the following: (A) *C. albicans* yeast phase, (B) *C. albicans* mycelial phase, (C) *C. parapsilosis*, (D) *C. krusei*, (E) *T. glabrata*, (F) *C. tropicalis*, (G) mannan deplete *C. albicans* (yeast phase).

Results

The SDS PAGE gel failed to show any consistent difference between the yeast and mycelial forms of *C. albicans* when stained by Coomassie blue. This confirms the work of Chaffin and Stocco.¹⁴ Immunoblotting *C. albicans* yeast phase antigens against rabbit antiserum raised against the same yeast phase yielded 21 bands of molecular weights 116 000 (band 1) to 33 000 (band 21). The same antiserum recognised only 14 bands in the mycelial phase (Tables 1 and 2). Antiserum raised against a predominantly mycelial pressate of *C. albicans* reacted with 17 bands in the same pressate and 17 bands in the yeast pressate. Eleven of the bands were recognised by both antisera in both the yeast and the mycelial phases. Two of these (bands 9 and 20) were absent from the mannan deplete prepara-

tion (Table 3). None of the bands was unique to the mycelial phase, whereas band 16 was detectable only in the yeast phase by the yeast antiserum.

Concanavalin A Sepharose B chromatography showed that 10% of the yeast phase and 6% of the mycelial phase proteins contained mannan. A total of five bands were consistently removed by mannan depletion. Four other bands were greatly depleted or removed (Table 3). The mannan deplete form was derived from the yeast phase of *C. albicans*.

Figs. 1 and 2 also show the reactivity of the antiserum, raised against *C. albicans*, to pressates of non-*albicans* species in the yeast phase. Cross reactivity was greatest for *C. parapsilosis*, the yeast antiserum recognising 20 bands and mycelial antiserum 14 bands of molecular weight 116 000–37 000. The 33 000 molecular weight protein was prominent in most species, with the exception of *C. parapsilosis*.

Bands of molecular weights less than 33 000 were not consistent and probably represent small fragments of mannoproteins.

Discussion

Previous studies^{2,3,14-17} attempting to identify mycelial specific antigens have given conflicting results. In this paper Western blotting was used because of its sensitivity⁴ and ability to break down a pool of antigens into its composite parts. With rabbit antisera raised against yeast, 21 antigenic bands were identified in the yeast phase of *C. albicans*. Ten of these were also present in the mycelial phase. At least one mannan containing band (number 16 of molecular weight 49 000) appeared to be unique to the yeast phase. Yeast specific proteins have been described by others.^{2,3} In contrast, all 16 bands in the mycelial pressate recognised by the antimycelial serum were recognisable in the yeast phase. There was therefore no evidence of any mycelial specific antigens.

The yeast phase of *C. albicans* can readily be prepared without any mycelial forms being present. In contrast, mycelial phases inevitably contain a small proportion of yeast cells.⁷ It is impossible to remove these. As a result, yeast specific antigens will be detectable in the electrophoresed "mycelial" pressate and the anti-"mycelial" serum may contain some antiyeast activity. This probably accounts for anomalous results such as bands 3 and 15 (present in both pressates but detected only by antiyeast serum) and band 6 (present only in the yeast phase but detected by both sera) and bands 4, 5, 12, 14, 17, and 18. Such bands may represent yeast specific proteins since, had they been common to both phases, they should be detectable with both antisera in both pressates. Mycelial specific antigens would not be masked in this manner, however, as there is no mycelial contamination of the yeast phase.

Mannan depletion removed bands 3, 4, 9, 15, and 20 consistently, which suggests that they contain appreciable amounts of mannan. Ten percent of the yeast phase and 6% of the mycelial phase proteins contained mannan. This is consistent with previous estimates of the mannan content of pressates⁵ and biochemical data which show that the yeast phase contains more mannan.¹⁸ Bands 16-19 were less consistently removed by mannan depletion, suggesting a much lower mannan content. The more mannan rich bands tend to be preferentially absorbed by the column.

Both antiyeast and antimycelial serum samples cross reacted with the yeast phases of various non-*albicans* species. This was most extensive between the antiyeast serum and *C. parapsilosis* (20 bands),

whereas only 14 bands were recognised by the antimycelial serum. Bands occurring in the same position as those in *C. albicans* are not necessarily the same proteins, although a protein of molecular weight 33 000 was prominent in most species except *C. parapsilosis*. The mannan containing proteins with molecular weights less than 33 000 showed a high degree of cross reactivity between species. They are probably the low molecular weight fragments of larger proteins and react with the *C. albicans* antisera because of their high mannan content. Although the mannoproteins constitute only a small proportion of the pressate, there can be no doubt of their immunogenicity.¹⁹ Excluding this activity, we found that cross reactivity was greater with *C. parapsilosis* and *C. krusei* than with *C. tropicalis* and *T. glabrata* among bands of equal or greater than 33 000 molecular weight.

Hasenclever *et al*²⁰ found that *C. albicans* type A and *C. tropicalis* were antigenically identical using whole cell agglutination. Whole cell agglutination, however, examines only those antigens on the external surface of the fungal cell. These contain mannan. The ultracentrifugation step of the preparation of a pressate is designed to remove as many as possible of such antigens, the sticky mannan being left in the pellet. It is therefore not surprising that immunoblotting suggests considerable cross reactivity between the small mannoproteins but only limited antigenic similarity between the other proteins.

Crossed immunoelectrophoresis has suggested the presence of mycelial specific antigens,^{2,16} but this may reflect the lower sensitivity of this assay system.¹⁶ Smail and Jones,¹⁶ using indirect immunofluorescence, showed that certain antigens were concentrated on the germ tubes of the mycelial phase but were also present in tiny amounts on the yeast surface. Perhaps these same antigens are present in relatively large amounts intracellularly in the yeast phase and so methods such as ours which disrupt the cells before blotting against rabbit antisera do not recognise them as unique to the mycelial phase. Consistent with this, Manning and Mitchell¹ failed to show any mycelial specific antigens when they disrupted their cells before two dimensional electrophoresis. It is possible that mycelial specific antigens are lost during solubilisation before electrophoresis since heat labile, germ tube specific antigens have been described by Sundstrom and Kenny.¹⁷ By means of indirect immunofluorescence, they also showed that these antigens were destroyed by dithiothreitol. In contrast, dithiothreitol extracts were used by Smail and Jones to show antigens that were preferentially expressed on the surface of germ tubes.¹⁶ It is possible that the mycelial phase pro-

duced in vivo is antigenically different from that formed in vitro, although they are morphologically similar.

In conclusion, Western blotting showed multiple yeast specific antigens but no mycelial specific antigens. Any antigen which was specific to the mycelial phase should be recognised by the antimycelial serum and would not be detectable in the yeast presate or by the antiyeast serum. The absence of such an antigen using this highly sensitive technique mitigates against any need to use the mycelial phase as the basis of a serological test for invasive candida.

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