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HSP 90, yeasts and *Corynebacterium jeikeium*

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SUMMARY

Recovery from disseminated candidosis is associated with seroconversion to a 47 kDa breakdown product of the Heat Shock Protein (HSP) 90 of *Candida albicans*. Cloning, sequencing and epitope mapping has allowed the delineation of the immunodominant epitopes LKVIRKNIVKKMIE and STDEPAGESA. Monoclonal and polyclonal antibodies specific to these epitopes are used to show that all strains of *C. albicans* tested produce HSP 90 in both the yeast and mycelial phases. Homologous proteins are demonstrated in *Saccharomyces cerevisiae*, *Candida parapsilosis* and *Corynebacterium jeikeium* but not in *Torulopsis glabrata*. Evidence is presented for the existence of two distinct HSP 90s in *C. albicans*. The first of these is expressed constitutively whilst the second is produced on heat shocking the yeast from 23 to 37 °C.

INTRODUCTION

In mammalian systems it is becoming increasingly obvious that a family of proteins with an apparent molecular weight of 90 kDa on SDS–PAGE electrophoresis and pronounced homology at DNA and protein levels plays a vital role in cell function. They have been implicated in steroid receptors including the receptors for progesterone, oestrogen, androgen and glucocorticoid [1–6].

These 'heat shock proteins' (HSP 90) were originally recognized in the yeast *Saccharomyces cerevisiae* [7] as two genes HSP 82 and HSC 82. Mutants with defects in both genes are not viable, whereas possession of either of the two genes alone is sufficient for growth at normal temperatures [8].

A similar gene has been identified and cloned from *Candida albicans* [9]. A 47 kDa antigenic breakdown product has been implicated in human infection. Patients who recovered from disseminated candidosis, patients with oral candidosis and AIDS and patients with chronic mucocutaneous candidosis had antibody against it [10–12]. Epitope mapping was performed by the Geysen technique, in which multiple overlapping peptides covering the whole length of the derived amino acid sequence were synthesized on polyethylene pins [13]. This showed that patients who recovered from disseminated candidosis and patients who had AIDS and oral candidosis each produced antibody against a peptide of sequence LKVIRK which was 315 amino acid residues from the carboxy terminus. Seroconversion to the 47 kDa antigen and recovery from infection was associated with the production of antibody against this epitope. Antibody against

the epitope DEPAGE which was eight amino acids from the carboxy terminus of the protein was associated with a positive immunoblot against the 40 kDa band of *C. albicans*.

The delineation of exact epitopes within candidal HSP 90 makes it possible to generate antibodies against synthetic peptide epitopes which can be neutralized by adsorption with the corresponding peptide. This paper reports monoclonal and polyclonal antibody production against the epitope LKVIRKNIVKKMIE and polyclonal antibody production against the epitope STDEPAGESA. These reagents are then used to define the temperature at which *C. albicans* expresses HSP 90 and the degree of cross-reaction with homologous antigens produced by the yeasts *S. cerevisiae*, *Candida parapsilosis* and *Torulopsis glabrata*. Previously, hyperimmune antiserum against *C. albicans* cross-reacted well with *C. parapsilosis* on immunoblots and detected circulating *C. parapsilosis* antigen by reverse passive latex agglutination (RPLA) [14, 15]. In contrast it cross-reacted poorly with *T. glabrata* on immunoblots and failed to detect circulating *T. glabrata* antigen (15, 16].

False positive reactions for *C. albicans* antigen have been detected during infection due to *Corynebacterium jeikeium* [17]. The level of antigen correlated with the successful treatment of the infection by vancomycin and seroconversion to an antigenic band at 52 kDa [17, 18]. This suggests that the circulating immunodominant antigen in *C. jeikeium* infection is homologous to Candidal HSP 90. The monoclonal and polyclonal antibodies described here demonstrated HSP 90 in *C. jeikeium*.

METHODS

Organisms

The strains of *C. albicans* in this study were: (1) a clinical isolate, previously cloned, which was responsible for the outbreak of systemic candidosis at the London Hospital [9, 10, 19]; (2) the serotype A strain NCPF 3153; (3) the serotype B strain NCPF 3156 and (4) the outbreak strain from the Belfast neonatal unit [20]. Both the *C. parapsilosis* and the *Corynebacterium jeikeium* were previously described clinical isolates [18, 21]. The *T. glabrata* was strain NCPF 3240 and the yeast *S. cerevisiae* was strain S150.

Media

Yeasts were grown overnight at 23, 30 or 37 °C, on glucose/peptone agar containing 2% (w/v) Bacto Dextrose, 1% (w/v) Bacto Peptone and 2% (w/v) Bacto Agar (Difco). The mycelial form of *C. albicans* was obtained by growth in a defined minimal medium supplemented with amino acids as described by Lee and colleagues [22]. *Corynebacterium jeikeium* was cultured on Columbia blood-agar (Oxoid), at 23 or 37 °C for 48 h aerobically.

Microbial antigen preparation

Organisms on plates were harvested in sterile distilled water. The cell suspension was centrifuged at 6000 g for 20 min. The pellet was resuspended in its own volume of sterile distilled water and placed inside an Xpress (LKB). It was fragmented at −20 °C and centrifuged at 12000 g for 20 min. The supernatant

('pressate') was collected and stored at −20 °C. It was standardized to a protein concentration of 10 mg ml^{-1}.

Immunoblotting

Each microbial pressate was heated at 100 °C for 2 min with cracking buffer (2·6% sodium dodecyl sulphate, 1·3% 2-mercaptoethanol, 6% glycerol, 0·2% bromophenol blue, 50 mM Tris hydrochloride, pH 6·8). Thirty μl of pressate was loaded into each well of a 10% (w/v) polyacrylamide slab gel. Electrophoresis was performed at a constant current of 40 mA. Transblotting onto nitrocellulose, in a buffer containing 20% methanol, 25 mM Tris, 192 mM glycine, pH 8·3, was carried out as previously described [10, 11]. The nitrocellulose was blocked overnight at 4 °C in 3% (w/v) bovine serum albumin (BSA) in buffered saline (0·9% NaCl, 10 mM Tris, pH 7·4). It was incubated at room temperature for 2 h with primary antibody (monoclonal antibody at 1:250 or rabbit antisera at 1:40) diluted in buffered saline containing 3% BSA, 0·05% (v/v) Tween 20. After washing five times for 30 min in 0·05% Tween 20, 0·9% NaCl, the nitrocellulose was incubated for 1 h with alkaline phosphatase conjugated anti-mouse or anti-rabbit IgG immunoglobulin (Sigma) as appropriate. Again after washing, the nitrocellulose was incubated for 15 min with a buffer (100 mM Tris hydrochloride, pH 9·5, 100 mM NaCl, 5 mM $MgCl_2$) containing, per 10 ml, 66 μl of nitro-blue tetrazolium (NBT) and 33 μl of 5-bromo-4-chloro-3-indolylphosphate (BCIP). NBT and BCIP were each made up to 0·05 g ml^{-1} in 70% N,N-dimethylformamide.

Antibodies, where appropriate, were cross-absorbed with the corresponding synthetic peptide (50 μg/ml) at 4 °C overnight.

The following molecular weight markers were obtained from Amersham International (Prestained Rainbow Markers): myosin 200 kDa; phosphorylase b 92·5 kDa; bovine serum albumin 69 kDa; ovalbumin 46 kDa; carbonic anhydrase 30 kDa; trypsin inhibitor 21·5 kDa; and lysozyme 14·3 kDa.

Rabbit antisera

Hyperimmune antiserum against *C. albicans* was prepared by the subcutaneous injection of three New Zealand White rabbits with 25 mg *C. albicans* pressate mixed with 1 ml Freund's complete adjuvant. The pressate was prepared from the outbreak strain from the London Hospital. Immunization was repeated at 14 days and serum collected at 28 days. The three hyperimmune antisera were examined separately.

Peptides LKVIRKNIVKKMIE and STDEPAGESA were synthesized on an ABI peptide synthesizer model 431A and each conjugated through cysteine to keyhole limpet haemocyanin (KLH). New Zealand White rabbits were injected subcutaneously with peptide (100 μl of 1 mg/ml) with 0·2 ml Freund's complete adjuvant. A further injection was given 14 days later and sera collected at 28 days.

Monoclonal antibodies

An IgG mouse monoclonal antibody (CA-STR7-1) was raised against the LKVIRKNIVKKMIE-KLH conjugate. Balb/c mice were first injected subcutaneously with 50 μg immunogen in sterile Complete Freund's Adjuvant, and

Table 1. *Identification of potential HSP 90 homologues with antibody probes*

Antigen detected (kDa)	Antibody probe: species			
	LKVIRKNIVKKMIE		STDEPAGESA	Pressate
	Mouse monoclonal	Rabbit antiserum	Rabbit antiserum	Rabbit antiserum
C. albicans				
92	+	+	+	+
47	+	+	−	+
40	−	−	+	+
C. parapsilosis				
52	+	+	−	+
T. glabrata				
55	−	−	−	−
S. cerevisiae				
84	+	+	−	+
47	−	+	−	+
Cor. jeikeium				
86	−	+	−	−
52	+	+	+	+

then injected intraperitoneally with 50 μg immunogen in Incomplete Freund's Adjuvant at intervals of 14 days until seroconversion. Fusion was performed 4 days later after a final immunization of 50 μg immunogen intravenously in sterile physiological saline. Fusion, hybridoma screening, clonal selection and antibody analysis were performed according to standard protocols [23]. Screening for antibody against HSP 90 was by immunoblotting with *C. albicans* for antibody against the 47 kDa and 92 kDa bands. Positive hybridomas were re-cloned and re-assayed.

RESULTS

Yeasts

The rabbit hyperimmune antisera raised against the pressate of the outbreak strain of *C. albicans* grown at 37 °C, detected numerous antigenic bands when immunoblotted against pressates prepared from all four strains of *C. albicans* grown at 23 or 37 °C. Fig. 1 shows the results of the three hyperimmune rabbit antisera. Many bands, including those at 40 and 47 kDa, were present in pressates prepared from yeasts grown at 23 or 37 °C, indicating that they were constitutively expressed. The bands at 92 and 60 kDa appeared predominantly at 37 °C but not 23 °C indicating that they were heat inducible. The three rabbit antisera also reacted with a 52 kDa band in *C. parapsilosis* and bands at 84 and 47 kDa in *S. cerevisiae*. They did not cross-react with the 55 kDa antigen of *T. glabrata* (Table 1).

The rabbit hyperimmune antiserum raised against LKVIRKNIVKKMIE detected bands at 92 and 47 kDa in the yeast and mycelial phases of all four strains of *C. albicans* tested. Fig. 2 shows the antibody reaction before cross-absorption with LKVIRKNIVKKMIE and Fig. 3 confirms the disappearance of the bands at 92 and 47 kDa after cross-absorption. Fig. 2 demonstrates a corresponding band at 52 kDa for *C. parapsilosis* and the lack of activity against

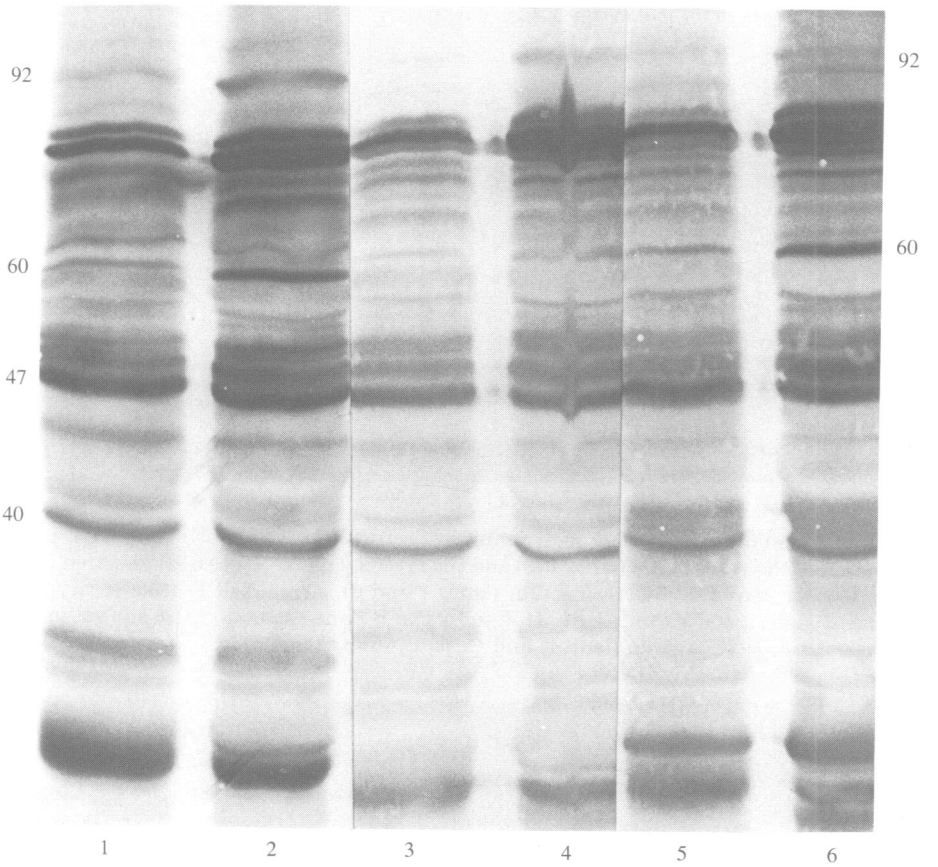

Fig. 1. Immunoblots of the *C. albicans* pressates (the London Hospital, outbreak strain) prepared from the yeast grown at 23 °C (lanes 1, 3 and 5) or 37 °C (lanes 2, 4 and 6) probed with three different hyperimmune rabbit antisera (lanes 1 and 2, rabbit 1; lanes 3 and 4, rabbit 2; lanes 5 and 6, rabbit 3).

T. glabrata. The band at 52 kDa in *C. parapsilosis* was removed by cross-absorption with the peptide. This antiserum also recognized bands at 84 and 47 kDa in *S. cerevisiae*, as did the rabbit antiserum raised against the pressate (Table 1).

The rabbit antiserum raised against STDEPAGESA detected bands at 40 and 92 kDa in all four *C. albicans* strains. This was independent of whether the strain was in the yeast or mycelial phase but in the case of the 92 kDa band dependent on heat shock. The 40 kDa band was expressed constitutively. The 92 kDa band was absent in yeasts grown at 23°, present weakly at 30 °C and strongly at 37 °C. Both the 92 kDa and the 40 kDa bands were removed by cross-absorption of the antiserum with the synthetic peptide. No such neutralizable bands were present in *S. cerevisiae*, *T. glabrata* or *C. parapsilosis* with this rabbit antiserum.

The monoclonal antibody probe (CA-Str7-1) confirmed the constitutive expression of the 47 kDa antigen in all strains of *C. albicans* tested. This was independent of both growth temperature and whether the strain was in the yeast or mycelial phase. It detected a 92 kDa band in isolates which were grown at 30

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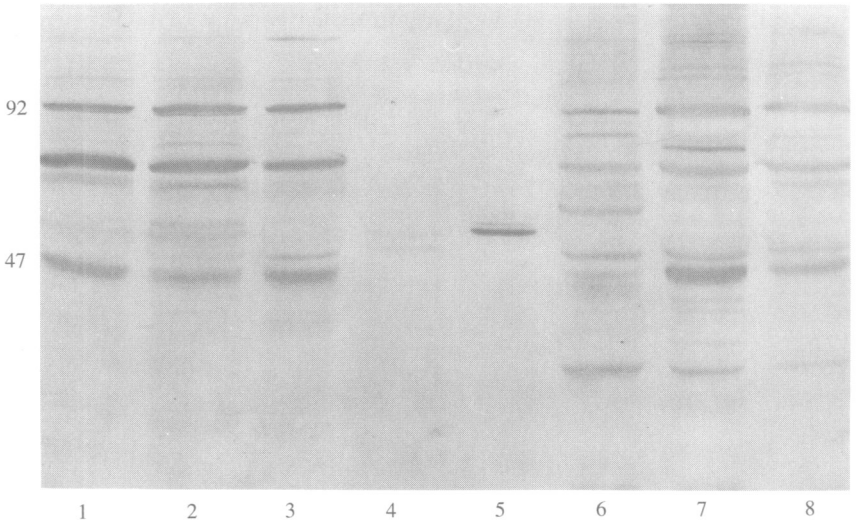

Fig. 2. Immunoblots, probed with rabbit antiserum to LKVIRKNIVKKMIE, of *C. albicans* in the yeast phase (lanes 1–3) and the mycelial phase (lanes 6–8) prepared from the London Hospital outbreak strain (lanes 1 and 6), strain NCPF 3153, serotype A (lanes 2 and 7), and strain NCPF 3156, serotype B (lanes 3 and 8). Also shown, in the yeast phase, is *T. glabrata* (lane 4) and *C. parapsilosis* (lane 5).

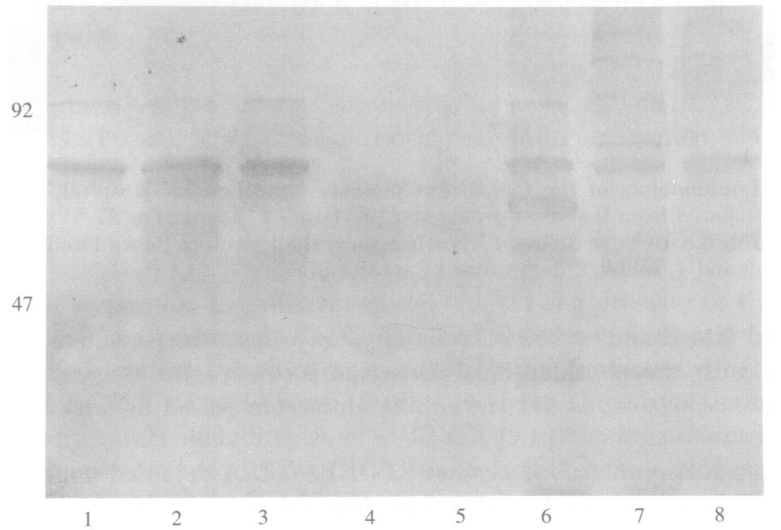

Fig. 3. As Fig. 2 but the rabbit antiserum has been cross-absorbed with LKVIRKNIVKKMIE.

and 37 °C but not at 23 °C (Fig. 4). Both bands were neutralizable by cross-absorbing the monoclonal antibody with the corresponding peptide. An additional band at 34 kDa was also detected by the monoclonal antibody.

In the case of *S. cerevisiae* no reaction was seen with the monoclonal when the yeast was grown at 23 °C but a band at 84 kDa was detected in yeasts grown at 37 °C. *C. parapsilosis* demonstrated a constitutively expressed band at 52 kDa. *T. glabrata* showed no reaction (Table 1).

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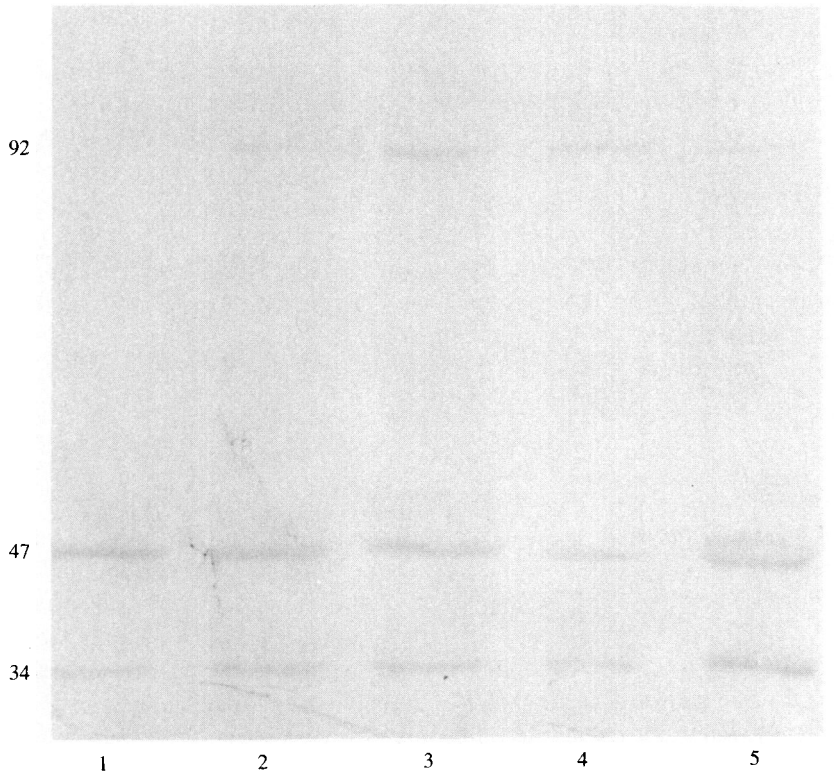

Fig. 4.Immunoblots of *C. albicans* probed with the monoclonal antibody CA-Str7-1. Pressates were prepared from the London Hospital outbreak strain, in the yeast phase, grown at 23 °C (lane 1), 30 °C (lane 2) and 37 °C (lane 3). Also shown is *C. albicans* NCPF 3153 in the yeast (lane 4) and mycelial (lane 5) phases grown at 37 °C.

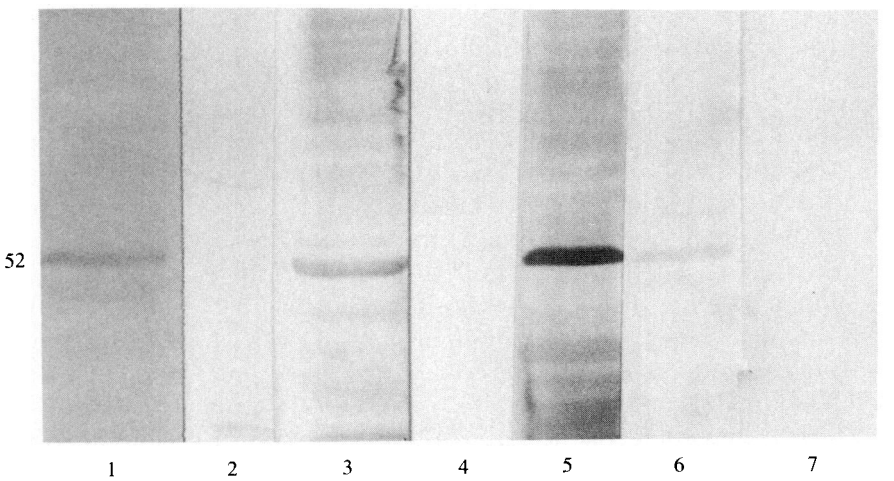

Fig. 5. Immunoblots of *C. jeikeium* probed with monoclonal antibody CA-Str7-1 (lanes 1 and 2), rabbit antiserum against LKVIRKNIVKKMIE (lanes 3 and 4), rabbit hyperimmune *C. albicans* antiserum (lane 5) and rabbit antiserum against STDEP-AGESA (lanes 6 and 7) both before (lanes 1, 3 and 6) and after (lanes 2, 4 and 7) cross-absorption with the corresponding peptides.

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Corynebacterium jeikeium

The rabbit hyperimmune antiserum against the pressate of *C. albicans* reacted with a band at 52 kDa. The *Corynebacterium jeikeium* 52 kDa band reacted with the rabbit sera raised against LKVIRKNIVKKMIE and STDEPAGESA and the monoclonal antibody CA-Str7-1 (Fig. 5). In each case this reactivity was neutralized by cross-absorption with the corresponding synthetic peptide. The rabbit serum against LKVIRKNIVKKMIE also detected a band at 86 kDa which could be removed by cross-absorption with the peptide (Table 1). The band at 52 kDa was present when the organism was grown at 23 or 37 °C whilst that at 86 kDa was only present at the higher temperature.

DISCUSSION

The monoclonal and polyclonal antibodies confirmed the ubiquity of HSP 90 and its breakdown product at 47 kDa in all the strains of *C. albicans* tested. The expression of the 47 kDa band was constitutive, being present at 23 and 37 °C, and independent of whether the strain was in the mycelial or yeast phase. The apparent heat inducibility of the 92 kDa band suggests the existence of two separate HSP 90's in *C. albicans*. The simplest explanation is that the first of these two HSP 90's is expressed at 23 and 37 °C and breaks down on a SDS–PAGE gel to a 47 kDa product. The second is only induced when the yeast is grown at 30 or 37 °C and is stable on electrophoresis, giving a band at 92 kDa. Two HSP 90's have been identified in *S. cerevisiae*, mouse, man, chicken and the mould *Achlya ambisexualis* [7, 8, 24–27]. In both the mouse and the *A. ambisexualis* they are thought to have different functions. In *S. cerevisiae* the deletion of one HSP 90 gene leaves the yeast viable whilst mutants with defects in both genes are non-viable. The mammalian homologue, which shares 60% identify, can effectively complement the double mutant [8].

Heat shock proteins at 84–85, 46–48 and 38–43 kDa have also been demonstrated by Dabrowa and Howard (1984) [28], being produced when *C. albicans* is heat shocked from 23 to 37 °C. They also showed that a germination deficient strain (300-SG) failed to produce the 46–48 kDa antigen [29]. Immunoblotting the HSPs with a rabbit candidal antiserum gave inconclusive results which were thought to be because the serum detected both HSPs and proteins normally synthesized by the cells [29]. The present study with rabbit candidal hyperimmune antiserum demonstrated an immunogenic heat inducible band at 92 kDa whilst that at 47 kDa was expressed constitutively. The inducibility of the 92 kDa band was confirmed by the polyvalent and monoclonal antibodies against LKVIRKNIVKKMIE and the polyclonal rabbit serum against STDEPAGESA. This latter serum also detected a further 40 kDa breakdown product of HSP 90 which was expressed constitutively.

The original dissection of the antibody response in disseminated candidosis identified six patterns (A–F) of response on immunoblots [10]. Response A occurred in patients infected by the strain causing the outbreak of systemic candidosis at the London Hospital. The antibody response was to 10 bands

including those at 92, 47, 40 and 34 kDa which the present study has demonstrated as being part of the HSP 90 complex. The band at 47 kDa was common to all six patterns of response and antibody against the 92 kDa was found in five of them. Response B which was the commonest outside the outbreak had antibody to bands at 92, 47 and 40 kDa. Affinity chromatography with the rabbit hyperimmune serum raised against *C. albicans* and the sera of patients with disseminated candidosis confirmed the presence of circulating candidal HSP 90 [11]. Direct antigen detection by a dot-blot immunoassay further showed that this cytoplasmic antigen of *C. albicans* circulated in disseminated disease [30]. In invasive candidosis, *C. albicans* HSP 90 circulates as both native HSP 90 and as its breakdown products of which the most important is the 47 kDa. The antibody response against immunoblots of *C. albicans* in patients who recover from disseminated candidosis is dominated by this HSP 90 complex. In these patients with antibody, 92% have it to the 47 kDa band and 27% to the 92 kDa [11].

In cases of *C. parapsilosis* infection recovery is associated with seroconversion to a 52 kDa antigen and circulating antigen can be detected by a RPLA test with hyperimmune serum raised against *C. albicans* [14, 21]. The present study suggests that this is due to a high degree of similarity between the HSP 90's of *C. albicans* and *C. parapsilosis* and that HSP 90 is again a dominant circulating antigen in infection due to *C. parapsilosis*. *T. glabrata* shows no cross-reaction with the HSP 90 antibody probes, a negative RPLA test in invasive disease and a lack of cross-reaction between the hyperimmune anti *C. albicans* serum and the immunodominant 55 kDa antigen of *T. glabrata* [16].

Infection with *C. jeikeium* has been associated with a false positive RPLA against *C. albicans* during the early stage of infection. Seroconversion to a *C. jeikeium* band at 52 kDa was demonstrated on recovery [17, 18]. The antibody probes against the HSP 90 of *C. albicans* cross-reacted with this band indicating that this was a breakdown product of *C. jeikeium* HSP 90. The cross-reaction occurred with the rabbit hyperimmune antiserum against the *C. albicans* pressate and the antibodies against LKVIRKNIVKKMIE and the carboxy terminal STDEPAGESA epitope. The carboxy ends of HSP 90s, where the sequences are known, tend to be the least conserved between species. Therefore this degree of cross-reactivity between the 52 kDa antigen of *C. jeikeium* and the 47 kDa antigen of *C. albicans*, demonstrating a high degree of homology at their carboxy ends, suggests a close similarity in the circulating peptides during infection. The rabbit serum against LKVIRKNIVKKMIE differed from the monoclonal in that it also detected a neutralizable band at 86 kDa in the *Corynebacterium jeikeium* pressate. This, in conjunction with the results from examining *S. cerevisiae*, suggests that the two antibodies, although raised against the same peptide, detect slightly different epitopes.

These results emphasize the importance of bacterial and fungal HSP 90 during systemic infection. The role of circulating HSP 90 in the pathogenesis of disease is presently unclear. It may mimic human HSP 90, which has been implicated in steroid receptors as well as being a possible 'molecular chaperon'. It is involved in the assembly and disassembly of proteins into higher-order structures [31]. It may also be present in such large amounts as to exert a function of its own. Interestingly in a mouse model of fatal candidal infection protection could be

produced by serum from patients who had recovered from disseminated candidosis and by a mouse monoclonal antibody to HSP 90 [32]

The HSP 90 of *S. cerevisiae* showed a high degree of homology with that of *C. albicans*, yet *S. cerevisiae* is generally believed to be non-pathogenic for mammals [9]. This is probably due to the far poorer ability of *S. cerevisiae* to colonize the gastrointestinal tract and other organs [33] rather than small differences in HSP 90 itself. In *S. cerevisiae* increasing the level of HSP 90 alone, by introducing the HSP 90 gene on a multicopy plasmid vector, does not lead to thermal tolerance [34]. Therefore, although it is heat inducible, it does not alone account for thermal tolerance and may play other roles in the physiology of the cell. In *A. ambisexualis*, a water mould with sexual reproduction, HSP 90 has been identified as part of the steroid receptor complex and is induced by the female steroid hormone antheridiol [25]. When added to vegetatively growing cultures of the male mating type, antheridiol induces formation of distinctive antheridiol branches. These reports, in association with the observation of a lack of the 46–48 kDa antigen in a germination defective variant of *C. albicans* [29], suggest HSP 90 may be involved in the reproduction of yeasts.

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