Cell Wall Composition of the Mycelial and Blastospore Forms of *Candida albicans*

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SUMMARY

Cell walls were obtained from the mycelial and blastospore forms of *Candida albicans*; these were delipidized and separated into alkali-soluble and alkali-insoluble fractions. The detailed composition of these fractions was determined in organisms grown on different media at 37° and on the same medium at different temperatures (blastospores 30°, mycelium 40°). The composition of the wall of each form was found to be constant, irrespective of growth conditions, except for some variation in the amounts of mannose and glucose in wall hydrolysates. The alkali-insoluble fraction from the mycelial form contained 3 times as much chitin as that from blastospores and only about one third as much protein. The protein from these two fractions showed marked differences in amino acid composition. Differences between the two morphological forms in the amounts of carbohydrate and protein in the alkali-soluble fractions were also found. The results are discussed in relation to other studies of cell-wall composition in the dimorphic fungi.

INTRODUCTION

An increased incidence of candidiasis in certain conditions, e.g. diabetes mellitus and hypoparathyroidism, has long been recognized; more recently it has been recorded as a complication of treatment with corticosteroids and with broad-spectrum antibiotics (for review see Seelig, 1966). *Candida albicans* has been shown to occur as a saprophyte in blastospore form in a high percentage of normal healthy individuals; figures varying from 15-40% have been given (Van Uden, 1960; Dubos & Hirsch, 1965). With tissue invasion, a change to a predominantly mycelial form occurs, and it has been suggested that this determines its pathogenicity (Hill & Gebhardt, 1956; Taschdjian & Kozinn, 1957; Rogers, 1957).

Several factors have been shown to affect the blastospore-mycelium balance in *in vitro* culture. Thus Nickerson (1954, 1963) and Nickerson & Falcone (1956) postulated that the mycelial form develops in cultures generating insufficient H⁺ or thiol groups to maintain reduction of cell-wall protein disulphide linkages or in the presence of chelating agents which inactivate a protein disulphide reductase. Widra (1964) also postulated that blastospore formation is dependent upon an adequate supply of reducing groups and has stressed the importance of adequate concentrations of available magnesium. Thus the development of the mycelial form is seen by these workers to be a result of the inhibition of normal cell division. To further understanding of the role of the mycelial form in susceptibility to *Candida* infection it seems

important to compare the composition and biochemical activities of the two forms. The present paper reports a comparative analysis of the cell-wall material of mycelial and blastospore forms of a single strain of *C. albicans*. In order that the material analysed should be as near as possible to the pathogenic type of growth all analyses were effected on organisms grown from fresh isolates obtained from a child with chronic widespread *Candida* infection.

METHODS

Organism. A strain of *Candida albicans* was used which was freshly isolated from mouth swabs of an 8-year-old child with a widespread infection of several years' duration, except that a few experiments were made, following the death of the patient, from freeze-dried cultures of initial isolates. Following isolation subculture was effected on to Sabouraud agar slopes and the growth harvested by suspension in sterile water for inoculation into media for large-scale growth.

Media. Media were devised based on those of Nickerson & Mankowski (1953), Roth & Goldstein (1961) and Widra (1964); for blastospore production medium A containing glucose (0.2%), sodium chloride (0.3%), Difco yeast extract (0.1%) and Difco Bactopeptone (5.0%) with incubation at 37°, and medium B as medium A but substituting Difco Neopeptone for the Bactopeptone and starch for glucose with incubation at 30°. Mycelial phase was grown on medium B with incubation at 40° and on 100% ox serum (medium C) with incubation at 37°. Ox blood was collected aseptically, allowed to clot overnight and the serum used after centrifugation.

All media were dispensed in a volume of 500 ml. in 1 l. flasks, inoculated heavily with a blastospore suspension and incubated for 18 hr in a New Brunswick Environmental Incubator Shaker (100 rev./min.).

Preparation of cell-wall fractions. Organisms were centrifuged down and washed 3 times by suspension in 0.05 M-tris (pH 6.8). Mycelial growth on the starch-Neopeptone medium at 40° was accompanied by some production of blastospores; these were removed by homogenizing a suspension of organisms in the tris buffer in an MSE glass homogenizer, followed by repeated filtration through a sintered-glass disc. After about 10 such filtrations the mycelial residue was free from blastospores. A heavy suspension of washed mycelium or blastospores in the tris buffer was put through a French press at 20,000 lb./in.² pressure. The product was examined by light microscopy for the extent of cell breakage and, when necessary, the process was repeated until at least 90% cell breakage had been achieved. The final suspension was centrifuged at 1400 g for 10 min. and then successively washed 3 times each at 0° with M-sucrose, 0.1 N-sodium chloride and distilled water, increasing the time and speed of centrifugation to a maximum of 2000 g for 20 min. This procedure gave clean wall material as judged by light microscopy and the absence of staining with methylene blue. The final product was freeze-dried and stored at -20°.

Lipid readily extractable was separated from the dried wall preparation (400 mg.) by shaking for 1 hr at 4° 3 times with 40 ml. lots of chloroform+methanol mixture (1+1, v/v). Chemically bound lipid was extracted by the method of Kessler & Nickerson (1959).

Experience showed that material reacting in the orcinol test for pentose could be extracted with tris buffer from the de-lipidized wall and thus all preparations were

extracted for 2.5 hr with 0.2 M-tris buffer (pH 7.8; 5 mg. de-lipidized wall/3 ml. buffer), conditions which were found to effect complete removal of this material which was probably due to traces of sucrose from the washing procedure. The wall material was then separated by the procedure of Kessler & Nickerson (1959) into an alkaline-insoluble fraction and alkali-soluble fractions 1 and 2.

Acid hydrolysis of cell-wall fractions. All tubes containing solutions for hydrolysis were evacuated to remove dissolved air and then sealed under nitrogen. All acid hydrolyses for determination of sugar components were effected in duplicate on 10 mg. samples together with a third hydrolysate containing a 5 mg. sample + 5 mg. of component to be estimated. All results were corrected for recovery of added sugar, since losses during hydrolysis were found to be considerable and variable. Acid hydrolysis conditions were as follows. (a) *Amino sugars*: 4 N-HCl (5 ml.) heated at 110° for 16 hr; HCl was removed in vacuum in a rotary evaporator, the residue dissolved in water, passed through Norit NK (Hopkin and Williams Ltd) on a sintered glass disc, the Norit washed with water, the combined filtrates adjusted to pH 7.0 and made to 25 ml. with water. (b) *Total reducing sugars* by the method of Johnston (1965). (c) *Amino acids*: 6 N-HCl (2 ml.) heated with wall fraction (10 mg.) for 18 hr at 105° and the hydrolysate treated as for amino sugars. (d) *Total phosphorus*: wall material (5 mg.) digested for 60 min. with 10 N-H₂SO₄ (0.4 ml.) at 130°, cooled and 30% H₂O₂ (0.1 ml.) added and then heated at 130° for 10 min. After cooling, distilled water (1.0 ml.) was added and after heating at 100° for 30 min. the orthophosphate formed was estimated by the method of Fiske & SubbaRow (1925).

Enzymic digestion of wall material. β -Glucanase was separated from a crude preparation of snail digestive juice (suc digestif d'*Helix pomatia* obtained from Industrie Biologique Française, Gennevilliers, Seine, France) by the method of Anderson & Millbank (1966). Fraction A was used and shown to be devoid of chitinase activity. Pronase and chitinase were obtained from Calbiochem Ltd. Alkali-insoluble wall fraction (100 mg.) was suspended in McIlvaine citrate phosphate buffer (pH 5.8; 50 ml.) and incubated with the β -glucanase (25.0 mg.) at 30° for 8 hr. The washed residue was suspended in 0.2 M-tris (pH 7.1; 50 ml.) and incubated with pronase (25 mg.) at 25° for 72 hr. A sample (5.0 mg.) of the residue was suspended in 0.05 M-phosphate buffer (pH 6.3, 5 ml.) and incubated with chitinase (10 mg.) at 36° for 22 hr. All incubation mixtures were shaken.

Analytical methods. Total reducing sugars were estimated by the method of Nelson (1944), glucose according to Marks (1959) except that 4.0 ml. of the 'working' solution and 0.4 ml. of the glucose-containing sample were used. The reaction was stopped after 12 min. by the addition of 8 N-HCl (0.25 ml.), giving a yellow colour which was read at 440 m μ (Dr M. Green, personal communication). Mannose was calculated from the difference between the values from the above two methods, also making allowance for hexosamine when present. Hexosamine was estimated according to Tracey (1955), total-N by the micro Kjeldahl method and α -NH₂ nitrogen according to Moore & Stein (1954). RNA was estimated by digestion of wall material (5 mg.) with RNase (1.5 mg.) in 0.2 M-tris (pH 7.8; 3 ml.) at room temperature for 40 min., followed by addition of 5% TCA (7 ml.) and heating at 90° for 15 min. After centrifugation, ribose was estimated on the supernatant solution by the method of Mejbaum (1939). Amino acids were determined in a single-column spherical-bead apparatus with Amberlite CG 110 resin and elution with citrate buffers at pH 3.5, 4.6 and 6.5.

Chromatography. Chromatography of sugars was effected on Whatman no. 1 paper with the following solvents: (a) *n*-propanol+acetic acid+water (7+1+2, by vol.); (b) *iso*-propanol+*n*-butanol+water (7+1+2, by vol.). Detection was with the alkaline silver nitrate reagent of Trevelyan, Procter & Harrison (1950). Ninhydrin-reacting materials were separated on Whatman no. 4 paper on 2-way chromatograms with the following solvents: (a) *n*-butanol+acetic acid+water (4+1+5, by vol.); (b) the phenol+ammonia mixture of Smith (1960).

RESULTS

Two factors which affect the yeast/mycelial morphology of dimorphic fungi are temperature of growth and medium composition (for review see Mariat, 1964). Both these factors also affect the morphology of *Candida albicans* and therefore the cell-wall composition of the two forms was studied when produced (a) under constant incubation temperature (37°) with different media (media A and C) and (b) with a common medium (medium B) but different incubation temperatures, 30° for blastospores and 40° for mycelium. Growth at the latter temperature gave a greater proportion of mycelium than at 37°.

Table 1. *Lipid fractions extracted from cell walls of blastospore and mycelial phases of Candida albicans as percentage of lyophilized whole wall*

Lipid fraction	Blastospore growth medium		Mycelial growth medium	
	A, 37°	B, 30°	B, 40°	C, 37°
Chloroform+methanol extract	0.55	3.32	1.9	5.5
Acidified ethanol+ether extract	—	7.3	3.14	—

Composition of cell wall from yeast and mycelial phases

Lipid. Typical results for the content of lipid extracted by chloroform+methanol from freeze-dried wall fractions and subsequent extraction with acidified ethanol+ether are shown in Table 1. The former lipid is seen to be variable in amount, being affected by medium composition and temperature. The tenfold increase in this fraction when the organism was grown in serum at 37° as compared with medium A at the same temperature may be a reflexion of the fact that the serum medium was the only medium with a significant lipid content. This result may be of interest, since *in vivo* growth is in the presence of such materials. Chemically bound lipid was only extracted from wall material grown on medium B; the amount found in blastospores grown at 30° was approximately twice that of mycelium grown at 40°. Since the reagents used in the extraction of this fraction may affect linkages between wall components (Phaff, 1963), this extract has been omitted from the described fractionation of wall material.

Fractionation of chloroform-methanol extracted wall. The percentage distribution between the alkali-soluble and alkali-insoluble fractions and the recovery of material during fractionation is shown in Table 2. The composition of these fractions as obtained from blastospores and mycelium is shown in Table 3 for growth on different media at 37° and in Table 4 for growth on medium B at different temperatures. Chromatographic study showed glucose and mannose to be present in the hydrolysates of all fractions; glucosamine only in those indicated in Tables 3 and 4, and

Table 2. *Alkali fractionation of cell wall of blastospore and mycelial phases of Candida albicans*

	Blastospore growth medium		Mycelium growth medium	
	A, 37°	B, 30°	B, 40°	C, 37°
	Recovery as % of lyophilized wall taken			
	87	100	92.5	99
	Composition of fractions as % of total wall recovered			
Alkali-insoluble	63.2	52	61.5	55.7
Alkali-soluble	36.8	48	38.5	44.5
Alkali-soluble 1	16.1	18.5	7.0	14.5
Alkali-soluble 2	20.7	29.5	31.5	31.0

Table 3. *Percentage composition of cell-wall fractions of Candida albicans grown in different media at 37°*

Blastospores were grown on medium A; mycelium was grown on medium C

Component	Cell-wall Fraction					
	Alkali-insoluble		Alkali-soluble 1		Alkali-soluble 2	
	Blastospores	Mycelium	Blastospores	Mycelium	Blastospores	Mycelium
Total reducing sugar	59.0	61.8	8.6	15.6	29.3	14.4
Glucosamine	2.7	9.9	Nil	0.9	Nil	2.6
Glucose	45.0	36.0	4.2	13.3	1.9	3.8
Mannose	14.0	25.8	4.4	2.3	27.4	10.6
Total-N	6.0	2.2	9.2	8.7	2.2	3.8
Protein*	36.8	9.0	57.5	53.8	13.8	22.5
Phosphorus	2.2	1.3	2.6	1.8	2.6	1.4
RNA	1.5	0.7	—	—	—	0.38

* Total N $\times 6.25$ after allowing for glucosamine-N.Table 4. *Percentage composition of cell-wall fractions of Candida albicans grown on medium B at different temperatures*

Blastospores were grown at 30°, mycelium at 40°

	Cell-wall fraction					
	Alkali-insoluble		Alkali-soluble 1		Alkali-soluble 2	
	Blastospores	Mycelium	Blastospores	Mycelium	Blastospores	Mycelium
Total reducing sugar	63.6	64.3	10.0	21.3	26.7	20.3
Glucosamine	2.9	10.6	Nil	Nil	Nil	Nil
Glucose	47.0	45.3	3.3	12.3	1.0	5.3
Mannose	16.6	19.0	6.7	9.0	25.7	15.0
Total N	3.8	2.2	9.6	7.3	1.5	3.6
Protein*	22.5	8.8	60.0	45.6	9.4	22.5
Phosphorus	0.6	0.6	0.5	1.4	0.6	0.7
RNA	0.54	0.17	—	—	0.53	0.35

* Total N $\times 6.25$ after allowing for glucosamine-N.

no other sugars were demonstrated. Total phosphorus values were consistently higher in wall fractions grown on media A and C than in those grown on medium B. This difference was not simply due to a higher medium concentration of phosphorus, since medium A contained 19.1 mg. P/100 ml. and medium B 14.5 mg. P/100 ml., while ox serum has a value of about 3–8 mg. P/100 ml.

Composition of alkali-insoluble fraction

The composition of this fraction from blastospore walls was virtually constant under the two sets of growth conditions; that of mycelium was also constant except for a change in the glucose:mannose ratio from 1.4:1 on medium C to 2.4:1 on medium B. The most striking differences between the two forms was in the glucosamine content of the hydrolysates, which in the mycelial fraction was several times greater than that from the blastospores, irrespective of growth conditions, and the appreciably higher level of protein in the latter material.

Nature of amino sugar binding. Chitin has been shown to be present in the cell wall of a number of yeast species, including *Candida albicans*, on the basis of chemical tests or X-ray diffraction studies (Roelofsen & Hoette, 1951; Houwink, Kreger & Roelofsen, 1951; Bishop, Blank & Gardner, 1960) and its presence in blastospores of *Candida albicans* inferred from glucosamine estimations by Kessler & Nickerson (1959). Korn & Northcote (1960) and Eddy (1958) showed that only a small proportion of the glucosamine present in baker's yeast cell-wall hydrolysates was derived from chitin. The form in which glucosamine was present in the alkali-insoluble fractions has been examined by extraction with 2% HCl and 3% NaOH as described by Korn & Northcote (1960) and by X-ray diffraction studies of the fractions as initially prepared and after digestion with β -glucanase and pronase. The extraction procedure showed that 96% of the glucosamine of both mycelial and blastospore fractions was resistant to extraction and thus had the solubility properties of chitin.

The results of enzymic digestion of the fractions is shown in Table 5. The fact that 75% of the glucan in the blastospore fraction was broken down and only 44% of that in the mycelial fraction may indicate a difference in wall structure between the two forms. Pronase digested 55% of the protein of the blastospore and 35% of the mycelial fraction. The final residue contained from 90 to 95% of the glucosamine of the original material. Chitinase treatment of this residue released as N-acetyl glucosamine 62% of the hexosamine from the mycelial and 43% from the blastospore fraction. X-ray diffraction studies of the original fractions and the residue after glucanase and pronase treatment showed the presence of chitin in material from both forms. These results indicate that the glucosamine of the alkali-insoluble fraction from both growth forms is present almost entirely as chitin, the level in the mycelial form being significantly greater than that of the blastospores.

Protein. The amino acid composition of acid hydrolysates of alkali-insoluble wall fractions from both growth forms is shown in Table 6. The most striking difference between the two materials is the occurrence of histidine as the principal basic amino acid in the blastospore fraction and its absence from the mycelial fraction, with arginine showing the opposite distribution. In addition, glutamic acid, proline, valine, methionine and lysine show major differences in their distribution between the two forms, and minor components, mainly unidentified, also show differences. Glucosamine appeared to be present in appreciably larger amounts in the mycelial fraction,

but the interpretation is difficult owing to incomplete separation from phenylalanine. These results indicate that the protein in this fraction is different in the two growth forms, although the insolubility of the material has made it impossible to determine whether a single protein or a mixture is present.

Table 5. *Digestion of the alkali-insoluble fraction of Candida albicans cell wall with β -glucanase, pronase and chitinase*

The wall fractions were treated successively with β -glucanase, pronase and chitinase under conditions described in the text. Values of components estimated are given as % of original fraction taken. The α -amino-N values have been corrected for glucosamine content.

Morphological form of culture	Material released by:					
	β -glucanase		Pronase		Chitinase: N-acetyl glucosamine	Acid hydrolysis of chitinase substrate: Glucosamine
	Glucose	Glucosamine	α -Amino-N	Glucosamine		
Mycelium	18.8	0.12	0.6	0.05	6.7	10.8
Blastospore	39.2	0.4	1.74	0.16	1.8	4.26

Table 6. *Amino-acid composition of acid hydrolysates of the alkali-insoluble fraction of cell walls of the blastospore and mycelial forms of Candida albicans*

Values are the mean of two determinations and are expressed as μ mole amino acid/1000 μ mole. The material analysed was obtained from growth on medium B under conditions as described in the text.

Amino acid	Mycelium	Blasto-spores	Amino acid	Mycelium	Blasto-spores
3-hydroxy proline*	2.2	—	Leucine	75.1	63.3
4-hydroxy proline*	29.0	—	Tyrosine	36.1	50.9
Aspartic acid	65.4	65.3	Glucosamine + phenylalanine†	140.5	47.1
Threonine	74.3	61.0	O‡	42.6	—
Serine	56.5	55.5	P‡	—	4.3
Glutamic acid	90.7	65.5	S‡	—	15.0
Proline	36.9	63.1	U‡	—	8.8
Glycine	73.6	69.3	Lysine	7.7	25.8
Alanine	107.0	88.8	Histidine	—	112.2
Valine	93.8	50.6	Arginine	53.4	—
Methionine	21.6	79.3	Recovery	89.9%	98.4%
Isoleucine	58.7	53.2			

* Identified only by absorption maximum at 440 m μ .

† Complete separation of glucosamine and phenylalanine was not achieved.

‡ Unidentified components.

Composition of alkali-soluble fractions

Fraction 1 (Tables 3 and 4). This fraction from both growth forms showed a high protein content. The composition of blastospores was constant irrespective of growth conditions; that of mycelium was also constant except for variation in the glucose: mannose ratio from 5.8:1 on medium C to 1.4:1 on medium B. The principal difference shown between the two forms was in the concentration of glucose, this being approximately 3 times greater in the mycelial form. Glucosamine was absent from this fraction in both forms.

Fraction 2 (Tables 3 and 4). The material from blastospores was of constant composition irrespective of growth conditions; that from mycelium was also relatively constant

except for variation in the mannose values, this being lower on medium C. Comparison of the two forms showed a considerably higher proportion of mannose in the blastospore fraction and of protein in the mycelial fraction. Glucosamine was absent or at low value in both forms. The most notable feature of these analyses is the low recovery (i.e. some 40%) of material as sugar, amino acid derivatives and RNA.

DISCUSSION

The composition of the cell wall of blastospores of *Candida albicans* as revealed by analysis of hydrolysates of wall fractions was constant when grown on different media and at different temperatures. The cell-wall composition of the mycelial form showed a similar constancy, except for variation in the values for glucose and mannose units present, according to the growth conditions used. Comparison of the composition of the cell walls of the two growth forms showed a qualitative similarity but considerable quantitative variations, particularly in terms of glucosamine, glucose, mannose and protein content. Such qualitative similarity, accompanied by quantitative variations in wall composition of dimorphic forms, is also seen in *Mucor rouxii* (Bartnicki-Garcia & Nickerson, 1962) and *Histoplasma capsulatum* (Kobayashi & Guiliacci, 1967). This suggests that the blastospore $\leftrightarrow$ mycelium change is directed by variation in the activity of enzymes which produce wall components rather than in the elaboration of new components. Comparative study of metabolic activities in the two forms of *C. albicans* is the subject of further work.

The presence of a higher value for chitin in the mycelial form of *Candida albicans* suggests a similarity in wall structure to many filamentous organisms. A higher value for chitin in the mycelial form of growth has not previously been recorded in the dimorphic fungi. Bartnicki-Garcia & Nickerson (1962) found similar amounts in the two forms of *Mucor rouxii*, and whilst Kobayashi & Guiliacci (1967) demonstrated a greater hexosamine content in the yeast form of *Histoplasma capsulatum*, they did not show the presence of chitin in this organism. This difference in chitin values between the two growth forms of *C. albicans*, together with the differences in susceptibility to digestion by β -glucanase and in the protein composition of the alkali-insoluble wall fraction, indicate that considerable differences are likely to be present in the tertiary structure of the polysaccharide-protein complexes of the cell wall. These may result in differences in permeability, with changes in resistance to host defence mechanisms, and may be in part responsible for the greater persistence of the mycelial form *in vivo*. The differences in amino acid composition of the proteins analysed are striking, in particular the presence of histidine, but no arginine in the blastospore fraction and the reversal of this distribution in the mycelial fraction. Kessler & Nickerson (1959) reported a predominance of acidic amino acids in a corresponding wall fraction from baker's yeast and suggested that the acidic groups may be of importance in the cell-wall structure through ester linkages between polysaccharide and amino acid residues. No such preponderance of acidic amino acids was found in the *C. albicans* fractions studied here.

The only other recorded comparative analysis of the two forms of this organism is that by Kessler & Nickerson (1959) in which the wall material from blastospores and a divisionless mutant derived from the same strain were studied. Both forms were grown on the same medium and at the same temperature (28°) for 48 hr. The analyses

showed that the wall fractions had virtually the same composition, irrespective of the growth form. The mycelium produced by this mutant was non-septate 'pseudo'-mycelium, whereas that produced by the organism and under the growth conditions used here was normal septate mycelium; this difference and the different environmental conditions required to induce mycelium production may account for the differences in composition between the two sorts of mycelium. Alternatively, the cell wall of a freshly isolated pathogenic strain of the organism may differ markedly from that of a laboratory-developed mutant strain. Differences are also apparent between the composition of the blastospore wall described here and that recorded by Kessler & Nickerson. The principal differences are the presence of markedly higher protein contents in all our fractions and of appreciable quantities of mannose in the alkali-resistant fraction. Growth temperature did not affect blastospore wall composition and is unlikely to explain these differences. The inclusion by Kessler & Nickerson of the extraction with acidified ethanol+ether may have affected the stability of the macromolecular complexes of the wall, otherwise the different growth media used or strain differences are the most likely cause of these differences. Nickerson (1963) suggested that the development of the mycelial form results from impaired cell division arising from lack of intracellular sulphhydryl groups which normally result from protein disulphide reductase activity. Where this is decreased or lacking the elasticity of the mannan-protein complex is considered to allow extension of the cell without division. The results recorded above indicate that a more complex mechanism may be involved in the blastospore $\leftrightarrow$ mycelium change, since recorded differences between the two forms indicate a requirement for increased chitin synthesis and changes in the type of protein produced in the development of mycelium.

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