Induction of the Mycelial Form of *Candida albicans* by Hydrolysates of Peptides from Seminal Plasma

By F. W. CHATTAWAY AND J. O'REILLY

Department of Biochemistry, The University, Leeds LS2 9LS

AND A. J. E. BARLOW AND T. ALDERSLEY

Department of Dermatology, Huddersfield Royal Infirmary, Huddersfield, Yorkshire

(Received 6 November 1975; revised 23 March 1976)

SUMMARY

Previous work led to the separation from seminal plasma of a peptide fraction which promoted a high rate of germination of blastospores of *Candida albicans*. It has now been shown that an acid hydrolysate of this material is also highly active. A minimal amino-acid mixture consisting of aspartic acid, lysine, histidine, threonine, proline and β -alanine gave 90 % germination in 4 h with 17 out of 28 strains examined. Glucose and inorganic phosphate were also required. Phosphate was not required for the activity of the original peptide fraction.

INTRODUCTION

The isolation from seminal plasma of material which, in the presence of 0.2 % glucose and at 37 °C, is highly active in inducing germination of blastospores of *Candida albicans* has been reported previously (Barlow, Aldersley & Chattaway, 1974). Further separation of the peptides present in the active material proved to be difficult. In this paper we report the retention of germ-tube producing activity following acid hydrolysis of the peptides and describe a minimal amino acids/salts medium which induces a high rate of germination in many strains of *Candida albicans*.

METHODS

Organism. Freeze-dried cultures of *Candida albicans*, Ca1 and Ca2, were used in all experiments. The germ-tube producing capacity of the final minimal amino acids/salts/glucose medium was assessed on fresh isolates of 28 other strains obtained from a variety of sites on the skin, from sputum and from stools.

Estimation of growth. The extent of germ-tube production in the 6 h following inoculation was determined under the conditions described previously (Barlow *et al.*, 1974). Germ tubes were defined as cells with a diameter of more than three times that of 'normal' blastospores (judged visually); counting was carried out in a haemocytometer. Occasionally, cells showed the development of two germ tubes and these were counted individually. The extent of germ-tube formation induced by a given medium with the same *Candida* strain showed some variation between experiments. Thus in 40 experiments involving strain Ca2 and the minimal amino-acids mixture described in Table 3, the mean yield of germ tubes in 4 h was 87.8 % $\pm$ S.D. 4.65 and in 6 h was 87.3 % $\pm$ S.D. 4.63. In determining the composition of this mixture it was necessary to assess the significance of relatively small changes in the response

Table 1. *Germ-tube production by Candida albicans strain ca2 from serum fraction C5 and seminal plasma fraction D2 and their acid hydrolysates*

Fraction	% Germ-tubes produced in 4 h
Serum C5	82
Serum C5 acid hydrolysate	78
Seminal plasma D2	93
Seminal plasma D2 acid hydrolysate	94

and so the results are expressed as a percentage of the response to the complete minimal amino-acids mixture determined at the same time in each separate experiment.

Assessments were made after 4h, which is the time of maximum germ-tube production for this strain. Mycelial development in *Candida albicans* is followed by the development of blastospores from the hyphae, which themselves do not form germ tubes but multiply by budding and cell division; thus the proportion of mycelium present will decrease with time even though the mycelial strand itself is still being extended. The time at which this later blastospore development occurs varies both with the medium and the strain used.

Total growth in periods up to 24 h was determined as described previously (Barlow *et al.*, 1974).

Seminal plasma. The sources of bovine and human semen and the separation of the germ-tube producing material were as described previously (Barlow *et al.*, 1974).

Acid hydrolysis. Material prepared from seminal plasma and serum was dialysed against water, freeze-dried and then heated with 6 M-HCl (0.2 ml/mg protein) at 110 °C for 18 h under nitrogen. HCl was then removed by repeated evaporation *in vacuo* over KOH; and the final product was dried, redissolved in water to the original volume, and adjusted to pH 7.4.

Amino-acid analysis. This was carried out using a LKB Biocal BC-200 single column (600 × 9 mm) with buffers of pH 3.20, 4.25 and 6.45 and Biorad Aminex A-6 (sulphonated polystyrene) resin at 55 °C.

Chemical determinations. Protein was assayed by the method of Lowry *et al.* (1951) using bovine serum albumin (Sigma) as a standard; phosphorus was estimated by the method of Young (1966); potassium by absorption spectroscopy; sodium by emission spectroscopy; and other trace elements by flame photometry. In each case, correction was made for reagent blank values.

RESULTS

Germ-tube production with acid hydrolysates of fractions from serum and seminal plasma

Samples of the fractions previously isolated from serum (fraction C5) and seminal plasma (fraction D2) (Barlow *et al.*, 1974) were hydrolysed with hydrochloric acid. After removal of the hydrochloric acid and reconstitution of the product to the original concentration, the hydrolysates showed no loss of germ-tube producing activity (Table 1). Such hydrolysates always show full activity even though their quantitative amino-acid compositions showed some variation—typical analyses are given in Table 2. The analysis of fraction C5 indicates that it has a similar composition to serum albumin; this fraction has previously been shown by column chromatography to be 98 % albumin (Barlow *et al.*, 1974). Fraction D2 hydrolysates were examined for the presence of inorganic cations: the only ones detected were magnesium (580 µg ml⁻¹) and potassium (16.7 µg ml⁻¹); phosphorus was absent.

Table 2. *Amino-acid analysis of acid hydrolysates of serum fraction C5 and fraction D2 from bovine and human seminal plasma*

Samples were hydrolysed as described in Methods. Results for seminal plasma fractions are the mean of two determinations.

Amino acid	Percentage composition		
	Serum fraction C5	Seminal plasma fraction D2	
		Bovine	Human
Asp	10.1	12.8	17.0
Thr	4.3	3.6	3.0
Ser	3.1	6.1	3.6
Glu	17.9	29.0	35.1
Pro	3.3	3.3	0.8
Gly	1.2	5.1	4.8
Ala	7.5	7.3	2.6
$\frac{1}{2}$ Cys	2.0	—	—
Val	7.1	4.3	3.4
Met	1.0	0	0
Ile	1.4	1.8	1.7
Leu	10.3	3.9	2.5
Tyr	3.0	2.1	1.0
Phe	6.9	2.4	1.1
Lys	12.0	10.4	13.5
His	3.5	2.7	8.4
Arg	5.2	4.8	1.8
β -Ala	0	1.6	ND

ND, Not determined.

Germ-tube production with amino acids/salts mixture

A synthetic mixture of amino acids, magnesium sulphate and potassium hydrogen phosphate, prepared in accordance with the determined composition of fraction D2 was as effective in germ-tube production as the acid hydrolysate. By elimination, the minimal amino acids/salts mixture was determined which gave the same germ-tube production in 4 h as the complete mixture. The constituents were maintained at their concentrations in the original hydrolysate. The results, and the requirement for potassium and phosphate ions, are shown in Table 3. Magnesium was not required. The omission of any one of the amino acids from the optimum mixture of aspartic acid, threonine, proline, lysine, histidine and β -alanine led to a fall of 7 to 15 % in germ-tube yield; except when aspartic acid was omitted, the fall was approximately 40 %. β -alanine was not detected in the fully active hydrolysate of the serum C5 fraction and its omission in these experiments gave a marginal fall in activity. Germ-tube yield was low if KH_2PO_4 was omitted, but K^+ could be substituted by Na^+ with only a 15 % fall; substitution of Cl^- for H_2PO_4^- gave very low yields. This was not due to an inhibitory effect of the Cl^- since germ-tube yield was normal when both KH_2PO_4 and Cl^- were present and therefore seems to be due to an absolute requirement for phosphate.

The requirements for germ-tube formation appear to be different from those for growth over a longer period of time. The minimal amino acids/glucose/ K_2HPO_4 medium gave only 1.4 mg dry wt cells/10 ml medium after 24 h at 37 °C compared with 10.8 mg/10 ml medium with Sabouraud glucose broth under the same conditions. The minimal medium did not support mycelial development over the longer time as the small amount of growth obtained at 24 h was almost entirely blastospores.

Table 3. *Determination of a minimal amino acids/salts mixture to give maximum germ-tube formation from blastospores of C. albicans strain ca2*

An amino-acid mixture corresponding to an acid hydrolysate of bull seminal plasma fraction D2 was used as the starting material. This mixture contained (nmol ml⁻¹): Asp 95.4, Thr 30.6, Ser 62.6, Glu 215.7, Pro 33, Gly 70.6, α -Ala 89.5, Val 35.2, Ile 14.7, Leu 32.1, Tyr 14.6, Phe 14.7, Lys 70.1, His 17.6, Arg 49.2, β -Ala 20.0; glucose (11 μ mol ml⁻¹) and KH₂PO₄ (428.3 nmol ml⁻¹) were added; and it yielded 90% germ-tubes in 4 h. The minimal amino-acid mixture required to give the same yield of germ-tubes was determined by elimination. The effect of omitting each amino acid from this minimal mixture and the requirement for K⁺ and H₂PO₄⁻ are shown. Other ions were added in equimolar quantities. The response to the complete minimal mixture was determined in each experiment and the results are given as a percentage of this value. Glucose was present in all experiments. All values are the mean of at least three experiments.

Amino acids						K ⁺	H ₂ PO ₄ ⁻	Other ions	% Germ tubes produced in 4 h
β -Ala	Asp	Thr	Pro	Lys	His				
+	+	+	+	+	+	+	+	—	100
+	+	—	+	+	+	+	+	—	86
+	+	+	—	+	+	+	+	—	90
+	—	+	+	+	+	+	+	—	57
—	+	+	+	+	+	+	+	—	93
+	+	+	+	—	+	+	+	—	84
+	+	+	+	+	—	+	+	—	85
+	+	+	+	+	+	+	—	Cl ⁻	30
+	+	+	+	+	+	—	+	Na ⁺	85
+	+	+	+	+	+	—	—	Na ⁺ Cl ⁻	24
+	+	+	+	+	+	+	+	Na ⁺ Cl ⁻	100
—	—	—	—	—	—	+	+	—	2
+	+	+	+	+	+	—	—	—	15

To assess the wider significance of the minimal medium as a stimulus for the initiation of mycelial development, the results obtained with the two strains were extended to 28 other strains. These 28 strains could be divided into three groups on the basis of the results. First, 17 out of 28 strains gave the same yield of germ-tubes after 4 h and 6 h as strain ca2. In the second group, 7 out of 28, maximum germ-tube production was reached after 2 h (range 54 to 100 % of that by strain ca2), and fell by variable amounts over the succeeding period (range 39 to 88 % of that by strain ca2). Thirdly, 4 out of 28 strains gave less than 30 % germ-tubes compared with strain ca2 at any time. Thus 66 % of all strains examined gave a high response and it may be that strains in the last two groups require higher concentrations of some or all of the components of the mixture, or possibly the addition of other amino acids, to increase the yield.

DISCUSSION

There have been numerous conflicting claims concerning the composition of growth media and the environmental conditions required for the development of the mycelial form of *C. albicans* and these have been summarized by Evans *et al.* (1974). The recent literature and our own experience suggest that the four factors of a slightly alkaline pH, a glucose concentration of 0.2 %, a temperature of at least 37 °C, and an inoculum of 10⁵ to 10⁶ cells ml⁻¹, are prerequisites for inducing a high rate of germination of blastospores. In addition there is a requirement for a nitrogen source and for salts. The nitrogen sources used have included various peptones; L-asparagine, provided that serum albumin was also present (Bernander & Edebo, 1969); serum and serum dialysates (Taschdjian & Kozinn, 1961); mixtures of amino acids (Taschdjian & Kozinn, 1961; Johnson, Guzmon & Agurlera, 1954; Mardon,

Balish & Phillips, 1969; Dabrowa, 1971; Lee, Buckley & Campbell, 1975); single amino acids such as glycine (De Palma, 1966), proline (Dabrowa, 1971) and L- α -amino-*n*-butyrate (Mardon, Hurst & Balish, 1971); and ammonium sulphate (Chattaway, Odds & Barlow, 1971; Evans *et al.*, 1974; Marriott, 1975). It is difficult to compare the various findings and to reach any general conclusions because of variations in the other factors mentioned above, differences in the method of assessing the extent of mycelial development and the fact that often only a single strain has been examined.

In our work, we kept the factors of temperature, medium pH, glucose concentration and inoculum size constant, and sought to determine the minimal nitrogen requirements for not less than 90 % germination of blastospores. A mixture of aspartate, threonine, proline, lysine, histidine and β -alanine at the same concentrations as are present in the hydrolysate of a peptide mixture prepared from seminal plasma satisfied this criterion. The total amino-acid concentration of $37 \mu\text{g ml}^{-1}$ was the minimum required for 90 % germination: this is lower than that of any nitrogen source in the literature cited, but it should be noted that our aim was to determine minimal requirements for the initiation of germination whereas most previous descriptions have been of growth media.

Lee *et al.* (1975) report that for eight strains a mixture of eight amino acids (total amino-acid concentration 4.47 mg ml^{-1}) gives a high rate of germination in 2 to 3 h. The amino acids were selected on the basis of appreciable hydrolysis of their β -naphthylamides by whole cells of *C. albicans*. Only lysine, proline and threonine were also present in our minimal amino-acid mixture. Undoubtedly there are additional requirements, both qualitative and quantitative, for further growth whether as blastospores or by mycelial extension. Thus Yamaguchi (1974) reported in a single strain at 30°C that mycelium developed in the presence of biotin at 0.1 ng ml^{-1} , but budding to blastospores occurred at 10 ng ml^{-1} . We did not find a requirement for added biotin for germination but many workers have found it essential for normal growth on synthetic media. Evans *et al.* (1974) tested five growth media for their ability to promote mycelial development and found that their three strains gave some mycelium formation in the early stages of growth, the peak of such development depending upon the doubling time of the organism in a particular medium. On this basis, our minimal medium, with its low growth potential, would be expected to give a high rate of germination. The presence of β -alanine in the minimal mixture suggested that it might be acting as a precursor of pantothenic acid, but substitution of this for the amino acid led to a greater fall in germ-tube development than was produced by the omission of β -alanine from the mixture. Phosphorus was not detected in the fraction D2 hydrolysate but the addition of phosphate was essential for activity of the hydrolysate. This may represent an important difference between utilization of the original peptide and the amino-acid mixture.

The requirement for phosphate confirms observations made by McClary (1952), Widra (1964), and Dabrowa (1971), but is at variance with those of Simonetti, Strippoli & Cassone (1974) who found, for a single strain, inhibition of germination by phosphate buffer (0.1 M), but up to 90 % germination in the presence of *N*-acetylglucosamine (2.5 mM) and Mn^{2+} (0.1 mM) in an imidazole-HCl buffer, pH 6.6. This work is at variance with ours in a number of respects, and with strains ca1 and ca2 the above germination medium gave only 30 to 40 % germination (unpublished results).

It is clear that low concentrations of the minimal mixture of amino acids described will give a 90 % germination rate in many strains of *C. albicans*, but it is probable that a wide range of amino acids may give a moderate degree of germination and the effects can vary from strain to strain.

We thank the Huddersfield Hospitals Management Committee and Leeds Regional Hospital Board for financial support for T.A. and for the purchase of equipment; Mr W. K. Hunter of the Milk Marketing Board for providing the bovine semen samples; and Dr E. G. V. Evans and Dr J. C. Gentles for providing freshly isolated cultures of *C. albicans*.

REFERENCES

- BARLOW, A. J. E., ALDERSLEY, T. & CHATTAWAY, F. W. (1974). Factors present in serum and seminal plasma which promote germ-tube formation and mycelial growth of *Candida albicans*. *Journal of General Microbiology* **82**, 261-272.
- BERNANDER, S. & EDEBO, L. (1969). Growth and phase conversion of *Candida albicans* in Dubos medium. *Sabouraudia* **7**, 146-155.
- CHATTAWAY, F. W., ODDS, F. C. & BARLOW, A. J. E. (1971). An examination of the production of hydrolytic enzymes and toxins by pathogenic strains of *Candida albicans*. *Journal of General Microbiology* **67**, 255-263.
- DABROWA, H. (1971). Germination in *Candida albicans*. In *5th International Conference in Human and Animal Mycology. Comptes rendus des Communications*, pp. 51-52. Paris: Pasteur Institute.
- DE PALMA, P. A. (1966). *Biochemical and structural variations in the cell wall of Candida albicans under conditions of controlled morphogenesis*. Thesis, Boston University, Massachusetts, U.S.A.
- EVANS, E. G. V., ODDS, F. C., RICHARDSON, M. D. & HOLLAND, K. T. (1974). The effect of growth medium on filament production in *Candida albicans*. *Sabouraudia* **12**, 112-119.
- JOHNSON, S. A. M., GUZMON, M. G. & AGURLERA, C. T. (1954). *Candida (Monilia) albicans*. Effect of amino acids, glucose, pH, chlortetracycline (aureomycin), dibasic sodium and calcium phosphates, and anaerobic and aerobic conditions on its growth. *Archives of Dermatology* **70**, 49-60.
- LEE, K. L., BUCKLEY, H. R. & CAMPBELL, C. C. (1975). An amino acid liquid synthetic medium for the development of mycelial and yeast forms of *Candida albicans*. *Sabouraudia* **13**, 148-153.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265-275.
- MARDON, D., BALISH, E. & PHILLIPS, A. W. (1969). Control of dimorphism in a biochemical variant of *Candida albicans*. *Journal of Bacteriology* **100**, 701-707.
- MARDON, D. N., HURST, S. K. & BALISH, E. (1971). Germ tube production by *Candida albicans* in minimal liquid culture media. *Canadian Journal of Microbiology* **17**, 851-856.
- MARRIOTT, M. S. (1975). Isolation and chemical characterization of plasma membranes from yeast and mycelial forms of *Candida albicans*. *Journal of General Microbiology* **86**, 115-132.
- MCCLARY, D. O. (1952). Factors affecting the morphology of *Candida albicans*. *Annals of the Missouri Botanical Gardens* **39**, 137-164.
- SIMONETTI, N., STRIPPOLI, V. & CASSONE, A. (1974). Yeast-mycelial conversion induced by N-acetyl-D-glucosamine in *Candida albicans*. *Nature, London* **250**, 344-346.
- TASCHDJIAN, C. L. & KOZINN, P. I. (1961). Metabolic studies of the tissue phase of *Candida albicans* induced *in vitro*. *Sabouraudia* **1**, 73-82.
- WIDRA, A. (1964). Phosphate directed Y-M variation in *Candida albicans*. *Mycopathologia et mycologia applicata* **23**, 197-202.
- YAMAGUCHI, H. (1974). Mycelial development and chemical alteration of *Candida albicans* resulting from biotin insufficiency. *Sabouraudia* **12**, 320-328.
- YOUNG, D. S. (1966). Improved method for the automatic determination of serum inorganic phosphate. *Journal of Clinical Pathology* **19**, 397-399.