Effect of carbon dioxide on the growth and form of *Candida albicans*

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Summary. The effect of CO₂ on the growth of 31 strains of *Candida albicans* was studied in serum and in a defined medium containing urea, ammonium chloride, asparagine, glutamine or acetamide as the nitrogen source. CO₂ 10% enhanced the mycelial growth of all strains when the medium contained an appropriate constituent to mediate its effect. The effect of CO₂ was most clearly demonstrated at 30°C when it induced a characteristic growth form consisting of a single swollen blastospore giving rise to a long, unbranched mycelial tube with few secondary blastospores; in atmospheric concentrations of CO₂ only blastospore growth occurred. Growth in the blastospore form was more rapid in CO₂ 10% than in air. Bicarbonate ions had no effect on mycelium formation. The result suggest that the induction of germ-tubes and mycelial growth is essentially a physical phenomenon caused by the intracellular accumulation of CO₂ in limited nutrient conditions, a view consistent with other reported laboratory and clinical findings.

Introduction

The literature describing the formation of germ-tubes and induction of the mycelial form of growth of *Candida albicans* is controversial and contradictory (reviewed by Odds, 1979). Much of this discrepancy seems to have arisen from studies employing only single strains of the organism or from the use of different criteria for assessing growth form. During a study of the growth of *C. albicans* in media of composition similar to that of human saliva, the finding of Odds and Abbott (1980) that urea serves as the sole source of nitrogen was confirmed, but it was also found to promote the formation of germ-tubes and subsequent growth in the mycelial form. Furthermore, this effect was greatly enhanced by incubation in atmospheres containing CO₂ 5–10%. In view of the prevailing disagreement in this field, the effect of CO₂ on several strains of *C. albicans* was studied and a simple, unequivocal method of assessing growth form was used.

Materials and methods

Strains of C. albicans

Thirty-one strains were used: six were from normal mouths; four were associated with denture stomatitis;

three strains each were from candidiasis affecting the tongue and mucous membrane of the cheek; four strains were associated with angular cheilitis; and three were from juvenile thrush. Two strains were from the normal vagina and two were associated with vulvovaginitis. Strains nos. 3135 and 6406 were from the Mycological Reference Laboratory, London, and the remaining two were laboratory strains known to be reliable producers of germ-tubes and chlamydospores respectively. All 31 strains had been preserved from 2 months to 7 years on slopes of corn meal agar at 4°C.

The strains were identified by tests for the formation of germ-tubes in serum (Taschdjian *et al.*, 1960), the production of chlamydospores in corn meal agar containing Tween 80 1% (Rosenthal and Furnari, 1958) and by the reactions in API 20C auxanogram strips (API Laboratory Products, Basingstoke, Hampshire). Confluent growth of each strain was obtained on plates of Sabouraud agar (Oxoid) incubated at 37°C for 24 h, and then harvested into sterile 0.1M phosphate buffer, pH 7.2. The suspension was centrifuged at 500 *g* and the deposited cells washed twice in phosphate buffer and once in sterile distilled water. The cells were counted in a Helber chamber, suspended in sterile distilled water at a concentration of 20 × 10⁶ cells/ml and stored at 4°C until used.

Media

Stock solutions of 0.2M K₂HPO₄, 0.2M KH₂PO₄, glucose 10%, MgSO₄ 5% and CaCl₂ 0.1% were prepared and sterilised by autoclaving at 121°C for 15 min. Media were freshly prepared by dissolving 1 g of the appropriate

nitrogen source, i.e., ammonium chloride, urea, asparagine, glutamine or acetamide in 50 ml of distilled water and adding, where required, 1 g of powdered bovine serum albumin or globulin. All chemicals were obtained from the Sigma Chemical Company Ltd, Poole, Dorset. Next, 3.1 ml of K_2HPO_4 solution, 1.9 ml of KH_2PO_4 solution and 1 ml of each of the glucose, $MgSO_4$ and $CaCl_2$ solutions were added and the volume made up to 100 ml. When the constituents were completely dissolved the media were sterilised by passage through a 0.22- μ m membrane filter (Sterivex; Millipore). The pH values of the media were measured with a Radiometer Ion 85 analyser and an Ingold semi-micro combined reference and pH electrode; they were within the range 6.8–7.2. Horse serum no. 3 (Wellcome Reagents Ltd) was used undiluted. All media were dispensed in 1-ml amounts in sterile 7-ml plastic, screw-capped bijoux vials (Sterilin Ltd, Feltham, Middx).

Glucose-agar plates consisted of Oxoid Agar No. 1 1% containing glucose 0.1%. They were inoculated as described by Joshi *et al.* (1975).

Assessment of mycelial growth

For each strain of *C. albicans*, 0.05 ml of suspension was added to two vials of the appropriate medium and incubated with loose caps for 18 h at the test temperature: one vial was incubated in air and the other in CO_2 10% in a Hotpack automatic CO_2 incubator.

Wet preparations of each of the paired cultures were made on the same slide and examined by phase-contrast microscopy. When mycelial growth in the culture incubated in CO_2 was grossly and unequivocally greater than that in the culture incubated in air the fact was recorded; lesser increases were ignored. The increased mycelial growth induced by CO_2 was often of a novel form consisting of long, unbranched mycelial tubes with few or no secondary blastospores. When this type of growth predominated over the usual coarse, branched mycelium with associated secondary blastospores it was recorded.

Glucose-agar plates were examined after incubation for 5 h at 37°C in air, in air plus CO_2 10%, in polycarbonate anaerobic jars (Don Whitley Scientific Ltd, Shipley, W. Yorks) filled with H_2 or H_2 90%, CO_2 10%.

Effect of CO_2 on growth.

This was assessed with 22 strains that grew predominantly as blastospores in the medium with ammonium chloride as the nitrogen source. Four 1-ml cultures of each strain were incubated in air or air plus CO_2 10% at the appropriate temperature. After 4, 8, 12 and 18 h, vials were removed and the apparent absorbance at 420 nm was measured in a Pye Unicam SP6-500 spectrophotometer with a cuvette of 1-cm light path.

Estimation of protein

Five 1-ml cultures of each of the strains in the ammonium chloride/bovine serum albumin broth were

incubated, with uninoculated broths for comparison, at 37°C for 18 h. The cultures were pooled, centrifuged and the supernate passed through a 0.45- μ m membrane filter (Millex; Millipore). The protein content of the supernates was measured with the reagents and protocol of the Sigma Chemical Co. kit no. 690. This method is a modification of that described by Ohnishi and Barr (1978) and reproducibility studies, quoted by Sigma, show it has a standard deviation of 0.3 mg/100 ml for a sample containing protein 25 mg/ml.

Results

Effect of CO_2 on growth in the mycelial form

CO_2 promoted mycelial growth of all the strains of *C. albicans* (table I). Except in media containing either urea or acetamide as the nitrogen source this effect was difficult to demonstrate at 37°C because growth in the mycelial form was already florid at atmospheric concentrations of CO_2 . In the numerous examinations made, enhancement of mycelial growth was apparent at 37°C but difficult to quantify and insufficient to satisfy the criterion adopted. A temperature of 37°C has generally been regarded as essential for germ-tube and mycelium formation but the effect of raising the partial pressure of CO_2 was more readily demonstrable at 30°C when germ-tubes and mycelium formed in abundance. A few strains grew in the mycelial form at 25°C in horse serum (fig. 1). There was a marked tendency for CO_2 to induce a particular type of mycelial growth consisting of a long, usually unbranched, mycelial tube originating from a swollen, roughly spherical blastospore with few or no secondary blastospores budding from the mycelium. Examples of strains isolated from the palate (P2) and from angular cheilitis (Acl) growing in media with asparagine and bovine serum albumin plus ammonium chloride, respectively, as the nitrogen sources are shown in figs. 2 and 3. In horse serum, branching was usual but secondary blastospores remained rare (fig. 1). Vacuolisation of the mycelial protoplasm (Gow and Gooday, 1984) is seen in fig. 4 which shows a strain isolated from a healthy mouth (N4) growing in the medium with urea as the source of nitrogen.

Media constituents

The results in table I show that raising the CO_2 partial pressure alone was insufficient to induce mycelium formation; CO_2 had no effect when the medium contained ammonium chloride as the source of nitrogen. The selection of compounds for testing arose from the finding that urea was a

Table I. The effect on 31 strains of *C. albicans* of increasing the CO₂ concentration from atmospheric to 10% with incubation at different temperatures in various media for 18 h

Medium or nitrogen source in medium	Temperature (°C)	Number of strains demonstrating		
		Increase in growth in the mycelial form	Increase in coarse, branched mycelium with attached secondary blastospores	Increase in long, unbranched mycelial tubes with few secondary blastospores
Horse serum	37	0	0	0
	30*	31	0	31
	25*	12	12†	0
Ammonium chloride	37	0	0	0
	30*	0	0	0
Bovine albumin + ammonium chloride	37	0	0	0
	30*	31	6	25
Urea	37	29	20	9
	30*	29	15	14
Asparagine	37	0	0	0
	30*	31	14	17
Glutamine	37	0	0	0
	30*	31	14	17
Acetamide	37	15	15	0
	30*	13	13	0

* Growth in control cultures in air almost entirely in the blastospore form.

† Few secondary blastospores.

Fig. 1. *C. albicans* (Mycological Reference Laboratory strain no. 3135) incubated in horse serum for 18 h at 25°C in CO₂ 10% showing branching mycelium but few secondary blastospores. Control culture in air consisted of blastospores only. Wet preparation. Bar = 50 µm.

Fig. 2. *C. albicans* (oral strain P2) incubated in the basal medium with asparagine as the nitrogen source for 18 h at 30°C in CO₂ 10%. The blastospore giving origin to the mycelial tube is swollen in comparison with the cluster of blastospores nearby. Growth in the control culture in air was entirely as blastospores. Wet preparation. Bar = 25 µm.

satisfactory nitrogen source for *C. albicans* and promoted mycelial growth in media similar in composition to that of saliva. Urea is a di-amide; therefore acetamide and formamide were tested but the latter compound supported only slow and feeble growth. Sheperd *et al.* (1980) showed that glutamine induced germ-tubes and so it, together with the

other amino acid with an amide side chain, asparagine, were included. Horse serum provided a reference medium and bovine albumin was used as a control for some of the numerous possible inducers in serum, e.g., urea.

Initial work with a medium containing urea as the nitrogen source showed that to induce good myce-

Fig. 3. *C. albicans* (oral strain Ac 1) incubated in the basal medium with ammonium chloride as the nitrogen source and bovine albumin as inducer for 18 h at 30°C in CO₂ 10%. Control culture in air consisted of blastospores only. Wet preparation. Bar = 100 µm.

Fig. 4. *C. albicans* (oral strain N4) incubated in the basal medium with urea as the nitrogen source for 18 h at 30°C in CO₂ 10%, showing vacuoles in the mycelial tubes and the original blastospores. Wet preparation. Bar = 20 µm.

lial growth it was important to keep the glucose concentration below 0.2% and the pH above 5.7. The medium used in this study was formulated to have an initial pH near neutrality with any of the nitrogen sources and to remain little changed after growth of *C. albicans*. The pH values of the 18-h cultures were all within ± 0.3 of the initial pH.

Adding sodium bicarbonate to any of the media, up to a concentration of 1%, had no effect on mycelium formation by any of the strains, a finding similar to that recently reported by Klotz *et al.* (1984) who also found that only gaseous CO₂ influenced the morphology of *Coccidioides immitis*.

Two bovine-serum-albumin powders, azo-albumin and egg albumin (Sigma A-2153, A-4503, A-2392 and A-5378 respectively) were effective inducers whereas crystallised and lyophilised bovine albumins and a globulin (Sigma A-4378, A-

7638 and G-5009) were not. No explanation of these differences can be offered. The ineffective preparation A-7638 is a more purified form of A-4503. Egg albumin was a potent inducer despite being crystallised and lyophilised. Some of the preparations were acidic but the pH of the medium was still above 6.8. Neither the effective albumin (A-4503) nor the ineffective albumin (A-7638) were reduced in concentration in cultures incubated for 18 h at 37°C in air or air plus CO₂ 10%. There was no statistical difference between the mean concentration of protein in the cultures and in the uninoculated controls. This confirms the findings of Bernander and Edebo (1969) and Barlow *et al.* (1974).

Effect of CO₂ on blastospore growth

Table II shows the effect of CO₂ on the growth of 22 strains of *C. albicans* that grew mainly as blastospores in the medium containing ammonium chloride as the nitrogen source. The ranges reflect the variations in growth rate of the individual strains and although the differences in mean absorbance are quite small they are consistently larger in the cultures incubated in CO₂ 10%. In only five of the 18-h cultures incubated at 37°C was growth less in CO₂ 10%. Analysis of the mean difference of the paired observations for each strain shows the differences to be significant ($p < 0.001$) for all incubation times at both temperatures. Clearly, CO₂

Table II. The absorbance of cultures of 22 strains of *C. albicans* growing in the basal medium + NH₄Cl 1% incubated in air and air + CO₂ 10%

Temperature (°C)	Time (h)	Atmosphere	Mean absorbance	Range
30	4	Air	0.104	0.089
		CO ₂	0.112	0.093
	8	Air	0.208	0.116
		CO ₂	0.220	0.117
	12	Air	0.314	0.135
		CO ₂	0.325	0.147
	18	Air	0.442	0.132
		CO ₂	0.454	0.140
37	4	Air	0.115	0.082
		CO ₂	0.124	0.082
	8	Air	0.226	0.104
		CO ₂	0.241	0.102
	12	Air	0.354	0.106
		CO ₂	0.369	0.112
	18	Air	0.472	0.047
		CO ₂	0.485	0.054

rates the metabolism of *C. albicans* as well as promoting the mycelial form of growth. This organism also grows more rapidly at 37°C than at 30°C.

Effect of CO₂ on germ-tube formation on glucose agar

To produce germ-tubes on glucose agar (Joshi *et al.*, 1975) the inoculated streak of *C. albicans* is covered with a coverslip. These authors stated that the effect of the coverslip was to create local anaerobiosis which promoted the formation of germ-tubes. With the 32 strains in this study it was confirmed that plates inoculated according to this technique and incubated in air produced germ-tubes, as expected, only beneath the coverslip. On plates incubated in air plus CO₂ 10% germ-tubes formed more abundantly in the uncovered streaked growth than under the coverslip. In anaerobic conditions growth was feeble and entirely as blastospores. In anaerobic conditions with CO₂ 10%, growth was still feeble; only an occasional short germ-tube was seen both under the coverslip and in the uncovered growth. These results strongly suggest that the effect of the coverslip in this technique is not the creation of anaerobiosis but the trapping of CO₂ produced by the metabolism of the organism which then promotes the formation of germ-tubes.

Discussion

At 30°C none of the compounds tested in this study (table I) induced germ-tubes and mycelial growth of *C. albicans*, yet simply raising the partial pressure of CO₂ converted them to potent inducers. Since these compounds were supporting satisfactory blastospore growth at this temperature, some general physical change seems a more likely cause of mycelial growth than the specific nutritional effects widely suggested in the past. The fact that albumin, a potent, reliable inducer, was unaltered in concentration in the cultures, as was previously observed by Bernando and Edebo (1969) and Barlow *et al.* (1974), supports this view. Furthermore, because albumin is effective at 37°C without extra CO₂ and without being reduced in concentration, it seems reasonable to suggest that the effect is physical and that atmospheric concentrations of CO₂ are sufficient to mediate it. Much of the controversy concerning the induction of germ-tubes seems to have arisen from attempting to explain the same physical phenomenon in nutritional and other terms. For example, Joshi *et al.* (1975) thought anaerobiosis caused by occlusion with a coverslip an essential feature for germ-tube production where-

as Lee *et al.* (1975) could produce germ-tubes only by aerating the liquid form of their amino-acid medium which failed to induce germ-tubes when dispensed as plates. These apparently contradictory papers can be reconciled by the explanation that, in both cases, the crucial feature was an increased concentration of CO₂. The results presented here strongly suggest that it is the trapping of CO₂ under the coverslip and not anaerobiosis that induces germ-tubes in the technique of Joshi *et al.* (1975). Similarly, since CO₂ is more than twenty times more soluble than O₂ at 37°C, the results of Lee *et al.* (1975) are more logically explained by an increased concentration of CO₂ in the aerated liquid cultures over that at the surface of an agar plate than by a corresponding increase in O₂.

There appears to be a rough inverse relationship between the growth temperature and the concentration of CO₂ required to induce mycelial growth suggesting that the internal concentration of CO₂ is the critical factor, whether achieved by raising the external pressure or internal metabolism. Because bicarbonate ions were without effect, it appears that the dissolved gas is involved. The way in which CO₂ and the inducer compounds are corporately concerned is a matter for conjecture. In the case of albumin, a known carrier of various molecules, some loose binding of CO₂ may occur creating an environment of increased CO₂ tension around the organisms. The albumin preparations that failed to induce germ-tubes remained ineffective with CO₂ 10%, which is in keeping with the notion that induction involves reaction with CO₂. The amide compounds tested, which, unlike albumin, are metabolised, seem more likely to function by raising the CO₂ level within the fungal cells.

Gow and Gooday (1984) have shown that the swollen blastospores of origin and the elongated mycelial cells contain large vacuoles; the cytoplasm is little more than a thin rim between the vacuole and the cell wall. This arrangement would be advantageous in poorly nutrient conditions, which many believe important in germ-tube induction, because the cytoplasm is maximally spread for absorption of nutrients and the elongating mycelium effects physical removal to possibly more plentiful conditions. These vacuoles may be formed in response to raised CO₂ partial pressures and their contents may be largely dissolved CO₂. Once a large vacuole had formed in a blastospore, considerations of surface tension and surface to volume ratio would preclude budding to form a roughly spherical cell of smaller diameter than the mother cell. A cylindrical cell of smaller diameter than the parent cell could form and this could elongate

indefinitely without altering its surface to volume ratio. Gow and Gooday (1984) found that secondary budding and branch formation could only occur from mycelial cells whose vacuoles had become refilled with cytoplasm. This favours the idea that the vacuoles form in response to CO₂ partial pressure because in this work, long unbranched mycelial tubes were a striking feature of *C. albicans* growing in CO₂ 10%.

Although there are several reports of concentrations of CO₂ above those in the normal atmosphere affecting the morphology of fungi in general, a recent extensive review of the literature concerned with the morphogenesis of *C. albicans* (Odds, 1985) cites only one report of CO₂ altering the growth form (Mardon *et al.*, 1969). This work involved a single, variant strain of *C. albicans* that did not form germ-tubes or chlamydospores and was grown in a medium of pH 4.5 containing glucose 2.5% incubated at 28°C in CO₂ concentrations twice that of oxygen. Despite doubts about the identity of the strain used and differences in the important parameters of pH and glucose concentration, the conclusion that CO₂ promotes mycelial growth is fully endorsed by the results presented here. At the conclusion of his excellent review Odds (1985) states that notwithstanding the numerous papers devoted to the subject the factors affecting the morphogenesis of *C. albicans* remain largely speculative and yet the explanation could be paradoxically simple. The results of this work and other papers where the

possible effect of CO₂ has not been considered suggest that CO₂ tension could provide that simple explanation.

Clinically, the association between candidiasis and localised accumulations of CO₂ is remarkably good. Frame *et al.* (1972) have shown that CO₂ is continually diffusing through the skin and the rate of diffusion increases three fold through wet skin. Comparable measurements for mucous membranes do not appear to have been made but are unlikely to be less than those for wet skin. Aly and Maibach (1983) state that when the skin is covered with an occlusive dressing the concentration of CO₂ under the dressing equilibrates with that of the underlying tissues in a few hours and amounts to 5–7%. Examples of occlusion leading to candidiasis range from clinical experiments to the wearing of a wristwatch (reviewed by Odds, 1979), but probably the clearest and simplest involve the covering of the palate with a denture or orthodontic plate. In many cases of denture candidiasis and in probably all of the examples of children wearing orthodontic appliances there is no evidence of predisposing debilitation. Ceasing to wear the appliance cures the candidiasis. Shielding of the palatal flora from the forces of mechanical removal and cleansing is, clearly, one factor in these infections but the trapping of CO₂ with consequent stimulation of the growth of *C. albicans* in general and the promotion of the mycelial form of growth in particular is, surely, another.

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