Effect of Temperature on Saprophytic Cryptococci: Temperature-induced Lysis and Protoplast Formation

By R. DABBAGH*, N. F. CONANT, H. S. NIELSEN†
AND R. O. BURNS‡

Department of Microbiology and Immunology, Duke University School of Medicine,
Durham, North Carolina 27710, U.S.A.

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SUMMARY

The saprophytic cryptococci, *Cryptococcus diffluens*, *Cryptococcus laurentii* and *Cryptococcus albidus* which are characterized by inability to grow at 37 °C and above, were shown to lyse at non-permissive growth temperatures. Incorporation of radioactive precursors of protein, RNA and DNA by *C. diffluens* suggested that protoplasmic growth continued at 37 °C. Incorporation of 20 % sucrose in the growth medium retarded lysis at the non-permissive temperature and direct microscopic examination of cells under these conditions revealed aberrations in the walls and protoplast extrusion. Similar wall aberrations which were induced by the inhibitor 2-deoxyglucose suggest that aberrant wall biosynthesis occurs at non-permissive growth temperatures.

INTRODUCTION

Growth of the human pathogen *Cryptococcus neoformans* is restricted at temperatures above 39.4 °C; saprophytic members of the genus *Cryptococcus* fail to grow at temperatures of 37 °C and above.

A number of investigations employing experimental animals have correlated the *in vitro* temperature-growth relationship with body temperature and survival or pathogenicity of the cryptococci (Mager & Aschner, 1947; Kuhn, 1949; Benham, 1956; Bergman, 1967; Linares & Baker, 1971). It is clear from these studies that the pathogenicity of *C. neoformans* is partly based on its ability to grow at 37 °C. However, the molecular basis of pathogenicity remains obscure. The pathogenicity of other species of the genus which either grow poorly or not at all at 37 °C is questionable.

The present investigation was initiated to gain insight into the mechanism underlying the inability of the saprophytic members of the genus *Cryptococcus* to grow at elevated temperatures.

METHODS

Organisms. The organisms used were from the collection of N. F. Conant. *Cryptococcus diffluens* (N. F. Conant stock number 2971) was received from the Communicable Disease Center, Atlanta, Georgia, U.S.A. in 1960. *Cryptococcus laurentii* and *C. albidus* (N. F. Conant stock numbers 3035 and 3507, respectively) were received from the Communicable Disease Center in 1968. All these organisms exhibited typical characteristics.

Cultivation of organisms. Stock cultures were maintained in Sabouraud's medium, of

* Present address: Department of Microbiology, College of Medicine, University of Baghdad, Republic of Iraq.

† Present address: Allarmed Laboratories Inc., 2203 Convoy Court, San Diego, California 92111, U.S.A.

‡ To whom reprint requests should be sent.

which fresh stocks were prepared weekly, and a minimal and an enriched medium were routinely used. The minimal medium contained (per litre): sucrose, 15 g; ammonium sulphate, 3 g; dibasic potassium phosphate, 7 g; monobasic potassium phosphate, 3 g; thiamin hydrochloride, 1 mg; trace element solution (containing 1.5 g $\text{MgCl}_2 \cdot 2\text{H}_2\text{O}$, 1.5 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.75 g CuSO_4 per litre), 1 ml. The enriched medium contained (per litre): Bacto yeast extract, 10 g; K_2HPO_4 , 7 g; KH_2PO_4 , 3 g; thiamin hydrochloride, 1 mg; pre-sterilized 50 % glucose solution, 20 ml.

Inocula were prepared by transferring a loopful of fungus from a slant of Sabouraud's agar to 100 ml of the desired medium contained in a 250 ml Erlenmeyer flask. The culture was placed in a New Brunswick Model G-76 gyratory water bath at room temperature for 18 to 24 h and then the contents were adjusted turbidimetrically to 1.5×10^7 cells/ml unless otherwise specified. One ml of this suspension was used as inoculum for each 100 ml of culture medium.

Quantification of growth. Turbidimetric measurements were made at 420 nm with a Bausch and Lomb Spectronic 20 colorimeter. Direct colony counts were made on appropriately diluted samples of cells with the use of a 'Bright-Line' (American Optical) counting chamber. Viable counts were made by plating on Sabouraud's agar and incubating at 27 °C for two days.

Radioactive measurements. The incorporation of [^{14}C]isoleucine, [^{14}C]uracil, and [^3H]thymidine into whole cells was measured by adding 1 ml samples of culture containing the appropriate labelled compound to 1 ml portions of 2 N-perchloric acid. After standing in the cold for 15 min each cell suspension was filtered through a Millipore filter (0.45 μm) which had previously been wetted with 2 N-HCl. The filter was washed 2 or 3 times with 2 N-HCl followed by a wash with 95 % ethanol, dried under an infrared lamp for 15 to 20 min, and then placed in a scintillation vial containing 15 ml of scintillation fluid (4.5 l toluene, 15.2 g 2,5-diphenyloxazole, 378 mg 1,4-bis-[2-(5-phenyloxazolyl)]-benzene. The samples were counted in a Beckman Model LS-233 liquid scintillation spectrometer.

Photomicroscopy. Photomicrographs were taken with a Wild (Heerbrugg, Switzerland) phase-contrast microscope equipped with an automatic shutter. Kodak Panatomic X 35 mm film was used. Cells were mixed with equal parts of a 2 % solution of Ficoll, before mounting under coverslips; the Ficoll tended to decrease their random motion and permitted sharper photographs.

Reagents and chemicals. [^{14}C]uracil and [^{14}C]isoleucine were purchased from Schwartz Bioresearch Inc., Orangeburg, New York, U.S.A. [^3H]thymidine was obtained from the New England Nuclear Corporation. All chemicals were reagent grade.

RESULTS

Preliminary experiments demonstrated that *C. diffluens*, used for most of the work, failed to grow at temperatures above 35 °C, whereas *C. neoformans* failed to grow at temperatures above 39 °C. These restrictive temperatures are consistent with the limits reported by others (Kligman, 1951).

In order to assess more adequately the qualitative differences in cultures of *C. diffluens* growing at permissive and non-permissive temperatures, cultures actively growing at 27 °C were rapidly shifted to 37 °C. Figure 1(a) shows the total cell count of a culture of *C. diffluens* growing at 27 °C in minimal medium and of an identical culture which was shifted to 37 °C early in the logarithmic phase of growth. As can be seen in this Figure the total cell count at 27 °C reached approximately 10^8 /ml, which is the characteristic

Fig. 1. (a) Total cell number in cultures of *C. diffluens* growing in minimal medium; (b) number of viable cells in cultures of *C. diffluens* growing in minimal medium; (c) turbidimetric quantification of *C. diffluens* growing in minimal medium. ●, Growth at 27 °C; ○, growth after shift to 37 °C. Details of the procedure are given in the text.

limit of growth for *C. diffluens* under these conditions; the count remained constant for 12 h, after which a gradual decline was observed. Growth of the culture which was shifted to 37 °C was severely restricted; cell multiplication continued for about two h. Afterwards the cell number remained constant for 6 to 8 h at which time a decline was observed. Figure 1(b) shows the results of viable cell counts during this same experiment. Comparisons of Fig. 1(a) and 1(b) show that the total cell count and the viable cell count followed approximately the same pattern but, as might be expected, the viable count was somewhat lower than the total count. Figure 1(c) represents the absorbancy of the same cultures during the course of growth. This Figure shows that the relationship between cell numbers and absorbancy was constant during growth at 27 °C but that the absorbancy showed a relatively larger increase after the shift to 37 °C. This difference in the relationship between cell number and absorbancy is explained by the difference in size of the cells growing at 27 °C and those exposed to 37 °C. Measurements revealed that after the shift in temperature the volume of the cells increased approximately 27-fold, i.e. the diameter of the 27 °C cells, which was 2 μm , progressively increased to 6 μm when the cultures were shifted to 37 °C.

It is evident from these experiments that growth inhibition at 37 °C was not exerted immediately. One explanation of the delayed effect of temperature is that accumulation of a toxic substance occurs at the non-permissive temperature. This possibility was ruled out by testing the ability of both concentrated culture filtrates and extracts of cells obtained from cultures of *C. diffluens* at 37 °C to inhibit the growth of *C. diffluens* at 27 °C. Samples of these were added singly and in combination to cultures of *C. diffluens* at various phases of growth at 27 °C. No growth inhibition was observed. Also, the same samples were used to determine if the response of cultures of *C. diffluens* which had been shifted to 37 °C could be altered. No effect of the 37 °C-derived materials was noted. It is concluded that toxic products were not responsible for the observed growth inhibition.

The rates of incorporation of radioactive precursors of protein, RNA and DNA were determined in cultures growing at 27 °C and in cultures which had been shifted to 37 °C. Fig. 2 depicts the incorporation of (a) [^{14}C]isoleucine, (b) [^{14}C]uridine and (c) [^3H]thymidine into perchloric acid-insoluble counts during the growth of *C. diffluens* at 27 °C and after the shift to 37 °C. The results show that the synthesis of the macromolecules at 37 °C always continued until the onset of lysis, and strongly suggest that generalized protein,

Fig. 2. Incorporation of (a) $[^{14}\text{C}]$ isoleucine, (b) $[^{14}\text{C}]$ uridine and (c) $[^3\text{H}]$ thymidine by cultures of *C. diffluens* growing in minimal medium at (●) 27 °C and (▲) after a shift to 37 °C. The arrows represent the time of the temperature shift and the addition of label. The amounts of labels were: L-isoleucine, 0.266 $\mu\text{mol/ml}$ (164 mCi/mmol); uracil, 10 $\mu\text{mol/ml}$ (40 $\mu\text{Ci}/\mu\text{mol}$); thymidine, 5 $\mu\text{mol/ml}$ (100 $\mu\text{Ci}/\mu\text{mol}$). Total cell numbers were determined by microscopy and are shown by continuous lines; incorporation of radioactive label is shown by broken lines.

RNA and DNA synthesis was not impaired at the higher temperature and that the cessation of synthesis was a consequence of cell lysis. It should be emphasized that the technique employed here would not reveal a perturbation of the synthesis of a specific macromolecule; this is especially so with regard to the synthesis of protein and RNA, though since ribosomal RNA constitutes the predominant RNA species it can be concluded that its synthesis is not affected at the higher temperature.

The above results suggest that the increased cell size is caused by the continuation of relatively balanced protoplasmic growth in the absence of cell division, rather than solely by the accumulation of vacuoles or storage granules within the cell. It seemed therefore that the lysis of the cells which occurred at 37 °C might be explained in terms of the

Fig. 3. Effect of addition of a carbon source (sucrose) to starved cells of *C. diffluens* at 37 °C. For details, see text. ○, Cells in minimal medium without carbon source; ●, Cells in minimal medium with carbon source. Viable cell numbers were determined as outlined in Methods.

uncoordinated synthesis of the protoplasm and the wall. Thus cell lysis could be a direct consequence of cell enlargement rather than a response to some active lytic principle induced at the high temperature.

To test this hypothesis the following experiment was performed. *Cryptococcus diffluens* was grown at 27 °C on minimal medium and the exponentially growing cells were harvested aseptically and washed once with sterile medium from which the carbon source was omitted. The washed cells were then suspended in the same medium and aerated at 27 °C for 4 h to allow the cells to deplete any residual carbon source. This cell suspension was then divided into two cultures. Sucrose was added to one, then both cultures were placed in water-bath shakers at 37 °C and viable cell numbers monitored. Figure 3 shows that in the culture without a carbon source the cell count remained constant, and direct microscopic examination of the two cultures revealed that the cells in the sucrose medium were enlarged whereas those in the medium without sucrose maintained the appearance of cells obtained from cultures grown at 27 °C. These results suggest that lysis only occurs in the presence of an energy source and may therefore be a consequence of attempted growth at the high temperature. This view is supported by a correlation between the growth rate of *C. diffluens* and the time required for the onset of lysis after the temperature shift. *Cryptococcus diffluens* has a generation time of 5 h in minimal medium and 2 h in enriched medium; lysis occurred 8 to 10 h after the temperature shift in minimal medium, and 4 to 6 h after in enriched medium.

Collectively, the foregoing results strongly indicate that the lysis of *C. diffluens* at 37 °C occurs because of cell enlargement. Cell division does not occur during this period and therefore it was conjectured that some facet of wall biosynthesis or bud formation was perturbed at the higher temperature. If this is true, an increase in the tonicity of the medium would protect the cells from lysis at 37 °C. The effect of an increased osmotic pressure was tested by growing cultures of *C. diffluens* at 27 °C in minimal medium containing 20 % sucrose to the mid-exponential growth phase and then shifting them to 37 °C. The total cell count was determined. It was found that the increased osmotic

Fig. 4. Incorporation of (a) $[^{14}\text{C}]$ isoleucine, (b) $[^{14}\text{C}]$ uridine, (c) $[^3\text{H}]$ thymidine by *C. diffuens* at (●) 27 °C and (▲) 37 °C in minimal medium containing 20 % sucrose. The temperature shift and addition of label were at 18 h. The amount of label is as in Fig. 2. Total cell numbers were determined by microscopy and are shown by continuous lines; incorporation of radioactive label is shown by broken lines.

pressure tended to stabilize the cells; upon shifting the cultures to 37 °C the lysis noted previously was delayed (Fig. 4).

The availability of a method for stabilizing *C. diffuens* at the non-permissive temperature presented the opportunity to verify one of the conclusions reached in this study. Results indicated that the synthesis of protein, RNA and DNA continued at 37 °C in non-stabilized cultures until the onset of lysis and it was concluded that the cessation of synthesis of these macromolecules was a direct consequence of lysis. The validity of this conclusion was determined by repeating the incorporation experiments with stabilized cultures. Incorporation of radioactive precursors was followed in cultures of *C. diffuens* in minimal medium with 20 % sucrose both at 27 °C and following a shift to 37 °C. Results of these experiments are shown in Fig. 4. Contrary to the original conclusion, these results indicate that macromolecular synthesis ceased well before onset of the delayed lysis of the stabilized cultures. They suggest that it is not only lysis which prevents the continued synthesis of protein, RNA and DNA. However, these results may not be representative of macromolecular synthesis. For example, the enlargement of cells which occurs at 37 °C might trigger processes of macromolecular turnover so that the incorporation observed represents net incorporation in which synthesis and degradation of the three pertinent macromolecules are balanced. Another explanation for these observations is that upon enlargement of the cells the externally supplied compounds do not readily enter them. The accompanying paper (Dabbagh, Conant & Burns, 1974) provides evidence to support the latter contention.

Fig. 5. Cells of *C. diffluens* grown in minimal medium at 27 °C. The photographs show cells at different stages in the cell cycle.

Fig. 6. Cells of *C. diffluens* from minimal medium after 6 h at 37 °C.

Fig. 7. Lysed cells of *C. diffluens* from minimal medium after 12 h at 37 °C.

Fig. 8. Pore formation in *C. diffluens*. Cells were taken from minimal medium containing 20 % sucrose 12 h after a temperature shift from 27 to 37 °C. The arrows indicate the sites of pore formation.

Fig. 9. Formation of sphaeroplasts and protoplasts in cultures of *C. diffluens* stabilized with 20 % sucrose. Sphaeroplasts are shown by the arrows. Cells were obtained from minimal medium containing 20 % sucrose approximately 18 h after a shift from 27 to 37 °C.

The foregoing results have shown that when shifted to the non-permissive temperature, cells of *C. diffluens* continue to divide for a relatively short time. The cells enlarge, and then lyse. The lysis which is a consequence of protoplasmic growth can be markedly delayed by increasing the tonicity of the medium. The most attractive hypothesis to explain these observations is that the budding process is prevented at the higher temperature, and in particular, that some aspect of wall biosynthesis is involved.

To gain additional insight into the effect of non-permissive temperatures of *C. diffluens*, a detailed microscopic examination of cells exposed to non-permissive temperatures was performed. Cultures of *C. diffluens* growing at 27 °C for 18 h in minimal medium were shifted to 37 °C. During the first 6 to 8 h, changes in both the size and shape of the cells were observed. They increased from 2 to 6 μm in diameter. The cells changed from spherical to ellipsoidal, and granularity and vacuolation increased. After approximately 8 to 10 h at 37 °C, cell lysis was noted. These changes are illustrated in Figs. 5 to 9.

The morphology of cells from a medium which contained 20 % sucrose was also examined. As in the non-stabilizing medium the cells enlarged during the first 6 to 8 h at 37 °C. However, unlike the case described above lysis did not occur at 10 h; instead gaps, or

Fig. 10. Cells of *C. laurentii* at 27 and 37 °C. (a) Cells from minimal medium at 27 °C; (b) protoplasts released from cells at 37 °C in minimal medium containing 20 % sucrose; (c) sphaeroplasts and protoplasts from sucrose-stabilized cells at 37 °C; (d) ghosts and cell wall debris in cultures shifted to 37 °C in the absence of 20 % sucrose.

pores, developed in the walls (Fig. 8). Protoplasts were later seen escaping through these pores and what appeared to be naked protoplasts were frequently observed (Fig. 9).

Similar experiments were performed with cultures of *C. diffluens* growing in enriched medium with additional sucrose (20 %) at 37 °C. The results are qualitatively similar to those obtained when using the minimal medium but morphological changes developed more rapidly – pores were observed after 6 h.

To assess whether similar events occurred on shifting other saprophytic species of *Cryptococcus* to non-permissive temperatures, *C. laurentii* and *C. albidus* were examined microscopically. The growth characteristics of these two strains are similar to those of *C. diffluens*, i.e. they grow well at 27 °C but fail to grow at 37 °C. The generation time of *C. laurentii* and *C. albidus* in minimal medium at 27 °C is 4 h, whereas *C. diffluens* has a generation time of 5 h. *Cryptococcus laurentii* and *C. albidus* were grown at 27 °C in minimal medium containing 20 % sucrose, shifted to 37 °C and microscopic examination made. Figure 10 shows the morphological transitions which occur in *C. laurentii* at 37 °C, and Fig. 11 shows similar changes with *C. albidus*. In both, the cells enlarge, pore formation occurs and protoplasts are formed, as in *C. diffluens*.

Fig. 11. Cells of *C. albidus* at 27 and 37 °C. (a) Cells from cultures grown at 27 °C in minimal medium; (b) cells from cultures shifted to 37 °C in minimal medium without 20 % sucrose; (c) cells from cultures shifted at 37 °C in minimal medium containing 20 % sucrose.

Fig. 12. Effect of 2-deoxyglucose on *C. diffluens* at 27 °C. Cells were obtained from cultures grown in minimal medium containing 0.25 mg 2-deoxyglucose/ml.

All of the observations presented thus far have been interpreted in terms of a temperature-induced perturbation of wall biosynthesis, and further support for this contention could be gained by demonstrating that the action at 27 °C of a known inhibitor of wall biosynthesis could mimic the effect of the high temperature (37 °C). Since 2-deoxyglucose inhibits fungal wall biosynthesis (Johnson, 1968), it was added to cultures of *C. diffluens* growing at 27 °C in minimal medium plus 20 % sucrose and the morphology of the cells examined microscopically (Fig. 12). It is evident that pores very similar to those produced at 37 °C are present, illustrating that the direct inhibition of wall synthesis in *C. diffluens* did produce abnormalities similar to those induced at the non-permissive temperature.

DISCUSSION

The results show that the inability to grow at 37 °C, used for the classical characterization of the saprophytic cryptococci, results from temperature-induced lysis. Most of the work described was performed with *C. diffluens* as a model system; limited results obtained with two additional saprophytes, *C. laurentii* and *C. albidus*, strongly suggest that the failure of these organisms to grow at 37 °C was also because of temperature-induced lysis. Results with the three species suggest that the lysis is a consequence of an imbalance of protoplasmic growth and the biosynthesis of wall, probably at the site of bud formation. Increased tonicity of the growth medium led to aberrant walls and protoplasts by impeding the lysis of the earliest osmotically-fragile forms produced at the higher temperature. The inability to observe these forms in non-stabilized cultures suggests that cells with even incipient pores are particularly subject to lysis, and that a continuous wall is necessary to prevent lysis in hypotonic environments.

The nature of the pores observed in stabilized cultures of *C. diffluens* is of particular interest. The question arises as to whether they always occur at a specific site. It was demonstrated that lysis is a consequence of growth rather than autolysis. This conclusion, coupled with the observation that only one pore per cell was observed, suggest that the pores may represent abortive budding sites. This contention is supported by the facts that actively growing cells of *C. diffluens* possess a single bud, that the site of most active cell-wall biosynthesis in a budding yeast is at the bud site (Johnson & Gibson, 1966), and that photomicrographs of pore-bearing cells of *C. diffluens* show that the pores vary in size.

The view that wall biosynthesis in *C. diffluens* is perturbed at 37 °C is further substantiated by comparing the morphological consequences of exposing cells to 37 °C and of treatment with the inhibitor 2-deoxyglucose at 27 °C. It is known that 2-deoxyglucose blocks wall synthesis in yeast and also causes protoplast formation (Heredia, De La Fuente & Sols, 1964; Berliner & Reca, 1970). Osmotically stabilized cells of *C. diffluens* possess pores when treated with 2-deoxyglucose at a permissive temperature (27 °C); these pores are smaller, however, than those induced at 37 °C. The reason for this difference may be that the cells treated in this manner do not enlarge to the extent which is typical of cells exposed to 37 °C. This failure to increase in size is probably due to an ambivalent effect of the 2-deoxyglucose, i.e. it blocks the phosphorylation of glucose and therefore retards growth (Biely & Bauer, 1967).

Because of the apparent correlation between temperature-induced lysis and aberrant wall biosynthesis in *C. diffluens*, certain aspects of wall biosynthesis were investigated; the preliminary results of this investigation are presented in the following paper (Dabbagh, Conant & Burns, 1974).

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