Interactions of *Candida albicans* Yeast Cells, Germ Tubes and Hyphae with Human Polymorphonuclear Leucocytes *in vitro*

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(Received 26 September 1983)

Suspensions of *Candida albicans* yeast cells, germ tubes and hyphae with biomass standardized by ATP measurement were compared for their relative susceptibilities to phagocytosis and intracellular killing by human polymorphonuclear leucocytes. All three forms were ingested to a similar extent, but significantly fewer yeast cells were killed intracellularly after ingestion than were filamentous forms of the fungus. Ketoconazole pretreatment significantly enhanced the susceptibility of hyphae, but not of germ tubes, to phagocytosis and intracellular killing. The opsonic requirements of the yeasts and filamentous forms for efficient phagocytosis and killing differed.

INTRODUCTION

Polymorphonuclear leucocytes (PMNs) are one of the major cell types involved in resistance to infection by the opportunistic dimorphic fungus *Candida albicans* (Rogers & Balish, 1980), and the interactions between PMNs and *C. albicans* have been extensively studied *in vitro* (Louria & Brayton, 1964; Yamamura & Valdimarsson, 1977; Kagaya & Fukazawa, 1981; Richardson & Smith, 1981). The results of some of these studies have suggested that the ability of *C. albicans* to produce germ tubes (early hyphal outgrowths) inside phagocytic cells may be a mechanism that allows the fungus to escape intracellular killing (Louria & Brayton, 1964; Stanley & Hurley, 1969). Little quantitative investigation has, however, been made of the interaction of phagocytic cells and pre-formed *C. albicans* germ tubes and hyphae, despite the observation that these forms may be found together with budding yeast cells in lesions *in vivo* (Rogers, 1966) and must therefore be exposed pre-formed to phagocytic cells *in vivo*.

This situation has arisen partly because of the difficulties inherent in manipulating filamentous forms of *C. albicans in vitro*, and partly because of problems of quantifying and standardizing the biomass of different morphological forms of the fungus for comparative assays of phagocytosis and intracellular killing. In addition, quantification of phagocytosis has proved difficult since the large size of germ tubes and hyphae precludes their total phagocytosis by single leucocytes, so that visual assessment of ingestion and killing becomes a very subjective process. Where phagocytosis of filamentous forms has been studied, standardized comparison between different forms has been absent (Diamond *et al.*, 1978) or the methods used for quantification of phagocytosis have been subjective (Scherwitz & Martin, 1979).

ATP bioluminescence photometry has been used for the estimation of viable biomass in a number of biological systems (Holm-Hansen, 1973), and for standardization of the viable biomass of *C. albicans* and other pathogenic fungi (Odds, 1980). This method therefore offers a useful basis for the standardization of biomass of suspensions of *C. albicans* in its different morphological forms, regardless of the shape of the suspended cells. The development of radio-metric assays for quantification of phagocytosis and killing of fungi (Yamamura *et al.*, 1977) has provided a more objective means for assessing these processes, and they can be applied to all morphological forms of *C. albicans*.

Abbreviations: EMEM, Eagle's minimal essential medium; PMN, polymorphonuclear leucocyte; SDB, Sabouraud dextrose broth.

This paper reports the results of a comparative study of the subceptibility of different forms of *C. albicans* to phagocytosis and intracellular killing by human PMNs *in vitro*, with fungal biomass standardized in terms of ATP concentration, and phagocytosis and killing measured radiometrically. The effects of some serum factors and the antifungal drug ketoconazole on these processes were also evaluated.

METHODS

Candida albicans isolate and preparation of filamentous forms. *Candida albicans* 73/055, originally isolated from a routine vaginal swab, was maintained at room temperature on Sabouraud dextrose agar (Oxoid). Yeast-form inocula were prepared in Sabouraud dextrose broth (SDB), comprising mycological peptone (Oxoid), 10 g l⁻¹ and D-glucose, 40 g l⁻¹, and incubated overnight at 30 °C in an orbital shaker set at 160 r.p.m. Germ tubes and hyphae were grown in Eagle's minimal essential medium (EMEM; Gibco) containing Earle's salts, L-glutamine and 20 mM-HEPES pH 7.4, and incubated statically in air at 37 °C for 2 and 6 h respectively. The initial yeast cell concentration in all cultures was 10⁶ ml⁻¹. Under these conditions more than 95% of the inoculum formed germ tubes, and by 6 h only well-developed hyphae with branches and without secondary yeast cell forms were seen microscopically. The fungi were harvested by centrifugation at 2500 g for 5 min, washed twice in distilled water and resuspended in EMEM + 20 mM-HEPES, pH 7.2. The average lengths of germ tubes and hyphae were 25 µm and 100 µm as determined with a calibrated eyepiece graticule and phase-contrast microscopy.

Determination of ATP concentrations of C. albicans suspensions. The viable biomass of *C. albicans* suspensions was estimated by ATP photometry essentially as described by Odds (1980). Briefly, 0.25 ml samples of fungal cultures were added to 2.5 ml volumes of TEMA buffer (0.05 M-Tris, 0.002 M-sodium EDTA, 0.05 M-MgSO₄, adjusted to pH 7.75 with acetic acid) that was preheated in test tubes in a boiling water bath. The mixtures were heated in the boiling water for 4 min and then cooled in ice-water. The ATP concentrations in the mixtures were determined by reference to a pure ATP standard solution with an integrating bioluminescence photometer (Lab-Line Instruments, Melrose Park, Illinois, USA) and partially purified luciferin-luciferase reagents.

Preparation of PMNs and sera. PMN-rich leucocyte suspensions were obtained from venous blood by the dextran sedimentation method described by Bridges *et al.* (1980). Leucocytes were washed twice in 10 ml EMEM + 20 mM-HEPES pH 7.2 and were resuspended in this medium to a concentration of approximately 10⁷ cells ml⁻¹.

Human serum was obtained from clotted venous blood donated by healthy volunteers. Some samples of serum were heated in a water bath to 56 °C for 30 min – a procedure designed to inactivate the C₃ component of the complement cascade (Wilson & Miles, 1964). Control and anti-*C. albicans* rabbit sera were obtained from New Zealand White rabbits. The anti-*C. albicans* antisera were raised against a mixture of cell wall and cytoplasmic antigens of yeast cells, germ tubes or hyphae prepared from *C. albicans* 73/055. Rabbits were immunized on three consecutive days of the week over a three week period, with increasing doses of antigen administered intravenously and subcutaneously. Blood was obtained from the animals three weeks after the last immunizing dose.

All sera were tested for the presence of anti-*C. albicans* precipitins by counter-immunoelectrophoresis against the homologous cytoplasmic extract (Odds *et al.*, 1975). Human sera, foetal calf serum (Gibco) and control rabbit sera were all negative in these tests. Antisera raised against *C. albicans* growth forms all had an anti-*C. albicans* precipitin titre of 1:32. All sera were stored in 1 ml lots at -20 °C.

Phagocytosis and killing assays. The method adopted for measurement of ingestion and intracellular killing of *C. albicans* was essentially that described by Bridges *et al.* (1980). It involved comparison of [³H]uridine uptake by the fungus in the presence and absence of PMNs. Phagocytosed *C. albicans* cells are unable to take up [³H]uridine, and the reduction in label uptake in the presence of PMNs, compared with control uptake, was considered to be proportional to the extent of phagocytosis. Intracellular killing was measured similarly by comparing uptake of [³H]uridine in *C. albicans* cells released from PMNs by sodium deoxycholate treatment with a control, phagocyte-free *C. albicans* suspension. Although this method may detect uptake of [³H]uridine by cells whose function is impaired although they are not killed, in practice a good correlation was found by Bridges *et al.* (1980) and in our preliminary experiments with data from subjective microscopic assessment of phagocytosis and intracellular killing.

PMN/*C. albicans* mixtures were incubated with continuous end-over rotation (30 r.p.m.) in 2 ml plastic vials (Sarstedt, Leicester, UK) at 37 °C for 1 h before uridine uptake was measured for the entire contents of the vials. After 1 h incubation in the presence of [³H]uridine, cell-associated radioactivity was collected on pre-wetted 25 mm diameter GFB filters (Whatman) which were then dried overnight at 80 °C. Radioactivity was counted in 3 ml of Fisofluor I (Fisons) in a Packard PL Tri-Carb scintillation counter. All assays were performed in duplicate in the presence of 5% control human serum unless otherwise stated.

Phagocytosis and intracellular killing were calculated according to the formula:

Percentage phagocytosis or killing = 100 × [(control c.p.m.) - (test c.p.m.)]/control c.p.m.

Fig. 1. Variation in phagocytosis and killing of *C. albicans* yeast forms (a), germ tubes (b) and hyphae (c) by human PMNs at different ratios of *C. albicans* to PMNs. Suspensions of *C. albicans* were standardized by ATP bioluminescence photometry. Each point represents the mean of four separate determinations; the bars represent SD. □, Phagocytosis; ■, killing.

The proportion of ingested *C. albicans* cells that were killed inside PMNs was calculated according to the formula:

Proportion killed (%) = $100 \times \text{Percentage of } C. albicans \text{ cells killed} / \text{Percentage of } C. albicans \text{ phagocytosed}$
Data were analysed statistically by the Mann-Whitney U test and results are expressed as means $\pm$ SD.

To determine the extent to which ingested yeast cells escaped phagocytosis by production of germ tubes, *C. albicans* yeast cell/PMN mixtures were sampled after 1 and 2 h incubation at 37 °C and examined by phase-contrast microscopy.

RESULTS

Preliminary experiments with *C. albicans* 73/055 yeast cells grown overnight in SDB showed that phagocytosis and intracellular killing were optimal at a blastospore:PMN ratio of 75 ng *C. albicans* ATP to 2×10^5 PMNs, an approximate numerical ratio of 2–3 yeast cells per PMN as judged by haemocytometer counts (Fig. 1a). Further assays at this optimal ratio showed the extent of variation in data for phagocytosis and intracellular killing by PMNs obtained from seven different donors (mean percentage phagocytosis = 75 ± 7 , mean percentage killing = 48 ± 10) and from the same donor in eight replicate assays at different times (mean percentage phagocytosis = 72 ± 8 , mean percentage killing = 40 ± 13). There was a clear linear relationship between the ATP content of yeast cells and the cell number judged microscopically: the association between [^3H]uridine uptake and cell number/ATP concentration was also linear over the range tested.

Phagocytosis and intracellular killing of yeast cells, germ tubes and hyphae

The extent of ingestion and killing of *C. albicans* germ tubes and hyphae was essentially the same over the range 50–100 ng ATP per assay (Figs 1b and 1c), and there was a fall-off in phagocytosis and killing above 100 ng ATP per assay for all three *C. albicans* growth forms tested (Fig. 1). Although the percentage of fungi phagocytosed was broadly similar for all three forms, the proportion of ingested yeast cells that were killed at the optimal 75 ng ATP per assay was statistically significantly lower than the proportion of ingested germ tubes or hyphae that were killed. The mean proportions of organisms killed were: yeast cells, 58.1; germ tubes, 99.1; hyphae, 100.8 ($P < 0.001$ for yeast cells vs germ tubes and yeast cells vs hyphae).

Yeast cells grown for 6 h in EMEM at 30 °C showed essentially the same extent of phagocytosis and intracellular killing as yeast cells grown overnight in SDB. For yeast cells of both types, approximately 10% had formed germ tubes within PMNs after 2 h incubation.

Effect of ketoconazole pretreatment of *C. albicans* on phagocytosis of germ tubes and hyphae

The imidazole antifungal drug ketoconazole at a concentration of 10^{-7} M inhibited hyphal development of *C. albicans* 73/055 after normal initial formation of germ tubes. The effect of PMNs on germ tubes and hyphae that had been grown in the presence of 10^{-7} M-ketoconazole

Table 1. *Phagocytosis and intracellular killing of C. albicans germ tubes and hyphae grown in the presence and absence of ketoconazole*

Germ tubes and hyphae were grown in the presence or absence of ketoconazole for 2 and 6 h respectively. The ratio of *C. albicans* to PMNs was 75 ng ATP : 2×10^5 cells. The data represent the means of six separate experiments $\pm$ SD.

Ketoconazole addition	Phagocytosis (%)		Killing (%)	
	Germ tubes	Hyphae	Germ tubes	Hyphae
None	59 $\pm$ 18	38 $\pm$ 16	64 $\pm$ 9	40 $\pm$ 14
10^{-7} M	62 $\pm$ 20	85 $\pm$ 8	54 $\pm$ 12	81 $\pm$ 4

Table 2. *Effects of serum factors on phagocytosis and intracellular killing of C. albicans yeast cells, germ tubes and hyphae*

The different sera were added to a final concentration of 5% (v/v). The data represent means of four separate determinations $\pm$ SD.

Serum addition	Phagocytosis (%)			Killing (%)		
	Yeasts	Germ tubes	Hyphae	Yeasts	Germ tubes	Hyphae
Normal human	68 $\pm$ 13	60 $\pm$ 13	68 $\pm$ 13	41 $\pm$ 13	42 $\pm$ 14	63 $\pm$ 18
None	27 $\pm$ 21	21 $\pm$ 20	36 $\pm$ 13	29 $\pm$ 13	18 $\pm$ 29	10 $\pm$ 8
Heat treated	57 $\pm$ 7	30 $\pm$ 5	13 $\pm$ 3	15 $\pm$ 12	26 $\pm$ 15	31 $\pm$ 12
Foetal calf	40 $\pm$ 12	45 $\pm$ 31	22 $\pm$ 14	14 $\pm$ 19	24 $\pm$ 8	27 $\pm$ 4
Normal rabbit	57 $\pm$ 22	21 $\pm$ 4	33 $\pm$ 6	38 $\pm$ 32	40 $\pm$ 21	39 $\pm$ 15
Rabbit anti-Y*	69 $\pm$ 13	20 $\pm$ 11	37 $\pm$ 9	47 $\pm$ 24	27 $\pm$ 20	41 $\pm$ 20
Rabbit anti-G*	70 $\pm$ 16	42 $\pm$ 17	54 $\pm$ 17	65 $\pm$ 19	45 $\pm$ 10	68 $\pm$ 14
Rabbit anti-H*	74 $\pm$ 11	32 $\pm$ 12	38 $\pm$ 18	38 $\pm$ 29	40 $\pm$ 17	58 $\pm$ 9

* Y, yeasts; G, germ tubes; H, hyphae.

was therefore investigated. Germ tubes grown for 2 h in the presence of ketoconazole were morphologically indistinguishable from control, untreated germ tubes, and they were phagocytosed and killed to a similar extent after the ketoconazole had been removed by washing (Table 1). By contrast, hyphae grown for 6 h in the presence of ketoconazole were unbranched, shorter, more pleomorphic and phase bright as compared with untreated hyphae, and they were phagocytosed and killed to a significantly greater extent than control hyphae after the ketoconazole had been removed by washing (Table 1).

Effect of serum factors on phagocytosis and killing of C. albicans by human PMNs

Phagocytosis of all three morphological forms of *C. albicans* tested was significantly lower in the absence of human serum than in its presence (Table 2). Heat pretreatment of serum had little effect on the phagocytosis of yeast cells, but significantly reduced phagocytosis of germ tubes and hyphae. Germ tubes and hyphae were phagocytosed to a similar extent in control and foetal calf serum but phagocytosis of yeast cells in foetal calf serum was significantly lower ($P < 0.01$) than in control serum (Table 2).

Omission of serum from the test system reduced the extent of killing of yeast cells, germ tubes and hyphae, but the effect was statistically significant ($P < 0.01$) only for the hyphae (Table 2). Yeast cells and hyphae were killed significantly less in heat-treated and foetal calf serum, but the reduction in killing of germ tubes was not statistically significant.

Although the data for phagocytosis and killing of all three forms of *C. albicans* tested were generally higher in the presence of homologous and heterologous rabbit anti-*C. albicans* antisera, there were no statistically significant differences between test and control experiments (Table 2). The overall percentage phagocytosis figures in the assays with rabbit serum were lower than those obtained for PMNs in the presence of human serum.

The absence of any significant effect of anti-*C. albicans* antiserum on the phagocytosis and killing of yeast cells was confirmed in experiments in which the precipitin titres of the rabbit

antisera were reduced by absorption with homologous or heterologous antigen or by dilution with precipitin-negative antiserum. Reduction of antibody titre by any of these means had no significant effect on the extent of phagocytosis and killing (data not shown).

DISCUSSION

The combination of ATP photometry for the standardization of fungal biomass and a radiometric assay for phagocytosis and killing allowed an objective comparison of the susceptibility of different morphological forms of *C. albicans* to phagocytosis by human PMNs *in vitro*. When germ tubes and hyphae were tested in this system, results were more variable than those for yeast cells; in particular, in some assays values for phagocytosis were less than those for intracellular killing. In the majority of cases the discrepancies were only of a few percent, and were probably inevitable in a system with fairly high replicate variance in which virtually all the ingested fungi were also killed. The possibility that the high percentage killing figures for germ tubes and hyphae were due to the presence of undisrupted PMNs in the assay mixtures was considered, but microscopic examinations of the suspensions after sodium deoxycholate treatment showed that all the PMNs were lysed. Diamond *et al.* (1978) reported that human PMNs could damage pseudohyphal forms of *C. albicans* even though the fungus was not completely ingested, and this observation may help to explain the apparent anomaly in the data in our study.

We have shown that, when standardized on the basis of ATP concentration, *C. albicans* yeast cells, germ tubes and hyphae were phagocytosed to a similar extent, particularly at low *C. albicans*:PMN ratios. Yeast form cells were, however, less effectively killed than were germ tubes and hyphae except at relatively high *C. albicans*:PMN ratios. The higher susceptibility of germ tubes and hyphae to intracellular killing may be due to a number of factors. Although these filamentous forms may not be completely phagocytosed (Diamond *et al.*, 1978) it is possible that localized damage at one site on the surface of germ tubes or hyphae may be sufficient to lead to the death of the fungal element. The continuity between different sections of *C. albicans* hyphal forms afforded by the septal pore (Scherwitz *et al.*, 1978; Gow *et al.*, 1980) could contribute to this action. The thickness and composition of *C. albicans* hyphal and yeast form cell walls differ (Chattaway *et al.*, 1976) and cell wall structure may be important in resistance of the fungi to intracellular killing. In particular, the hyphal tip may be especially sensitive to the action of hydrolytic enzymes as this is probably the weakest site on the cell wall.

Variation in the composition and arrangement of the cell wall layers of *C. albicans* yeast cells, germ tubes and hyphae may also affect the intensity of the metabolic burst involved in the killing of ingested fungi, as has been suggested to account for the variation in the extent of killing of *C. albicans* and other *Candida* species by macrophages (Sasada & Johnston, 1980). The differences in opsonic requirements of these morphological forms for both phagocytosis and killing could also relate to differences in cell surface composition and the availability of receptors and binding sites for serum complement and antibody. Yamamura & Valdimarsson (1977) reported that complement was required for the optimal killing of *C. albicans*, and Diamond *et al.* (1978) found that yeast cells and pseudohyphae differed in their opsonic requirements. Some of our data also suggested differences in opsonic requirements between the two morphological forms (Table 2), which may again reflect differences in the surface properties of the different morphological forms.

Although previous authors have suggested that the ability of *C. albicans* yeast cells to resist intracellular killing is due to their ability to produce germ tubes and then grow out of phagocytic cells (Stanley & Hurley, 1969), the results of our study suggest that processes other than germ tube production may be involved in the initial resistance to intracellular killing. If, however, ingested yeast cells survive the initial contact with the hydrolytic and oxidative killing mechanisms of PMNs they then seem likely to form germ tubes capable of thrusting out of PMNs as the ultimate means of escape. It has been suggested that, after phagocytic ingestion, yeast cells resistant to intracellular killing may be selected from a heterogeneous population and that such selection may be relevant in the maintenance of infection *in vivo* (Richardson & Smith,

1981). It is possible that the small proportion of yeast cells seen to produce germ tubes intracellularly in this study may represent members of a resistant population. Once such germ tubes have been produced, however, they may again be susceptible to the phagocytic and killing actions of other PMNs, although the possibility exists that germ tubes produced by resistant yeast cells may also be intrinsically more resistant to phagocytosis and killing.

The imidazole antifungal drug ketoconazole had a marked effect on the susceptibility of hyphae to phagocytosis and killing by PMNs, although it had little effect on the interaction of germ tubes and PMNs. De Brabander *et al.* (1980) reported that in mixed cultures of yeast cells and leucocytes, addition of ketoconazole to the system enhanced eradication of the fungus. In ketoconazole-free systems, a proportion of the ingested yeast cells survived and was able to multiply. In our studies, ketoconazole slowed the growth rate and elongation rate of hyphae, suggesting that its effect on morphology may be secondary to a generally inhibitory effect on *C. albicans* metabolism. Even so, the drug clearly affected the morphology of the fungus and may thus have altered surface properties important in resistance to attack by PMNs. Reduction in the growth rate of the fungus, enabling more efficient phagocytosis and killing by PMNs, may also explain the observed differences between the activity of this compound *in vitro* and *in vivo* (Odds *et al.*, 1980).

Although our observations indicate differences in resistance to killing by PMNs of the different morphological forms of *C. albicans*, the limitations of this information should be recognized. PMNs represent only one aspect of the host response to *C. albicans* invasion, and the response by PMNs *in vivo* may be more complex than that tested *in vitro*. For example, Denning & Davies (1973) found that *C. albicans* yeast cells and hyphae differed in their chemotactic properties towards phagocytic cells – a factor of considerable importance *in vivo*. Moreover, the suspension assay used in our study may not adequately represent the situation where *C. albicans* is growing on mucosal surfaces and access by phagocytic cells such as PMNs is limited.

A. Cockayne was supported by a Medical Research Council Studentship.

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