Interaction of *Candida albicans* with Neutrophils: Effect of Phenotypic Changes in Yeast Cell-surface Composition

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The susceptibility of four strains of *Candida albicans* to phagocytosis and intracellular killing by rabbit peritoneal neutrophils was investigated. Two of the strains, isolated from active infections, were known to synthesize a surface layer of mannoprotein fibrils in response to growth on 500 mM-galactose; the other strains, from asymptomatic carriers, lacked this capability. The presence of serum opsonins greatly enhanced phagocytosis of all four strains and, following opsonization, phagocytosis of an infective strain was equally rapid after growth on either 500 mM-galactose or 50 mM-glucose. In the absence of opsonins, galactose-grown infective strains were phagocytosed faster than either glucose-grown infective strains or galactose-grown carrier strains. These differences in phagocytic uptake were paralleled by differences in neutrophil chemiluminescence response. Intracellular killing of galactose-grown infective strains was only half that of glucose-grown infective strains or galactose-grown carrier strains after incubation for 60 min. Pretreatment of neutrophils with extracellular polymeric material, which contains the surface fibrils, completely inhibited intracellular killing. These results indicate that production of the fibrillar layer promotes yeast virulence by increasing resistance to intracellular killing, although it may enhance phagocytosis in locations where opsonic activity is poor.

INTRODUCTION

Polymorphonuclear leucocytes (neutrophils) play a major role in host defence against the opportunistic yeast pathogen, *Candida albicans* (Rogers & Balish, 1980), and there have been many reports on interactions between *C. albicans* and human or animal neutrophils *in vitro* (reviewed by Odds, 1988). Some of these studies focused on the relative susceptibilities of different strains (Richardson & Smith, 1981) or morphological forms (Scherwitz & Martin, 1979; Cockayne & Odds, 1984) of the fungus to phagocytosis and intracellular killing. Others concentrated on the biochemical processes involved and established that the myeloperoxidase–H₂O₂–halide oxidative system is the principal candidacidal mechanism within neutrophils (Lehrer & Cline, 1969; Diamond *et al.*, 1980; Wagner *et al.*, 1986). There have also been a number of investigations on the effect of yeast cell-wall mannoprotein on neutrophil and macrophage function (Wright *et al.*, 1981, 1983; Okawa *et al.*, 1986; Kolotila *et al.*, 1987). Wright *et al.* (1983), for example, showed that isolated mannoprotein (mannan) can bind to myeloperoxidase and thereby inhibit the candidacidal activity of neutrophils *in vitro*.

Previous work from this laboratory has demonstrated that growth of *C. albicans* in medium containing high concentrations of certain sugars, particularly galactose, can stimulate the production of a fibrillar mannoprotein layer on the yeast surface (McCourtie & Douglas, 1981, 1985). The fibrils, which can be recovered as extracellular polymeric material (EP) from culture supernatants, appear to enhance both yeast adhesion to epithelial cells and the virulence of the

Abbreviations: EP, extracellular polymeric material; HBS, HEPES buffered salt solution.

organism for mice (McCourtie & Douglas, 1984). Synthesis of the surface layer is strain-dependent; of nine strains of *C. albicans* tested, seven (isolated from active infections) showed considerable aptitude for this type of cell-surface modification, while two (obtained from asymptomatic carriers) did not. Other less pathogenic *Candida* species, as well as the non-pathogenic yeast *Saccharomyces cerevisiae*, also seem unable to synthesize the fibrillar layer (Critchley & Douglas, 1985).

Although the involvement of mannoprotein fibrils in *C. albicans* adhesion has been described (Critchley & Douglas, 1987*a, b*), the mechanism by which their synthesis enhances yeast virulence following intravenous injection has not been established. In the present study, we investigated the possibility that the fibrillar surface layer affords the yeast some protection against phagocytosis or intracellular killing by neutrophils. Both infective and carrier strains of *C. albicans* were examined under conditions which promote (growth on 500 mM-galactose) or inhibit (growth on 50 mM-glucose) fibril production in infective strains. The effect of isolated EP on neutrophil function was also evaluated.

METHODS

Organisms. Four strains of *C. albicans* were used in this study. Strains GDH 2346 (NCYC 1467) and GDH 2023 (NCYC 1468) were isolated at Glasgow Dental Hospital from patients with denture stomatitis. Strains GRI 681 (NCYC 1472) and GRI 682 (NCYC 1473) were obtained from routine cervical smears taken from asymptomatic women at Glasgow Royal Infirmary. All organisms were maintained on slopes of Sabouraud dextrose agar (Difco) and subcultured monthly. Every two months, cultures were replaced by new ones freshly grown from freeze-dried stocks.

Growth conditions. The yeasts were grown at 37 °C with shaking, in yeast nitrogen base medium (Difco) containing 50 mM-glucose or 500 mM-galactose as described by McCourtie & Douglas (1981). Organisms were harvested after 24 h (stationary growth phase) and washed twice in HEPES buffered salt solution (HBS), containing (g l⁻¹): NaCl, 8.0; KCl, 0.40; MgCl₂·6H₂O, 0.20; CaCl₂, 0.14; glucose, 1.0; HEPES, 2.39 (pH 7.4).

Opsonization of yeasts. Opsonization was achieved by incubating yeasts with 10% (v/v) normal rabbit serum at 37 °C for 30 min. The serum was stored at -70 °C.

Preparation of rabbit neutrophils. Peritoneal neutrophils were obtained from female New Zealand white rabbits. Saline (500 ml), containing 0.1% oyster glycogen (Sigma), was injected intraperitoneally and peritoneal exudate collected 4 h later (Lackie, 1977). The exudate was stored at 4 °C and used within 2 d of isolation. Before use, the cells were washed once in divalent-cation-free HBS, containing 1 mM-EDTA, and then in normal HBS. Contaminating erythrocytes were lysed after the first wash by brief exposure (5 s) to distilled water. To obtain a homogeneous population of single cells and to remove any fibrin clots, the neutrophils were passed through a 10 µm Nitrex filter (Plastok). Finally, cell numbers were standardized by counting with a haemocytometer. All neutrophil suspensions were more than 95% pure and cell viability was greater than 95% as determined by trypan blue exclusion.

Chemiluminescence measurements. Luminol-dependent chemiluminescence of neutrophils was measured (in mV) at 37 °C using an automated luminometer (Wallac-LKB 1251) connected to a microcomputer. Each assay mixture contained 10⁶ neutrophils, 10⁷ yeasts and 10⁻⁵ M-luminol in a total volume of 0.7 ml; this *Candida* to neutrophil ratio of 10:1 produced optimal chemiluminescence response (results not shown). Neutrophils were preincubated at 37 °C for 20 min before addition of the yeasts. At the start of the assay, luminol (70 µl) was added by an automatic dispenser. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma) was first prepared as a 0.01 M solution in dimethyl sulphoxide and then diluted 100-fold in HBS and stored at -20 °C until use. All mixtures were assayed in triplicate. Control mixtures in which neutrophils were incubated without yeasts, in the presence or absence of rabbit serum, produced a low, background, chemiluminescence response.

Phagocytosis of yeasts. The method used was essentially that of Leijh *et al.* (1977). Yeasts (10⁷ ml⁻¹ in HBS; 1 ml) were incubated with 1 ml neutrophil suspension (10⁷ cells ml⁻¹) and 0.2 ml rabbit serum (opsonized conditions) or 0.2 ml HBS (unopsonized conditions) in plastic bijoux bottles at 37 °C under gentle rotation (50 r.p.m.) for 60 min. This 1:1 ratio of *Candida* to neutrophils was found to be optimal for phagocytosis studies (results not shown). At intervals, portions of suspension (0.1 ml) were removed and diluted with 0.1 ml HBS. The number of extracellular yeasts lying totally free in the suspension was then determined using a haemocytometer. [Attached (but unphagocytosed) yeasts were never observed at 37 °C and the number of extracellular yeasts was therefore a valid measure of phagocytosis.] The figure obtained was used to calculate, by subtraction, the percentage of yeasts which had been phagocytosed. All phagocytosis experiments were repeated a minimum of three times. Control incubation mixtures containing yeasts and serum but no neutrophils indicated that the number of yeast cells

remained constant over the 60 min period. Clumping of yeasts or formation of germ tubes was not observed during the assay.

Yeast binding to neutrophils. This was measured at 4 °C, a temperature at which phagocytosis (of either opsonized or unopsonized yeasts) was inhibited by more than 90%. *Candida* suspension (2×10^7 yeasts ml⁻¹; 1 ml) was incubated with 1 ml of neutrophil suspension (10^7 cells ml⁻¹) and 0.2 ml rabbit serum (opsonized conditions) or 0.2 ml HBS (unopsonized conditions). At intervals, portions of the mixture were removed and the number of yeasts attached to 100 neutrophils was determined using a haemocytometer.

Intracellular killing of yeasts. Assay mixtures were prepared and incubated as described for phagocytosis experiments. At intervals during incubation, portions of the suspension (0.1 ml) were removed, added to 0.1 ml of filter-sterilized 1% saponin with thorough mixing, and left at room temperature for 5 min to allow lysis of the neutrophils and release of internalized yeasts. Duplicate serial dilutions of the lysate (in HBS) were plated on Sabouraud dextrose agar. After incubation of the plates at 37 °C for 18 h, the number of c.f.u. was determined as a measure of the number of viable *Candida* cells present. Intracellular killing was expressed as the percentage decrease in c.f.u. as compared with the initial value for the yeast suspension. Control incubation mixtures showed that yeast viability was unaffected by saponin or serum during the assay period.

Pretreatment of neutrophils with EP. In some experiments, neutrophils were pretreated with EP prepared from culture supernatants of *C. albicans* GDH 2346 as described by Critchley & Douglas (1987a). For assays of phagocytosis and intracellular killing, neutrophils (1 ml) were incubated at 37 °C for 30 min with EP (10 mg ml⁻¹) prior to addition of *Candida* suspension. Similarly, for chemiluminescence assays, neutrophils were incubated with EP (10 mg ml⁻¹) at 37 °C for 30 min before the addition of yeasts and injection of luminol. In control mixtures, neutrophils were pretreated with HBS instead of EP. Viability of the neutrophils was unaffected by pretreatment with EP as determined by trypan blue exclusion.

RESULTS

Phagocytosis of C. albicans

Phagocytosis of opsonized *C. albicans* GDH 2346 by rabbit peritoneal neutrophils was much greater than that of unopsonized yeasts of the same strain (Fig. 1). After 15 min, less than 25% of the opsonized organisms remained extracellular to the neutrophils whereas over 90% of the unopsonized yeasts were unphagocytosed. The rates of phagocytosis of opsonized organisms grown on different carbon sources were essentially similar. However, with unopsonized yeasts, organisms grown in medium containing 500 mM-galactose were phagocytosed more extensively than those grown on 50 mM-glucose (Fig. 1). Yeasts attached to the neutrophil surface were not observed in any of these assays.

Other strains of *C. albicans* were also examined for their sensitivity to phagocytosis after growth in high-galactose medium. With all of these strains, opsonized yeasts were phagocytosed rapidly and to a similar extent (Table 1). Unopsonized organisms were phagocytosed less rapidly, and strains isolated from active infections (GDH 2346 and GDH 2023) appeared to be more susceptible to phagocytosis than those (GRI 681 and GRI 682) obtained from asymptomatic carriers.

Yeast binding to neutrophils

Incubation at low temperature has been used to dissociate the attachment and ingestion phases of phagocytosis of another yeast pathogen, *Cryptococcus neoformans*, *in vitro* (Kozel &

Table 1. *Phagocytosis of C. albicans* strains grown in medium containing 500 mM-galactose as the carbon source

Values shown are means ($\pm$ SEM) from 3–6 separate experiments.

Strain	Percentage of yeasts remaining extracellular after 60 min	
	Opsonized	Unopsonized
GDH 2346	3.5 $\pm$ 0.5	71 $\pm$ 2
GDH 2023	2.8 $\pm$ 0.4	65 $\pm$ 3
GDH 681	3.2 $\pm$ 0.6	93 $\pm$ 2
GDH 682	2.9 $\pm$ 0.4	85 $\pm$ 2

Fig. 1. Phagocytosis of opsonized (○, ●) and unopsonized (□, ■) *C. albicans* GDH 2346 after growth in medium containing either 50 mM-glucose (○, □) or 500 mM-galactose (●, ■) as the carbon source. The results shown are representative of those obtained in at least three separate experiments.

Mastroianni, 1976). In the present study, ingestion of opsonized *C. albicans* GDH 2346 was severely inhibited at 4 °C, with an apparent uptake of less than 10% after 60 min, as compared with more than 95% uptake at 37 °C (results not shown). *Candida* attachment to neutrophils was measurable at 4 °C, and bound yeasts probably accounted for most of those reckoned, by the phagocytosis assay, to be ingested at this temperature. There was no difference in neutrophil binding between opsonized yeasts grown on 50 mM-glucose as a carbon source (8.6%) and those grown on 500 mM-galactose (9.1%). With unopsonized organisms, galactose-grown yeasts consistently bound in higher numbers (5.5%) than glucose-grown yeasts (3.5%), although the difference at this low level of attachment was not statistically significant.

Intracellular killing of yeasts by neutrophils

The ability of yeasts to resist intracellular killing was investigated using opsonized organisms only, because of the relatively poor uptake of unopsonized yeasts by neutrophils. Survival was dependent both on the strain of *C. albicans* and on the nature of the growth medium (Table 2). Growth of strain GDH 2346 on 500 mM-galactose produced yeasts with an enhanced capacity for survival; after incubation with neutrophils for 60 min, these organisms had a viability twofold greater than that of yeasts grown on 50 mM-glucose. A similarly increased resistance to intracellular killing was observed with galactose-grown yeasts of strain GDH 2023. Growth of the two carrier strains (GRI 681 and GRI 682) on galactose, on the other hand, produced no such increase; these organisms had a survival rate comparable with that of glucose-grown yeasts of strain GDH 2346 (Table 2).

Chemiluminescence studies

Stimulation of neutrophil phagocytic activity by yeasts was also investigated by measuring the generation of luminol-enhanced chemiluminescence (Allen & Loose, 1976). The chemiluminescence response of neutrophils to opsonized *C. albicans* GDH 2346 was much greater and more rapid than that to unopsonized organisms (Fig. 2*a, b*). There was essentially no difference in the response elicited by opsonized yeasts grown on different carbon sources (Fig. 2*a*). However, with unopsonized organisms (Fig. 2*b*), 500 mM-galactose-grown yeasts produced a

Fig. 2. Luminol-enhanced chemiluminescence emitted by neutrophils stimulated with opsonized (a) or unopsonized (b) *C. albicans* GDH 2346 grown in medium containing 500 mM-galactose (●) or 50 mM-glucose (○) as the carbon source. The data represent mean values from triplicate assays, and are typical of those obtained in at least three separate experiments.

Table 2. Intracellular killing of *C. albicans* strains by neutrophils after growth of the yeasts in medium containing 50 mM-glucose or 500 mM-galactose

Figures given are representative results from a minimum of three separate experiments. Control yeast suspensions incubated in the absence of neutrophils showed at least 95% viability after 60 min.

Strain	Carbon source	Percentage of viable yeasts after incubation for:	
		30 min	60 min
GDH 2346*	Glucose	40	15
GDH 2346	Galactose	51	31
GDH 2023	Galactose	65	33
GRI 681	Galactose	36	15
GRI 682	Galactose	36	16

* Glucose-grown yeasts of the other strains gave similar results, with percentage viabilities of 13–16% after 60 min.

bigger response than those grown in medium containing 50 mM-glucose. The ability of different strains of *C. albicans* to stimulate chemiluminescence is compared in Fig. 3, which shows results obtained with galactose-grown, unopsonized organisms only. The two strains capable of significant cell-surface modification during growth in galactose medium (GDH 2346 and GDH 2023) generated much more neutrophil response than either of the carrier strains, GRI 681 and GRI 682. These strain differences were not apparent when the yeasts were opsonized (results not shown).

Pretreatment of neutrophils with EP

Since EP isolated from culture supernatants of *C. albicans* GDH 2346 contains the fibrillar surface component produced by this strain in response to high concentrations of galactose

Fig. 3. Luminol-enhanced chemiluminescence emitted by neutrophils stimulated with four strains of unopsonized *C. albicans* grown in medium containing 500 mM-galactose as the carbon source. ■, Strain GDH 2346; ●, strain GDH 2023; □, strain GRI 682; ○, strain GRI 681. The data represent mean values from triplicate assays, and are typical of those obtained in at least three separate experiments.

Table 3. *Effect of pretreatment of neutrophils with EP on chemiluminescence response elicited by C. albicans GDH 2346*

Yeasts were grown in medium containing 500 mM-galactose. Values shown are means $\pm$ SEM from triplicate assays, and are typical of those obtained in at least three separate experiments.

Pretreatment of yeasts	Pretreatment of neutrophils	Chemiluminescence response	
		Maximum peak value (mV)	Total summed counts (mV)
Unopsonized	HBS	479 $\pm$ 9	10296 $\pm$ 91
Unopsonized	EP	300 $\pm$ 3	5131 $\pm$ 96
Opsonized	HBS	558 $\pm$ 5	11665 $\pm$ 77
Opsonized	EP	349 $\pm$ 23	6897 $\pm$ 440

(McCourtie & Douglas, 1985), the effect of EP on neutrophil function was investigated. Pretreatment of neutrophils with EP did not significantly affect phagocytosis of opsonized *C. albicans* GDH 2346 grown on either 50 mM-glucose or 500 mM-galactose. However, pretreatment did result in complete inhibition of intracellular killing of opsonized yeasts. After incubation for 60 min with EP-treated neutrophils, both glucose-grown and galactose-grown organisms showed 100% viability; the corresponding figures for yeasts incubated with control neutrophils pretreated with HBS were 8% and 16% viability, respectively. Neutrophil activity as measured by chemiluminescence response was also affected by pretreatment with EP. With both opsonized and unopsonized yeasts, EP-treated neutrophils showed a decrease in response of up to 50% as compared with control, HBS-treated neutrophils (Table 3).

DISCUSSION

The results of this study demonstrate that synthesis of mannoprotein fibrils by *C. albicans* in response to high concentrations of galactose promotes phagocytosis of the organism by rabbit

neutrophils but, at the same time, increases its resistance to intracellular killing. These differences in neutrophil response were observed in experiments with infective strains (GDH 2346 and GDH 2023) which are able to synthesize the fibrillar layer, and not in assays with carrier strains (GRI 681 and GRI 682) which do not have the same capacity for galactose-induced cell-surface modification. Phagocytosis was greatly enhanced by serum opsonization of the yeasts and differences in sensitivity due to fibril production were detected only with unopsonized organisms. However, increased resistance to intracellular killing resulting from this change in candidal surface composition was observed with opsonized yeasts.

Phagocytosis of opsonized micro-organisms is a process in which recognition between phagocytes and their target is mediated by antibody or complement or both. Neutrophils and macrophages possess receptors for the F_c moiety of IgG and for activated forms of C3 (Stendahl, 1983). In the absence of serum opsonins, on the other hand, phagocytes appear to recognize micro-organisms by lectin-carbohydrate interactions which promote strong attachment but are less efficient at initiating ingestion. Lectin-like interactions are thought to mediate attachment of *C. albicans* to vaginal and buccal epithelial cells via galactose-induced mannoprotein fibrils (Critchley & Douglas, 1987*a, b*). The results reported here suggest that, in the absence of serum opsonins, the same fibrils also promote yeast binding to neutrophils by a related mechanism. Phagocytosis of unopsonized *Candida* species has previously been demonstrated with mouse alveolar and peritoneal macrophages (Warr, 1980; Kolotila *et al.*, 1987) and there is evidence that yeast-macrophage binding depends on a lectin-like macrophage receptor specific for glycoproteins terminating in mannose or fucose residues (Warr, 1980; Stahl & Gordon, 1982).

Although fibril production by *C. albicans* enhances non-opsonic phagocytosis, our data show that it also substantially increases yeast resistance to intracellular killing. The candidacidal activity of neutrophils depends primarily on the myeloperoxidase- H_2O_2 -halide system (Lehrer *et al.*, 1983). However, neutrophils possess a second oxygen-independent mechanism involving the operation of intrinsically antimicrobial proteins and peptides, some of which are highly active against *C. albicans* (Selsted *et al.*, 1985). The fibrillar surface layer of resistant yeasts might exclude or inhibit some or all of these neutrophil components. This would be consistent with a previous finding that yeast cells with this type of surface modification are also more resistant to enzymic digestion by Zymolyase (McCourtie & Douglas, 1981).

An alternative explanation is suggested by the work of Wright *et al.* (1981, 1983) who have shown that myeloperoxidase-mediated killing of *C. albicans* requires binding of the enzyme to target yeasts and can be antagonized by mannoprotein solubilized from the yeast cell wall. Since the fibrillar mannoprotein layer synthesized by resistant yeasts is released from the cell surface relatively readily (McCourtie & Douglas, 1981), it too might inhibit candidacidal activity by interfering with the binding of myeloperoxidase to the cell wall proper. In support of this conclusion we have shown that pretreatment of neutrophils with EP, which contains fibrillar material, dramatically suppresses intracellular killing as determined either by a viable count or by the chemiluminescence assay. Although Ballart *et al.* (1987) have reported a linear correlation between chemiluminescence response and candidacidal activity of neutrophils, some anomalies were observed in the present study. Galactose-induced synthesis of surface fibrils by infective strains resulted in enhanced chemiluminescence (observed with unopsonized organisms) but also increased intracellular survival. However, there did appear to be some correlation between chemiluminescence and the phagocytic activity of neutrophils towards unopsonized yeasts.

Overall, our results indicate that the capacity of some strains of *C. albicans* for cell-surface modification is likely to be an important virulence attribute. Fibril production can be induced *in vitro* by growth on galactose but may be controlled by a variety of other, as yet undefined factors *in vivo*. One consequence of this surface change – an increased susceptibility to non-opsonic phagocytosis – could, paradoxically, be disadvantageous to the yeast in tissues such as the renal medulla where opsonic activity is poor (Silverblatt *et al.*, 1979). On the other hand, fibril production would undoubtedly confer significant benefits in terms of adhesion to epithelia (Douglas, 1987) and evasion of phagocytic killing. Release of fibrils from the yeast surface at localized sites of infection could produce high concentrations of mannoprotein with consequent severe effects on phagocyte function. Within the neutrophil, the binding of fibrils to

myeloperoxidase might permit survival of the yeast long enough to allow germ-tube formation and outgrowth. However, prolonged survival following such an escape mechanism would require that the germ tubes produced under these conditions were just as resilient as the parent yeast (Cockayne & Odds, 1984).

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