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Interaction and intracellular killing of *Candida albicans* blastospores by human polymorphonuclear leucocytes, monocytes and monocyte-derived macrophages in aerobic and anaerobic conditions

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SUMMARY

Polymorphonuclear leucocytes (PMN), monocytes and monocyte-derived macrophages were capable of interacting with opsonized *C. albicans* in both aerobic and anaerobic conditions. Superoxide anion release by these cells was inhibited in anaerobic conditions while lysozyme release and phagocytosis were equally efficient in both aerobic and anaerobic conditions. All cell types tested were capable of intracellular killing of *C. albicans* and this appeared to be maximum at 6 h for monocytes and macrophages and 24 h for PMN. Monocytes killed the lowest number of organisms, 1×10^6, and the killing was similar for aerobic and anaerobic conditions. In contrast, PMN and macrophages demonstrated greater killing of *C. albicans* in aerobic conditions compared with anaerobic conditions; PMN killed $1{\cdot}9 \times 10^6$ organisms and macrophages 3×10^6 when incubated anaerobically. Inhibitors of oxygen metabolism decreased intracellular killing of *C. albicans* by macrophages and PMN in aerobic but not anaerobic conditions. The oxygen reaction products involved in the killing of *C. albicans* appeared to be different however: macrophage killing was decreased by superoxide anion and hydrogen peroxide inhibitors. PMN killing was decreased by superoxide anion, hydrogen peroxide, hypochlorous acid and hydroxyl radical inhibitors. The present study shows that although monocytes, macrophages and PMN function similarly in their interaction with *C. albicans*, they appear to use different oxygen reactive products for the intracellular killing of *C. albicans*.

Keywords neutrophils monocytes phagocytosis killing *Candida albicans*

INTRODUCTION

Candidiasis is most commonly observed in the immunocompromised host, constituting a significant percentage of all infections and as a cause of death [1–3]. The incidence of infection with the pathogenic yeast *C. albicans* appears to have increased in the last two decades and the normal host appears also to be at some risk [4,5]. The presence of hyperglycaemia as a consequence of diabetes, steroid use or hyperalimentation as well as the transformation of yeast into mycelial phase, increase the risk and gravity of infection [6].

Polymorphonuclear leucocytes (PMN) and monocytes represent the first line of defence against bacterial and fungal infections [7,8]. Recent studies involving animals with granulocytopenia and monocytopenia have shown that PMN and monocytes are necessary for combating fungal infection [9,10]. The mechanisms used to defend the host include phagocytosis, lysosomal enzyme release and the generation of reactive oxygen metabolites [11]. It has been shown that if phagocytosis of *C. albicans* by PMN and monocytes was inhibited, then the numbers of organisms killed was decreased [12,13]. Subsequent studies dealing with fungi have provided strong evidence that both complement deposition and subsequent ingestion are necessary for the control of infection [14,15].

Although several investigators have demonstrated that PMN and monocytes are capable of killing *C. albicans*, the part played by the oxygen-dependent and oxygen-independent mechanisms appears to be controversial [16,17]. Studies of patients with chronic granulomatous disease and myeloperoxidase deficiency have shown that phagocytic cells from these patients killed *C. albicans* poorly, thus providing strong evidence that the oxygen-dependent pathways are used in killing [12,16,18,19]. In addition, Diamond & Krzesicki [20] have shown that the extracellular killing of fungal organisms by normal PMN was mediated through the oxygen-dependent pathway. However Yamada *et al.* [21] found that *C. albicans* was not susceptible to oxygen metabolites generated by two different cell-free systems.

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Given the evidence that the phagocytosis of fungal organisms is important for the control of infection and the controversial evidence for the involvement of oxygen-dependent killing mechanisms, the aim of this study was to measure the intracellular killing of *C. albicans* and to determine the effect of environmental changes from aerobiosis to anaerobiosis on the interaction and killing of *C. albicans* by human PMN, monocyte and monocyte-derived macrophages.

MATERIALS AND METHODS

Isolation of blood leucocytes

Peripheral blood from healthy adult volunteers was mixed with preservative-free heparin (1000 U/20 ml, Leo Laboratories, UK) and the leucocytes isolated by Ficoll–Isopaque centrifugation [22]. The mononuclear cells were reserved and the PMN were further separated from contaminating erythrocytes by sedimentation in 3% dextran (BDH Chemicals, Poole, UK) and hypotonic lysis of residual erythrocytes using Tris-buffered NH_4Cl [23]. Both cell types were counted and viability assessed by Trypan blue exclusion. The monocytes and PMN were resuspended at 5×10^6 cells/900 μl in Hanks balanced salt solution (HBSS) with HEPES but without phenol red (GIBCO, Paisley, UK) and allowed to adhere to glass coverslips in 24-well tissue culture plates (Costar, CA, USA) for 1 h at 37°C (PMN) or 2 h at 37°C (monocytes) in an atmosphere of 5% CO_2/95% air. The wells were then washed to remove non-adherent cells. Monocytes cultured aerobically for 5 days in HAMS F-12 medium (GIBCO) containing 5% fetal calf serum (FCS) showed a two-fold increase in H_2O_2 released in response to PMA when compared with uncultured monocytes, therefore these cells were referred to as 'macrophages'. The macrophages were then washed from the coverslip with phosphate-buffered saline (PBS) and resuspended at 5×10^6 cells/ml and allowed to adhere to glass coverslips.

C. albicans *preparations*

C. albicans, a clinical isolate from a patient with oral candidiasis, was kindly supplied by Mr R. Nash, London Hospital Medical College. *C. albicans* were grown on Sabouraud's dextrose agar plates (SAB) (Oxoid, UK) aerobically at 37°C before suspension in PBS (Oxoid) at an optical density of 1·0 to give a concentration of $1\text{–}2 \times 10^8$/ml. The yeast was pre-opsonized with 20% normal human serum from a single donor (containing IgG antibodies to *C. albicans* blastospores as measured by ELISA) for 30 min while unopsonized organisms were incubated with equivalent volumes of HBSS.

Aerobic/anaerobic incubation

All reagents for the assays were incubated at 37°C either in an aerobic incubator (Astell-Hearson, UK) or an anaerobic chamber (Don Whitley, UK) for 24 h before use. Cell monolayers were transferred to the anaerobic chamber or aerobic incubator for 1 h before use in the assays. PMN survival was not affected by 24 h incubation in anaerobic conditions as viability at time zero was 99% (5×10^6 cells) for both aerobic and anaerobic conditions, while viability at 24 h was 90% ($4{\cdot}5 \times 10^6$ cells) for aerobic incubations and 85% ($4{\cdot}2 \times 10^6$ cells) in anaerobic incubations.

Superoxide assay

The superoxide assay was performed in 96-well plates, containing 5×10^5 PMN/well [24]. A 200 mM solution of cytochrome C (Sigma, Poole, UK) was added to each well in the presence or absence of superoxide dismutase (SOD) (Sigma) at a concentration of 2 mg/ml. Opsonized organisms (10 μl) were added to each well and the plates were incubated for 1 h. Following centrifugation the optical density was read on a Titertek Multiscan MCC, at 540 nm (Flow Laboratories, UK). The protein content of each well was then estimated using a Bio-Rad dye assay (Bio-Rad, UK). The nmoles of the superoxide release was calculated by the following equation: (absorbance at 540 nm $\times$ 100)/6·3 $\times$ mg cell protein.

Lysozyme (muramidase) assay

Eight microlitres of the supernatants from the above incubation (superoxide assay samples) or 8 μl of hen egg white lysozyme standards were added to 2·5 mm diameter wells cut in Petri dishes containing 10 mg *Micrococcus lysodiekticus* in 20 ml of 1% agarose in 0·067 M phosphate buffer, pH 6·25 [25]. The plates were then incubated for 24 h at room temperature and the diameter of the resulting zones of bacterial clearance was measured using a $\times$ 10 magnifying eyepiece with a 20 mm grid. The amount of lysozyme present in the samples was calculated by correlation of the zones of clearance with logarithmic concentration of lysozyme in the standards (5–500 mg/ml).

Phagocytosis assay

Opsonized *C. albicans* (100 μl) was added in triplicate to each well of a 24-well tissue culture plate, which contained a coverslip with adherent PMN with or without oxygen metabolism inhibitors. After 1 h incubation, the PMN were washed from the coverslip by vigorous pippeting with 1 ml of PBS three times. The coverslips were then observed microscopically to ensure the cells had been detached. The cells were centrifuged at 40 ***g*** for 4 min, the supernatant containing the unbound organisms was removed and the cell pellet lysed with 1 ml of ice cold water. The number of organisms in this fraction was then counted microscopically using a haemocytometer and phagocytosis was expressed as the number of organisms ingested.

Trypan blue exclusion

C. albicans were heat killed by incubating at 56°C for 1 h. The organisms were then opsonized as already described. Opsonized live and heat-killed *C. albicans* were then incubated with phagocytic cells adherent to coverslips for 30 min. Trypan blue (0·4%) was added and the number of organisms staining blue was counted.

Cell killing assay

C. albicans killing was measured by a colony-forming assay. Cell monolayers were incubated as for the phagocytosis assay. After 1, 6 and 24 h of incubation the cells were pelleted and then lysed with 1 ml H_2O to release the internal organisms. The suspensions were then centrifuged and the ingested organisms counted (this figure would be the total number of colonies formed if all the ingested organisms were alive). The organisms were then serially diluted with sterile PBS before being spread on SAB plates. These were incubated aerobically for 4 days and the number of colonies was counted. The number of organisms killed was then calculated as:

Table 1. Release of superoxide anion and lysozyme following incubation with *C. albicans*

		Superoxide anion (nmoles/mg protein)		Lysozyme (μg/mg protein)	
Cell	Stimulant	Aerobic	Anaerobic	Aerobic	Anaerobic
Monocyte	None	ND	ND	4·3±1·5	5·2±0·7
	OPC	18·7±11·2	ND	18·3±8·2	17·2±5·5
Macrophage	None	ND	ND	8·0±2·1	8·3±1·7
	OPC	39·2±8·9	ND	34·7±10·9	39·2±12·4
PMN	None	ND	ND	2·7±0·4	3·1±1·2
	OPC	27·8±10·3	1·2±3·2	31·9±11·5	30·4±19·8

ND, not detectable; OPC, opsonized *C. albicans*.
Results are expressed as the mean±s.d. of four experiments.

Number of organisms killed =
Number of organisms phagocytosed −
number of colonies counted

Inhibitors of oxygen metabolism
Superoxide dismutase, catalase, thiourea and methinione (all from Sigma) were added after 1 h incubation of the adherent cells and again just before adding the organisms. To ensure that the candidal killing resulted from the incubation with the PMN and monocytes and not as a result of the adverse environmental conditions, colony counts of yeast incubated in the absence of PMN for each time point were performed.

RESULTS

Superoxide anion and lysozyme release
Each cell type released superoxide anion in response to *C. albicans* when incubated aerobically but no release was detected when the cells were incubated anaerobically (Table 1). Monocytes released the lowest amount of superoxide anion: 18·7±11·1 nmol, whilst macrophages released the maximum with 39·2±8·9 nmol measured. No increase in superoxide release was detected for any cell type beyond 1 h (data not shown). In contrast, the phagocytes released equal amounts of lysozyme when incubated in aerobic or anaerobic conditions, e.g. monocytes released 18·3±8·2 μg lysozyme aerobically and 17·2±5·5 μg anaerobically.

Phagocytosis
The uptake of opsonized *C. albicans* was efficient for all cell types and was not different under aerobic or anaerobic conditions at 1 h (Table 2). Monocytes ingested the lowest percentage of *C. albicans* at 69–74%, while macrophages ingested the largest at 90–98%. This uptake appeared to be maximal as no further increase in uptake was found after 3 h or 6 h (data not shown). Phagocytosis by all cell types was low (20%) when unopsonized *C. albicans* were used.

Trypan blue exclusion
To ensure that we were counting intracellular *C. albicans* and not cell bound organisms, a Trypan blue dye test was carried out with live and heat-killed organisms. The number of phagocytic cells positive for phagocytosis was no different for live or heat-killed organisms (data not shown). Uningested, heat-killed *C. albicans* were 100% positive for Trypan blue uptake whilst live organisms were less than 1% positive. After phagocytosis, however, there was no Trypan blue uptake by ingested *C. albicans* whether live or heat-killed organisms were used.

Table 2. Phagocytosis of opsonized *C. albicans**

	Organisms ingested† (%)	
Cell type	Aerobic	Anaerobic
Monocyte	74·7±13·7	69·1±10·5
Macrophage	98·0±14·8	90·4±14·0
PMN	83·2±14·2	81·2±6·9

* 5×10^6 cells were incubated with 1×10^7 organisms.
† Results are expressed as the mean±s.d. of four experiments.

Intracellular killing of C. albicans
Monocyte and macrophage killing of *C. albicans* at 1 h was low with 10% of the organisms ingested ($1–1·5 \times 10^6$ organisms) being killed when incubated either aerobically or anaerobically at 1 h (Fig. 1). Monocytes incubated for 6 h increased their killing of *C. albicans* to $2·5 \times 10^6$ organisms which was 20% of

Table 3. Effect of oxygen metabolism inhibitors on the intracellular killing of *C. albicans**

	10^6 *C. albicans* killed†	
Inhibitor	Macrophage	PMN
None	5·83±1·09	6·31±1·22
SOD (250 U/ml)	2·45±1·32	3·15±0·77
Catalase (5000 U/ml)	3·49±0·82	3·53±0·49
Thiourea (100 mM)	5·20±0·61	4·85±0·64
Methionine (50 nM)	5·52±0·72	3·70±0·83

* Intracellular killing was measured at 6 h.
Results are expressed as the mean±s.d. of four experiments.

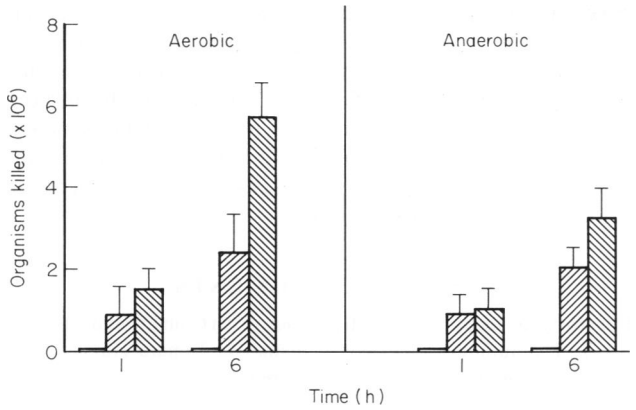

Fig. 1. Monocyte and macrophage intracellular killing of *C. albicans*. Bars show mean ± s.d. of four separate experiments. The initial inoculum of *C. albicans* was $1-1{\cdot}5 \times 10^7$ organisms per well. □, Control; ■, monocytes; ▨, macrophages.

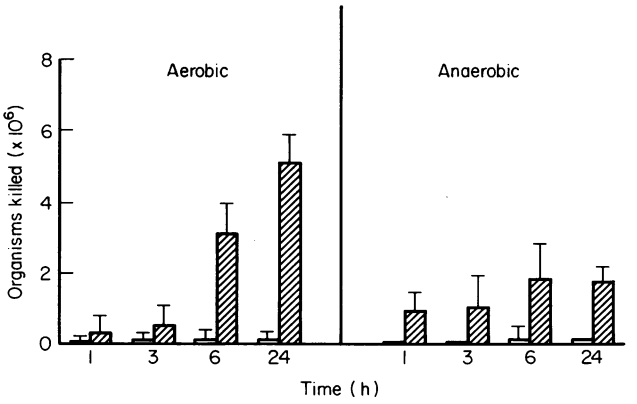

Fig. 2. Intracellular killing of *C. albicans* by PMN. Bars show mean ± s.d. of four separate experiments. The initial inoculum of *C. albicans* was $1-1{\cdot}5 \times 10^7$ organisms per well. □, *C. albicans*; ■, PMN + *C. albicans*.

the ingested organisms for both aerobic and anaerobic incubation. In contrast, macrophages incubated anaerobically at 6 h killed 3×10^6 organisms while macrophages incubated aerobically were able to kill significantly ($P < 0{\cdot}005$) more, 6×10^6 organisms. Neither monocytes nor macrophages showed a further increase in killing at 24 h (data not shown). PMN killing of *C. albicans* at 1 h and 3 h was low at $0{\cdot}5-1 \times 10^6$ organisms, representing 10% of the organisms ingested both aerobically and anaerobically (Fig. 2). Maximum killing by PMN was observed at 24 h and the difference in aerobic and anaerobic killing was also largest at this time with $5{\cdot}5 \times 10^6$ organisms killed aerobically and $1{\cdot}9 \times 10^6$ organisms killed anaerobically ($P < 0{\cdot}005$). The differences in killing observed were not simply due to intracellular digestion by phagocytic cells and therefore the failure to be able to count them at the end of the killing assays. The extracellular organisms and the intercellular organisms were both counted and, when added together, gave totals close to the number of organisms added (< 2% losses). These data suggest that little complete digestion of the organisms had occurred by this time.

Effect of oxygen metabolism inhibitors on intracellular killing

As both PMN and macrophages showed an increased capacity to kill *C. albicans* in aerobic conditions it was decided to investigate which oxygen-dependent metabolic products might be responsible for killing. No decrease in anaerobic killing was found for any of the inhibitors, showing that the anaerobic killing was truly oxygen independent (data not shown). Macrophages showed decreased killing of *C. albicans* when preincubated with SOD and catalase, e.g. in the presence of SOD, killing was decreased from $6{\cdot}83 \times 10^6$ to $2{\cdot}45 \times 10^6$ organisms ($P < 0{\cdot}005$) (Table 3). PMN also showed decreased killing on the addition of SOD and catalase but, in contrast to the macrophage, they also showed a similar decrease for methionine ($P < 0{\cdot}001$) and a slight decrease (from 6×10^6 to 5×10^6) was observed with thiourea ($P < 0{\cdot}05$).

DISCUSSION

The present study has examined the anti-fungal activity of human PMN, monocytes and monocyte-derived macrophages for *C. albicans* in aerobic and anaerobic conditions. No superoxide anion was detected with any cell type under anaerobic conditions. This was not due to the inability of the cells to respond as superoxide was detected when cells from the same population were incubated aerobically. In contrast, each cell type released lysozyme in response to opsonized *C. albicans*, with equal efficiency for aerobically and anaerobically incubated cells. All cell types also showed high levels of phagocytosis which did not differ in aerobic and anaerobic incubations. Thus, any difference in the intracellular killing detected for aerobic and anaerobic conditions was not simply due to different levels of fungal ingestion.

Previous reports have shown that patients with defective oxygen metabolism have reduced ability to control fungal infection [13,16,26]. In the present study, the intracellular killing of *C. albicans* by monocytes was poor. However, this did not appear to be due to the low numbers phagocytosed as monocytes ingested about 77% of organisms added. It is also possible that the *C. albicans* were not internalized by the monocytes but were instead cell-associated and thus not exposed to lysosomal enzymes. However, we do not think this is the case as after phagocytosis of heat-killed *C. albicans* by all cell types, the dead organisms failed to take up Trypan blue suggesting that the organisms were truly intracellular and thus inaccessible to Trypan blue dye. More likely the failure of the monocytes to kill *C. albicans* efficiently may be related to the low amounts of oxygen metabolites generated by the monocytes, as no difference in intracellular killing was observed for monocytes in aerobic or anaerobic conditions. Further evidence for the involvement of the oxygen metabolites in *C. albicans* killing was obtained when it was observed that both PMN and macrophages released large amounts of superoxide and also killed increased number of fungus. Also, in contrast to the monocyte, intracellular killing by PMN and macrophages was more efficient in aerobic conditions when compared with anaerobic conditions. This suggests that if *C. albicans* is found in areas of low oxygen tension then control by PMN and macrophages would be difficult, leading to a more prolonged or severe infection.

During the respiratory burst a variety of products are generated which include superoxide anion, hydrogen peroxide

and hydroxyl radicals, also with MPO a variety of additional products [27]. A number of studies have shown the involvement of different products in the killing of various organisms [7,12,26,28]. The inhibitors employed were: SOD, catalase and thiourea which inhibit the products of oxygen metabolism superoxide anion, H_2O_2 and hydroxyl radical respectively. Reactive products can also be generated by MPO and hydrogen peroxide. Although this pathway would be partly assessed by the inclusion of catalase, methionine, an inhibitor of the MPO pathway at a later stage of hypohalide formation, was also included. In the present study it was found that PMN incubated aerobically demonstrated a decreased killing of *C. albicans* when SOD, catalase and methionine were included in the assay. Thiourea also reduced the killing of *C. albicans*, but this decrease was not so great as that given by SOD and catalase. These results suggested that superoxide anion, H_2O_2, hydroxyl radicals and hypohalides are all capable of killing *C. albicans*, but that killing via hydroxyl radical is less efficient. Similar findings were observed by Diamond & Krzesicki [20] who showed that killing of *C. albicans* was inhibited by SOD, cyanide (an MPO pathway inhibitor) and 1,4 diazobyicyclo (2,2,2,) octane, a singlet oxygen inhibitor. However, they failed to observe any inhibition of killing with mannitol or benzoate (hydroxyl radical inhibitors). The differences in these findings for the hydroxyl radical compared with our own may result from the fact that their experiments were investigating extracellular killing of *C. albicans* pseudohypae, while the present study was investigating the intracellular killing of the yeast form of the fungus.

In contrast to PMN, macrophage killing was only reduced by inhibitors of superoxide and hydrogen peroxide. Although macrophages showed the largest respiratory burst in response to *C. albicans* and also ingested more organisms than the other two cell types, they did not show significantly increased killing compared with PMN. The reason for the failure of macrophages to kill greater numbers of organisms than PMN may be due to low levels of myeloperoxidase and thus a limited ability to form products such as hypohalides. The importance of hypohalide products has been further shown in a recent study where inhibitors such as methionine and tryptophan decreased the killing of *C. albicans* [29].

It would appear that *C. albicans* can also be killed by O_2-independent PMN mechanisms because intracellular killing was observed in monocytes, macrophages and PMN incubated anaerobically. This killing was totally unaltered by the inhibitors of oxygen metabolism tested. Lehrer [16] showed that PMN from MPO-deficient patients killed *C. albicans* efficiently and suggested that killing could be attributed to cationic proteins. Similar conclusions were reached by Brummer & Stevens [30] who showed that the killing of *Blastomyces dermatitidis* was unaffected by the presence of oxygen metabolite inhibitors such as SOD, catalase and sodium azide. Candidates for involvement in oxygen-independent killing would include the defensins, in particular the neutrophil peptide 4 [31].

In general, monocytes killed less organisms than PMN which is consistent with previous studies [18,32,33]. Macrophages, even though they ingested a larger number of organisms, killed similar numbers to PMN. This indicates that macrophages are limited in the number of organisms they can kill, not by their phagocytic activity, but by their ability to generate oxygen metabolites to which *C. albicans* appears to be susceptible. It is also important to note that killing in anaerobic conditions and thus by non-oxygen-dependent pathways, was much lower than that observed in aerobic conditions, showing that oxygen-dependent pathways were involved in the intracellular killing of *C. albicans*. Therefore, it may be that in tissues or wounds with poor blood supply and low oxygen tension, the infectivity of *C. albicans* may increase as a resul of the increased virulence of this organism.

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