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Release of *Candida albicans* yeast antigens upon interaction with human neutrophils *in vitro*

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Candida albicans is the leading cause of invasive candidosis. As conventional tests do not reliably detect invasive infection, attention has turned to the detection of *C. albicans* antigens circulating in blood. As antigen tests for invasive candidosis could be improved if *C. albicans* antigens released upon phagocytosis were defined, this study was undertaken to characterise antigens released during the interaction of yeasts and human neutrophils *in vitro*. An enzyme immunoassay developed previously to detect what were believed to be predominantly *C. albicans* cytoplasmic antigens in patients with invasive candidosis was used to follow the neutrophil-mediated release of yeast antigens. Serum opsonisation enhanced antigen release, which was rapid and essentially complete by 1 h. When fresh *C. albicans* yeasts were added to medium from cultures of neutrophils plus yeasts or neutrophils plus latex beads, additional yeast antigens were released. Medium from neutrophils plus yeasts or from yeasts alone had similar immunoblot patterns with rabbit antibodies to a *C. albicans* cytoplasmic antigen preparation, with the reactive antigens generally being of higher mol. wt than the reactive antigens in the antigen mixture used for preparation of the antiserum. The two supernates also had similar immunoblot patterns with rabbit anti-*C. albicans* cell-wall mannan antibodies. These results suggest that yeast surface antigens are released quickly during phagocytosis by neutrophils. Detection of such yeast surface antigens, possibly together with selected yeast cytoplasmic antigens, should improve the sensitivity of *C. albicans* antigen assays.

Introduction

The opportunist fungal pathogen *Candida albicans* is the leading cause of invasive candidosis. Invasive candidosis is difficult to diagnose, firstly because clinical symptoms are often non-specific and, secondly, because conventional laboratory tests are not sufficiently sensitive. For example, culture of *C. albicans* from superficial body sites does not correlate well with infection. Moreover, blood cultures and tests to detect yeast-specific antibodies lack both sensitivity and specificity [1, 2]. Clearly, better tests for the detection of invasive *C. albicans* infections are needed.

A recent report described an enzyme immunoassay (EIA) for the detection of what were presumed to be *C. albicans* cytoplasmic (CACP) antigens present in blood during invasive candidosis [3]. The EIA is

based on antisera raised against antigens in a *C. albicans* cytoplasmic extract prepared from disrupted yeast cells. Gel electrophoresis of CACP antigen showed five major proteins with mol. wt between c. 36 kDa and 44 kDa. The EIA was shown to have a sensitivity of 70% and a specificity of 85–90% for patients at risk for invasive *C. albicans* infections.

If *C. albicans* cytoplasmic antigens are present in the circulation during invasive candidosis, it is thought to be due to antigen release following phagocytosis by neutrophils and other phagocytic cells [2, 4]. However, the time course and nature of the antigens released remains largely undefined, as only a few *C. albicans* cytoplasmic antigens present in patient sera during invasive infection have been identified. These include enolase and a heat-shock protein [5, 6]. It should also be noted that earlier work had indicated that *C. albicans* surface-accessible antigens, i.e., mannan or mannoproteins, are also present in the circulation of patients with invasive disease [7]. We believe that defining the *C. albicans* antigens that may circulate

during invasive infection would lead to a more sensitive diagnostic test for this disease. Therefore an in-vitro approach was used to study the release of *C. albicans* antigens following the interaction of yeast cells with isolated human neutrophils.

Materials and methods

Yeasts

C. albicans clinical strain 60553 was propagated in liquid culture as described previously [3]. Freshly grown yeasts were washed twice at 4°C with a volume of sterile, phosphate-buffered saline (PBS; 20 mM sodium phosphate buffer, pH 7.4, and 140 mM NaCl) equal to the original culture. Cells were resuspended in 0.5 volume of Hank's Balanced Salts Solution (pH 7.0–7.5; Gibco Laboratories) containing 20 mM HEPES (Sigma) and 20 mM sodium bicarbonate (buffered HBSS). Yeasts were counted in a haemocytometer, with 1 cfu being a mother cell plus any attached buds. Yeasts were pre-opsonised according to the method of Solomkin *et al.* [8]. Cells were resuspended at 2×10^7 cfu/ml in buffered HBSS pre-warmed to 37°C and containing autologous serum 10%. After incubation at 37°C for 20 min on a rocking platform (four times/min), the cells were collected by centrifugation at 225 *g* for 5 min at 4°C. The cells were washed once at 4°C with a volume of PBS equal to the opsonising medium and resuspended to *c.* 10^8 cfu/ml in buffered HBSS. Viability was assessed with a trypan blue dye 0.1% solution [9]. Yeasts, pre-opsonised or not, were used in experiments at 10^7 cfu/ml.

Neutrophils

Peripheral blood was collected from donors who had given informed consent with venepuncture Vacutainer tubes (Becton Dickinson) containing EDTA 1.5 mg/ml as anticoagulant. A sample of blood was also collected without anticoagulant to provide autologous serum for yeast opsonisation. Neutrophils were separated from whole blood by differential centrifugation [10]. Four ml of anticoagulated blood were mixed at 4°C with 16 ml of isotonic NH_4Cl solution in 50-ml conical centrifuge tubes which were kept at 4°C for 15 min while red cell lysis occurred. White blood cells were collected by centrifugation at 160 *g* for 10 min at room temperature. The cells were resuspended in 10 ml of calcium- and magnesium-free (CMF) buffered HBSS (pH 7–7.5) and transferred to 15-ml conical tubes. Neutrophils were differentially pelleted by centrifugation at 55 *g* for 10 min at room temperature. Supernate was removed by suction and the neutrophils were resuspended in 5 ml of CMF-buffered HBSS and again centrifuged (55 *g*, 5 min). Neutrophils from all tubes were pooled in 0.75 ml of CMF-buffered HBSS and counted manually in Türk's fluid. Control counts on some preparations were performed by the Haematology Laboratory, Royal University Hospital, Saskatoon.

Neutrophils were diluted in CMF-buffered HBSS to 10^6 /ml and held at 4°C until use (1–8 h later). Viability was assessed by trypan blue dye exclusion.

Sonicated neutrophils were used to test for neutrophil antigens which cross-react in the CACP antigen EIA. Neutrophils were sonicated at 10^6 /ml in 3 ml of buffered HBSS containing autologous serum 10%. Cells were disrupted with a Braunsionic 1510 ultrasonic homogeniser with a 14×0.4 -cm probe and with sonic bursts of 350 W for 3×15 s and 1×30 s. Between bursts, the tube was placed in ice for 1 min. Microscopic examination after sonication showed no intact neutrophils.

Latex beads

Flouresbrite™ 2.16- μm plain latex beads (Polysciences Inc., Analychem Corporation) (2×10^7 /ml) were opsonised at 4°C for ≥ 12 h in buffered HBSS containing human immune serum globulin (Miles Canada Inc., Etobicoke, ON, Canada) 1 mg/ml. Beads were kept in suspension by rocking (four times/min). They were then collected by centrifugation (780 *g* at 4°C for 15 min) and washed twice at 4°C with buffered HBSS equal to the original incubation volume. They were resuspended at 2×10^7 /ml in buffered HBSS containing autologous serum 10%. After incubation for 2 h at 37°C (rocking four times/min), the beads were collected by centrifugation, washed once and resuspended in buffered HBSS. To compensate for the smaller volume of the beads compared to yeast cfu, the beads were used in experiments at 3×10^7 /ml.

CACP antigen EIA

The EIA developed to detect *C. albicans* cytoplasmic antigens in sera of patients with invasive candidosis [3] was used to detect *C. albicans* yeast antigens released upon interaction with neutrophils. Antigen was quantified by comparison with a standard curve constructed from CACP antigen prepared from *C. albicans* strain 60553 [3]. Throughout this work, antigen concentrations are expressed as [protein]/ml.

Antisera

Preparation of rabbit anti-CACP antigen antiserum was as described previously [3]. Rabbit anti-*C. albicans* cell-wall mannan antiserum was similarly prepared with *C. albicans* cell-wall mannan purified according to the method of Peat *et al.* [11]. Mouse antisera against two of the major CACP antigens (mol. wts 38 and 44 kDa) were prepared by immunising BALB/c mice with protein purified by two-dimensional polyacrylamide gel electrophoresis of CACP antigen [3]. Gel bands containing the 38-kDa or the 44-kDa protein were excised, dried and emulsified in PBS with a sterile glass rod. The immunising antigen was prepared in a Mulsichurn™ (Mulsijet, Elmhurst, IL, USA) [12] and

consisted of *c.* 1 μ g of protein in 0.25 ml of PBS emulsified with 0.25 ml of Freund's incomplete adjuvant, the emulsion was then dispersed by mixing with 0.5 ml of Tween 80 2% in PBS [13]. Mice were immunised intraperitoneally with 1 ml of antigen preparation. Mice received two booster injections at 2-week intervals and were terminally bled 3 weeks later. Antibody titres for the mouse antisera were determined by an antibody EIA in which adsorbed CACP antigen (1.5 μ g in 200 μ l of PBS/well in a 96-well plate) was the antibody-binding target and horseradish peroxidase (HRP)-conjugated AffinipureTM goat anti-mouse immunoglobulin IgG + IgM (H + L) antibodies (Jackson ImmunoResearch Laboratories; Bio/Can Scientific Inc., Mississauga, ON, Canada) were the indicator antibodies. Diluents and assay conditions paralleled successive steps of the CACP antigen EIA [3]. Titres of the mouse antisera were determined with an arbitrary cut-off of 0.2 units above background at an OD of 492 nm.

Time course of C. albicans antigen release

A 1-ml mixture of cells and medium in a 1.5 ml Eppendorf tube was used for each 15-min time point. Neutrophils were incubated with yeasts (10 cfu/neutrophil) at 37°C on a rocking platform (six times/min). Neutrophils and yeasts were also each incubated alone as controls. Culture medium was buffered HBSS with or without autologous serum 10%. After a tube was removed, it was centrifuged at 8800 *g* for 3.5 min at room temperature and the supernate was saved. If a supernate was to be tested by CACP antigen EIA the following day, it was stored overnight at 4°C; otherwise, storage was at -20°C.

Extracellular release of neutrophil enzymes and metabolites

One-ml mixtures of pre-opsonised *C. albicans* yeasts and neutrophils (10 cfu/cell), latex beads and neutrophils (30 beads/cell), neutrophils alone, and pre-opsonised yeasts alone were incubated at 37°C for 10 min in 1.5-ml Eppendorf tubes. After centrifugation (8800 *g* for 3.5 min) at room temperature, 0.9 ml of supernate was removed and divided into two portions; 50 μ l of fresh, pre-opsonised yeasts (0.5×10^7 cfu) were added to one aliquot, while 50 μ l of CMF-buffered HBSS were added to the other as a negative control. After re-incubation at 37°C for 30 min, yeast cells were removed by centrifugation as above. Supernates were stored at -20°C until tested. *C. albicans* antigen was quantified before and after addition of fresh yeasts by the CACP antigen EIA.

Immunoblotting

Pre-opsonised yeasts were incubated with neutrophils (10 cfu/cell) on a rocking platform (six times/min) at 37°C in 15- or 50-ml polypropylene tubes, containing

10 ml or 25–30 ml, respectively, of buffered HBSS. To provide control supernates, latex beads were incubated with neutrophils (30 beads/cell), and neutrophils and pre-opsonised yeasts were each incubated alone. Because neutrophils were prepared in CMF-medium, an equal volume of CMF-buffered HBSS was included in control tubes. Tubes were incubated for 1–2 h and centrifuged at 4°C at 280 *g* for 5 min (15-ml tubes) or 780 *g* for 10 min (50-ml tubes). Supernates were filtered through 0.45 μ m filters to remove remaining yeasts and neutrophils, and stored at -20°C until used.

Equal volumes of test and control supernates (10–18 ml) from a given experiment were each concentrated to *c.* 1 ml through a Diaflo ultrafilter in a 10-ml concentrator (mol. wt cut-off 10 000; Amicon). Concentrated supernates were transferred to Centricon-10 microconcentrators (Amicon, Toronto, ON, Canada). Diaflo filters were washed with 1 ml of PBS and washes were transferred to microconcentrators, giving a total of 2 ml for each supernate. Microconcentration and desalting (solvent exchange) with PBS in Centricon-10 microconcentrators were according to the supplier's protocol. Centrifugation was at 5000 *g* with an SS34 rotor in a Sorvall RC-5B centrifuge at 4°C for 80 min. Final, desalted, concentrated supernates (40–45 μ l) were stored at -20°C until use.

SDS-PAGE of concentrated supernates was performed under reducing conditions [14]. Electrophoresis was at 42 mA in a Hoefer SE600 water-cooled vertical slab gel unit (Hoefer Scientific Instruments; Fisher Scientific, Edmonton, AB, Canada) with stacking and separating gels of 4 and 10% acrylamide, respectively. The control lane contained 20 μ g of CACP antigen preparation. Equal volumes of desalted, concentrated supernates from the same experiment were added to the remaining lanes (antigen concentration was not determined).

Electrophoretic transfer was at 100 V for 1 h at 4°C in a TE Series Transphor Electrophoresis unit (Hoefer Scientific Instruments). Primary immunoblotting antibodies were rabbit anti-CACP antigen, or rabbit anti-*C. albicans* cell-wall mannan polyvalent antiserum (diluted 1 in 150), or mouse monospecific antiserum (diluted 1 in 200). Indicator antibodies were either HRP-conjugated AffinipureTM donkey anti-rabbit IgG (H + L) antibodies (diluted 1 in 2000) or HRP-conjugated AffinipureTM goat anti-mouse IgG + IgM (H + L) antibodies (diluted 1 in 1000) (Jackson ImmunoResearch Laboratories). The colour development reagent was 4-chloro-1-naphthol.

Results

Yeasts and neutrophils

Yeasts were 100% viable in buffered HBSS; therefore, cellular breakdown had not begun before the initiation

of experiments. One cfu was determined microscopically to contain from one to three cells (buds were considered as cells) with an average of 1.5 cells/cfu. Occasionally, a single hyphal strand was observed; therefore, $\geq 99\%$ of the cells were in yeast form in each experiment.

Neutrophils were obtained from blood by a differential centrifugation method which is rapid, yields neutrophil preparations of high purity and avoids potential adverse effects on neutrophil function [10]. An average neutrophil purity of $> 70\%$ was obtained. Other cells present were T and B lymphocytes (10–24%), eosinophils (2–11%) and monocytes (3–6%). The use of CMF-buffered HBSS during neutrophil isolation and in experiments was essential to reduce white cell clumping. If used within 2.5 h after preparation, neutrophils were 100% viable before incubation with yeasts. When pre-opsonisation of latex beads or yeasts was required, typically 8 h elapsed between venepuncture and the start of the experiment, and *c.* 1.5% of all cells present had lost viability.

Kinetics of phagocytosis

During the incubation of yeasts or latex beads with neutrophils, 10- μ l portions were removed and yeasts or latex beads within neutrophils were counted by light microscopy at 400 $\times$. Samples were taken every minute up to 5 min, and then at 7.5, 10, 15, 30, 45 and 60 min. Ingestion of yeasts or latex beads resulted in a distortion of neutrophil shape. In medium without serum, neutrophils showed little or no uptake of unopsonised yeasts or latex beads. In medium containing autologous serum 10%, phagocytosis was rapid, with yeasts clearly visible inside neutrophils within 2–3 min. By 15 min, many neutrophils had ingested several yeasts. Phagocytosis of pre-opsonised yeasts was also followed, as pre-opsonised yeasts were used to generate supernates for immunoblotting (the use of pre-opsonised yeasts ensured that reasonably efficient phagocytosis by neutrophils occurred, yet serum proteins were not present to complicate SDS-PAGE analysis of released yeast antigens).

Phagocytosis of pre-opsonised yeasts, in a medium lacking serum, occurred more slowly. In a representative experiment, 30 (49%) of 61 neutrophils had ingested 86 pre-opsonised yeasts after incubation for 1 h. The number of phagocytosed yeasts ranged from one to six, with the average being 1.4, representing *c.* 15% of the total yeasts. Neutrophils ingested more pre-opsonised latex beads than pre-opsonised yeasts, as after incubation for 1 h, 21 (70%) of 30 neutrophils had ingested at least one latex bead. The number of latex beads/neutrophil ranged from two to nine, with an average of three.

Time course and quantities of *C. albicans* antigen released

The largest release of *C. albicans* antigen occurred when yeasts were incubated with neutrophils in serum 10%. Considerable quantities of antigen were detected at 15 min, and release reached a maximum at 1 h (Fig. 1). Antigen release from pre-opsonised yeasts was slower, at a lower level, and reached a maximum slightly later. Overall, an even lower level of antigen release occurred from unopsonised yeasts incubated with neutrophils in buffered HBSS. The average amount of *C. albicans* antigen released when autologous serum 10% was present in the incubation medium was 4.5 times the average quantity released from pre-opsonised yeasts and 12.5 times that released from unopsonised yeasts (Table 1). Finally, only a low level of antigen release occurred from yeasts incubated alone in medium containing serum 10%. Release of antigens from pre-opsonised yeasts or unopsonised yeasts incubated alone in buffered HBSS without serum was similarly low (data not shown). Neutrophils release antigens that only minimally cross-react in the CACP antigen EIA, as values only slightly above background were obtained when this supernate was tested (Fig. 1). No additional reactivity was observed when supernate from sonicated neutrophils alone in buffered HBSS plus serum 10% was tested undiluted (data not shown).

Degradation of yeasts by extracellular neutrophil metabolites

It seemed unlikely that large amounts of *C. albicans* antigen could be released within 15 min from inside

Fig. 1. *C. albicans* antigens released over 2 h detected by the CACP antigen EIA. Supernates were diluted 1 in 8 to avoid prozoning; further dilution gave progressively lower CACP antigen values. ■, Yeasts plus neutrophils in buffered HBSS containing 10% serum; □, pre-opsonised yeasts plus neutrophils in buffered HBSS; ●, yeasts plus neutrophils in buffered HBSS; ▲, yeasts alone; or △, neutrophils alone in buffered HBSS plus serum 10%. Data shown are from a representative experiment and values have been corrected for the assay background of 0.240.

Table 1. Quantities of *C. albicans* antigen released from yeasts upon interaction with human neutrophils* under various conditions *in vitro*

Supernate	Number tested	Antigen released (ng/ml; average and SE)
Buffered HBSS + 10% autologous serum [†]		
Yeast plus neutrophil	3	7900 (3089)
Yeast alone	3	138 (131)
Pre-opsonised yeasts in buffered HBSS [‡]		
Yeast plus neutrophil	6	1741 (409)
Yeast alone	6	337 (51)
Buffered HBSS without serum [†]		
Yeast plus neutrophil	5	634 (173)
Yeast alone	5	215 (79)

*Neutrophils were obtained from three normal volunteers.

[†]Antigen release was monitored after incubation for 1 h.

[‡]In four cases, antigen release was monitored after incubation for 1 h. For the remaining two samples, incubation was for 1.5 and 2 h respectively.

Table 2. Release of solubilised *C. albicans* antigens by neutrophil metabolites

Cells in initial incubation	<i>C. albicans</i> antigen (ng/ml; average and SE)*			
	Number of tests	Initial supernate	Number of tests	Final supernate
Pre-opsonised yeasts plus neutrophils	7	547 (61)	8	1130 (58)
Latex beads plus neutrophils	8	0	4	1290 (124)
Neutrophils	8	0	3	485 (28)
Pre-opsonised yeasts	8	102 (9)	4	211 (22)

*After incubation for 10 min in buffered HBSS, pre-opsonised yeasts and neutrophils, latex beads and neutrophils, pre-opsonised yeasts alone, or neutrophils alone were removed. Fresh, pre-opsonised yeasts were added to the recovered supernate and incubated for a further 30 min. Antigen levels were assigned by EIA with CACP antigen to create a standard curve [3].

the neutrophil phagolysosomes (Fig. 1). Table 2 shows that during phagocytosis, neutrophils released metabolites which could solubilise antigens from non-phagocytosed yeasts. A further 583 ng of *C. albicans* antigen/ml was released when fresh yeasts were incubated in a supernate derived from a short incubation of yeasts with neutrophils. When fresh yeasts were incubated in a supernate from a comparable incubation of latex beads plus neutrophils, a further 1290 ng of *C. albicans* antigen/ml was released. Centrifugation conditions used to obtain initial supernates (8800 g, 3.5 min) also resulted in a release of neutrophil metabolites in the absence of phagocytosis, as incubation of fresh yeasts in supernate from neutrophils alone released an additional 485 ng of *C. albicans* antigen/ml. In comparison, only 109 ng/ml was released when yeasts were incubated in a supernate from yeasts initially incubated alone.

Mol. wt of C. albicans antigens released

Whether *C. albicans* antigens were released in degraded form was determined by using the CACP antigen EIA to quantify the amount of antigen able to pass through a concentration filter with a mol. wt cut-off of 10 kDa. Only 11% of EIA-reactive material in

the supernate after incubation of pre-opsonised yeasts plus neutrophils for 1 h was < 10 kDa in size.

Immunoblotting

Because of the small amount of *C. albicans* antigen released into the supernate following neutrophil phagocytosis of pre-opsonised yeasts, it was necessary to concentrate the supernates before immunoblotting. Immunoblotting with rabbit anti-CACP antigen antiserum highlighted a greater proportion of higher mol. wt immunoreactive material in yeast plus neutrophil supernate than in the CACP antigen preparation (Fig. 2). These immunoreactive components appeared as heavy, diffuse bands with mol. wts between 50 and 100 kDa. Antigens of the same mol. wt range appeared when the yeast supernate was tested, but fewer antigens were stained and the staining was less intense. A number of bands corresponding to cross-reactive neutrophil antigens appeared in the lane corresponding to latex bead plus neutrophil supernate. Immunoblotting with rabbit antiserum to *C. albicans* cell-wall mannan (Fig. 3) highlighted the same range of high mol. wt antigens in yeast plus neutrophil supernate as observed with rabbit anti-CACP antigen antiserum (Fig. 2), although a different banding pattern was

Fig. 2. Immunoblot of concentrated supernates with rabbit anti-CACP antigen antiserum. **C**, CACP antigen control. Equal volumes of concentrated supernates obtained after incubation for 1.5 h were added to remaining lanes: **Y + N**, pre-opsonised yeasts plus neutrophils; **Y**, yeasts alone; **LB + N**, latex beads plus neutrophils.

Fig. 3. Immunoblot of concentrated supernates with rabbit antiserum to *C. albicans* cell-wall mannan. Lanes and experimental conditions were as in Fig. 2.

present. Again, some cross-reacting material was observed in the supernate of latex beads incubated with neutrophils.

Concentrated supernates were also immunoblotted with mouse monospecific antisera against the 38- and 44-kDa CACP antigens (Fig. 4). The EIA-determined titre of antiserum from a single mouse immunised with the 38-kDa antigen was 1 in 800, while titres of antisera from three mice immunised with the 44-kDa antigen were 1 in 3200, 1 in 6400 and 1 in 12 800. Blotting with a pre-immune serum pool failed to reveal antigens in either the CACP antigen preparation or the concentrated supernates (data not shown). Immunoblotting with the mouse

Fig. 4. Immunoblots with mouse monospecific antiserum against the 38-kDa (**a**) and 44-kDa (**b**) CACP antigens. **C**, CACP antigen control. Equal volumes of concentrated supernate obtained after incubation for 1.5 h were added to the remaining lanes: **Y + N**, yeasts plus neutrophils; **Y**, yeasts alone; **LB + N**, latex beads plus neutrophils; **N**, neutrophils alone; **H**, buffered HBSS.

monospecific antiserum raised against the 38-kDa antigen showed that this antigen was not present in yeast plus neutrophil supernate, although the expected band appeared in the CACP antigen (Fig. 4a). A lower mol. wt band appearing in the yeast plus neutrophil lane was probably a cross-reactive neutrophil antigen, as a band of similar mol. wt was also present in the latex bead plus neutrophil lane. Immunoblotting with pooled monospecific antisera raised against the 44-kDa antigen showed binding with two antigens in CACP antigen; namely a 44-kDa major antigen and a 55-kDa minor antigen (Fig. 4b). Only a 55-kDa antigen was present in yeast plus neutrophil supernate. No reactive antigens were present in yeast-alone or neutrophil-alone supernates.

Discussion

Although the neutrophil preparations in the present study contained low levels of contaminating T and B lymphocytes and eosinophils, these cells play only a minor role in the phagocytosis and killing of *C. albicans* compared to neutrophils [15, 16]. Small numbers of monocytes were also present and these cells are efficient in the phagocytosis and killing of *C. albicans* yeasts [15–19]. However, as neutrophils comprised >70% of the phagocytic cells in the preparations, it was expected that the majority of *C. albicans* antigens were released because of neutrophil action.

When serum was present in the medium, phagocytosis was rapid and yeasts were visible inside neutrophils within a few minutes. Evans [20] has shown that a single phagocytic event can occur in as little as 2 s once a yeast cell and a neutrophil are in contact in a medium containing serum. When equal numbers of yeasts and neutrophils are incubated together, virtually all yeasts are phagocytosed by 15 min [9, 15]. Furthermore, neutrophils can ingest 7–11 yeasts/neutrophil during a incubation for 1 h in a medium containing serum 10% [8, 9, 20–22]. In the present system, neutrophils each ingested an average of 1.4 pre-opsonised yeasts, representing *c.* 15% of the total yeasts present. The fact that each neutrophil ingested only one or two pre-opsonised yeasts is probably because pre-opsonised yeasts were taken up more slowly than yeasts in the presence of serum, as a result of the washing step following opsonisation removing some opsonising serum factors from the yeast surface. It should also be noted that to ensure that phagocytosis was not limited by the number of yeasts present, a 10-fold excess of yeast cfu over neutrophils was used, resulting in a smaller percentage of yeasts being phagocytosed compared to the other studies discussed above.

When yeasts were incubated with neutrophils in a medium containing serum, substantial release of *C.*

albicans antigen occurred within 15 min (Fig. 1). When pre-opsonised yeasts or untreated yeasts were used, release of *C. albicans* antigen was slightly slower and did not reach the same level as with serum present (Fig. 1; Table 1). However, in either case it seems unlikely that the rapid release of antigen observed could occur from yeasts within intact neutrophil phagolysosomes. An alternative scenario is that surface antigens were released both from yeasts being phagocytosed and from the large number of external yeasts not yet undergoing phagocytosis. Surface solubilisation of yeast antigens probably begins immediately upon initiation of phagocytosis, as neutrophil degranulation occurs, resulting in the release of granule contents into the developing phagolysosome [23]. At this point, neutrophil metabolites and quickly solubilised yeast surface antigens are free to diffuse into the surrounding medium. Furthermore, 20–40% of human neutrophil phagolysosomes containing ingested yeasts maintain an open channel to the outside of the cell [24–26]. This means that granule contents and solubilised antigens from ingested yeasts can continue to diffuse into the surrounding medium. This idea is supported by the finding that components of neutrophil oxygen-dependent reactions are detectable in the medium surrounding stimulated neutrophils [27, 28]. These extracellular metabolites, including enzymes such as α -mannosidase [29], can then cause release of surface antigens from surrounding unphagocytosed yeasts.

Immunoblotting of concentrated yeast plus neutrophil supernate with rabbit polyvalent anti-CACP antiserum gave heavy, diffuse bands in the same mol. wt range as antigens in a yeast-alone supernate (Fig. 2). The yeast cell wall contains glycoproteins, particularly mannoproteins, some of which are loosely bound, and can be released and detected in culture media [2, 30]. Consequently, it seemed likely that the densely staining, diffuse antigen bands in the yeast plus neutrophil supernate as well as corresponding bands in the yeast-only supernate were cell-wall mannoproteins. Immunoblotting with anti-*C. albicans* cell-wall mannan antiserum (Fig. 3) provided additional evidence for this. The fact that the anti-CACP antigen antiserum and anti-mannan antiserum were each able to stain the yeast surface strongly in an immunofluorescence assay (unpublished data) confirms that both antisera were able to recognise yeast surface antigens. It is perhaps not surprising that the rabbit antibodies raised against the CACP antigens can react with yeast surface antigens, as cytoplasmic extracts prepared from mechanically disrupted yeasts are known to contain cell-wall glycoproteins [2, 3]. This, taken together with the immunoblot and surface-reactivity results, suggests that mannoproteins were rapidly solubilised from the yeast cell wall when yeasts were incubated with neutrophils.

Mouse monospecific antisera against the 38- and 44-

kDa CACP antigens were also employed to obtain evidence that *C. albicans* surface antigens were released upon interaction with neutrophils. As both the 38-kDa and 44-kDa CACP antigens used to prepare the mouse antisera were derived from a yeast cytoplasmic extract that had been subjected to two-dimensional SDS-PAGE [3], these antisera reacted well with the appropriate antigen in immunoblots of the control CACP antigen. Immunoblotting with antiserum raised against the 38-kDa antigen revealed no bands in the yeast plus neutrophil supernate or in the yeast-only supernate which were unique compared to the neutrophil control supernate. In contrast, immunoblotting with the mouse antiserum to the 44-kDa antigen highlighted a 55-kDa antigen in only the yeast plus neutrophil supernate. As the antiserum to the 44-kDa antigen also reacted with a 55-kDa antigen present in minor amounts in the CACP antigen preparation, the 55-kDa antigen may be a glycosylated version of the 44-kDa antigen, which is exported to and incorporated into the yeast cell wall, and released into the medium by neutrophil enzymes. If this is the case, the 55-kDa antigen is apparently not loosely bound on the yeast cell surface, as this antigen was not detected in immunoblotting of the yeast-only supernate.

The results of the present study support the hypothesis that interaction of yeasts with neutrophils results in rapid release of surface antigens from yeasts that are being phagocytosed and from yeasts that are in the vicinity of activated neutrophils. The evidence that yeast surface antigens such as mannoproteins are actively released following interaction with neutrophils supports our contention that such antigens should be targeted by assays designed to detect yeast antigens in the peripheral blood of patients with invasive infection. In this regard, it should be noted that the rabbit anti-CACP antibodies used in the EIA for invasive candidosis described earlier [3] probably already do this to an extent, as these antibodies can react with yeast surface antigens as shown by their ability to give a positive surface fluorescence antibody test with intact yeasts (unpublished data). Through defining the spectrum of yeast surface antigens released by neutrophil metabolites and recognised by the rabbit anti-CACP antiserum, we anticipate being able to develop a more sensitive and specific monoclonal antibody-based assay for the detection of *C. albicans* antigens released during invasive infection.

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