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Kinetics of Phagocytosis and Intracellular Killing of *Candida albicans* by Human Granulocytes and Monocytes

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The study of the phagocytosis and intracellular killing of *Candida albicans* by granulocytes and monocytes has been hampered by the budding and pseudomycelium formation of this yeast during a relatively short incubation period at 37°C and by the similar density of candida cells and phagocytes, which makes differential centrifugation impossible. In the present study, *C. albicans* was used after 5 days of preculture at 30°C, after which the number of candida cells remained constant during incubation at 37°C for 90 min. On this basis, phagocytosis and intracellular killing were limited to a period of 60 min. Phagocytosis of *C. albicans* by granulocytes and monocytes was measured with a hemocytometer, the number of extracellular candida being a measure of the ingestion of these microorganisms. After 60 min, 96% of the candida cells were ingested by normal human granulocytes and monocytes. This process was dependent on the opsonin concentration and temperature and was inhibited by mono-iodoacetic acid. Heat-inactivated serum was less active than fresh serum, reflecting the role of complement factors with respect to opsonization. Intracellular killing was measured by a microbiological assay. After 60 min of incubation of phagocytes together with *C. albicans* and serum, human granulocytes and monocytes killed 58 and 50% of the ingested candida, respectively. This process was inhibited by phenylbutazone. Phagocytes from patients with chronic granulomatous disease showed impaired intracellular killing.

Phagocytosis and intracellular killing of microorganisms by granulocytes and monocytes can be determined independently if the phagocytes can be separated from the noningested microorganisms after a period of phagocytosis (10). Because the density of *Candida albicans* is almost the same as that of granulocytes and monocytes, it is impossible to use differential centrifugation for this purpose.

Before microorganisms can be ingested by phagocytes, they must be opsonized by serum factors, but incubation of *C. albicans* with serum for even a short period leads to growth by budding and pseudomycelium formation. This causes clumping of the candida cells, which hampers accurate counting by a microbiological method. Therefore, killing of *C. albicans* is usually determined by the dye exclusion method of Lehrer and Cline (6), with the number of yeast cells stained with methylene blue after lysis of the phagocytic cells representing the intracellularly killed candida and the viable yeast cells being unstained. In a modification of this method described by Schmid and Brune (7), the methylene blue stains dead candida within intact phagocytic cells. The disadvantage of both methods is that they involve subjective microscopic evaluation.

In the present study a technique based on the original Maaløe method (O. Maaløe, Ph.D. thesis, Copenhagen, 1946) was developed to measure phagocytosis and intracellular killing of *C. albicans* by human granulocytes and monocytes.

MATERIALS AND METHODS

C. albicans. *C. albicans* (UC 820), held on an agar slant at 4°C and transferred every 14 days, were inoculated into 100 ml of nutrient broth no. 2 (code CM67, Oxoid, Ltd., London) containing 1% (wt/vol) glucose and cultured for 7 to 8 days at 30°C. After harvesting by centrifugation for 10 min at 1,500 × *g*, the pellet was washed twice with phosphate-buffered saline (pH 7.2) (hemagglutination Sörensen; Difco, Detriot, Mich.). The number of candida cells present in a suspension was determined in a hemocytometer with bright light; a candida unit was defined as a single yeast particle or a string of particles containing two or more yeast cells.

For the assays of phagocytosis and killing, a yeast suspension containing about 10^7 candida units per ml was prepared in Hanks buffered salt solution containing 0.1% (wt/vol) gelatin.

Viability of the suspended candida was tested by mixing equal volumes of the suspension and 4.10^{-4} M methylene blue in distilled water; after incubation for 3 to 5 min at room temperature, the number of stained (i.e., dead) candida was determined in a Bürker hemocytometer.

Cells. Leukocytes were obtained from heparinized blood derived from normal blood donors. A polymorphonuclear cell suspension was made by the dextran sedimentation method described elsewhere (10). After counting in a Bürker hemocytometer, a suspension of 1.0×10^7 granulocytes per ml in Hanks balanced salt solution containing 0.1% (wt/vol) gelatin was made; in this suspension >95% of the leukocytes were granulocytes. Blood monocytes were obtained by differential centrifugation of heparinized blood on a Ficoll-Hypaque gradient (1). The monocyte-lymphocyte layer was harvested and washed twice with buffered saline containing 0.5 U of heparin per ml, after which a cell suspension of about 1.0×10^7 to 1.5×10^7 monocytes per ml, 3.5×10^7 to 4.0×10^7 lymphocytes per ml, and about 5.0×10^5 granulocytes per ml in Hanks balanced salt containing 0.1% (wt/vol) gelatin was prepared.

To prevent attachment of monocytes to the glass surface, all experiments with these cells were done with siliconized glassware; the experiments with granulocytes were done in capped plastic tubes (16 by 100 mm; Falcon Plastics, Los Angeles, Calif.).

Opsonins. For opsonization of the *C. albicans*, serum prepared from blood of healthy donors with blood group AB was used. This serum was stored in 2-ml portions at −20°C.

Phagocytosis. Phagocytosis was performed by incubating 1 ml of candida suspension (10^7/ml) with 1 ml of the granulocyte or monocyte suspension (10^7 phagocytes per ml) and 0.2 ml of serum at 37°C under rotation (4 rpm). The number of extracellular yeast cells lying totally free in the suspension, as determined in a Bürker hemocytometer, was taken as the measure of phagocytosis. At given times a 0.1-ml sample of the candida-leukocyte suspension was taken and incubated with 0.1 ml of 3% acetic acid in distilled water for 3 min (to lyse the remaining erythrocytes and thus facilitate accurate counting of the candida), and the number of extracellular yeast cells was determined in a hemocytometer. Yeast cells attached to the surface of phagocytic cells were considered to be ingested, since we could not accurately determine whether these candida cells were still extracellular or ingestion had begun.

Intracellular killing. The decrease in the total number of viable candida cells at given times was taken as the measure of intracellular killing of *C. albicans*. Since phagocytosis of *C. albicans* by both granulocytes and monocytes is rapid, the number of extracellular microorganisms decreases rapidly, and, therefore, the course of the total number of viable candida cells is a good measure of the rate of intracellular killing.

The total number of viable candida within phagocytic cells could be determined only after lysis of the phagocytes. When granulocytes were used, this was done by adding to a cell sample an equal volume of distilled water containing 0.01% (wt/vol) albumin and mixing for 30 s; lysis of monocytes was performed by adding an equal volume of distilled water containing 0.01% (wt/vol) albumin to the cell sample and repeatedly alternating freezing in liquid nitrogen (−170°C) and quick thawing in a water bath at 37°C three times. The number of viable candida units was measured by plating serial dilutions of the lysate in phosphate-buffered saline (pH 7.2) on diagnostic sensitivity medium agar plates (Oxoid, Ltd., London). After incubation of the plates for 18 h at 37°C, the number of colony-forming units was determined as a measure of the number of viable candida cells.

Inhibitors. Mono-iodoacetic acid (BDH Laboratory, Poole, England) was used to inhibit phagocytosis (3). A stock solution (10 mM) was prepared with phosphate-buffered saline and stored at 4°C, and a final concentration of 1 mM was used in the tests. Phenylbutazone (Butazolidine, Ciba-Geigy, Switzerland) was used as an inhibitor of intracellular killing (8) in concentrations of 0.5 and 1 mM. Stock solutions of 10 mM phenylbutazone in Hanks buffered salt solution were prepared freshly before use.

Calculations. The amount of phagocytosis that took place after a certain period of time is expressed as the percentage of decrease in the initial number of extracellular candida counted in a hemocytometer. Intracellular killing after a certain period of time is expressed as the percentage of decrease of the initial number of colony-forming units determined by a microbiological assay. Values represent the mean and standard deviation of at least four experiments.

RESULTS

Effect of serum on growth of *C. albicans*. When a suspension of 5.10^6 candida cells per ml incubated overnight in broth at 37°C was reincubated with 10% AB serum for 1 to 3 h at 37°C under rotation (4 rpm), the number of candida units determined with a hemocytometer or by a microbiological assay decreased rapidly (Table 1). However, after incubation of the candida for 5 days in broth at 30°C, the number of candida cells remained constant for at least 90 min when reincubated with serum at 37°C (Table 1). These results led us to use candida grown for at least 5 days at 30°C for phagocytosis and killing tests over a period of 60 min.

Phagocytosis. Incubation of *C. albicans* in the presence of serum under slow rotation (4 rpm) showed that the phagocytosis of candida cells by both granulocytes and monocytes is a rapid process: after 15 min, less than 15% of the initial number of candida was still extracellular (Fig. 1). Incubation of *C. albicans* for 60 min with 10% serum, but without phagocytic cells, gave a slight increase, indicating that there was no detectable clumping of extracellular candida cells (Fig. 1).

TABLE 1. *Influence of incubation on C. albicans*[a]

Second incubation[b] (min)	First incubation after: 18 h at 37°C (colony count, ×10⁶ cells/ml)	5 days at 30°C: Hemocytometer count (×10⁶ cells/ml)	5 days at 30°C: Colony count (×10⁶ cells/ml)
0	4.2 ± 0.2	4.4 ± 0.1	4.3 ± 0.1
60	3.8 ± 0.5	4.2 ± 0.1	4.3 ± 0.3
90	1.5 ± 0.2	4.2 ± 0.3	4.1 ± 0.4
120	1.4 ± 0.5	3.4 ± 0.6	3.4 ± 0.4
180	0.6	0.4	0.8

[a] Values represent means of eight experiments.
[b] At 37°C under rotation (4 rpm) with 10% AB serum.

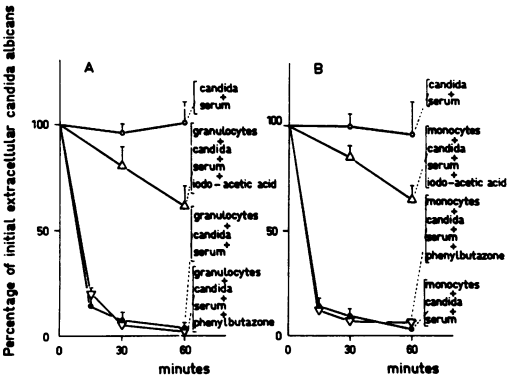

FIG. 1. *Phagocytosis of C. albicans by granulocytes (A) and monocytes (B) in the presence of serum. The effects of mono-iodoacetic acid and phenylbutazone are also shown, as well as the growth curve of candida in the presence of serum without cells.*

If 1 mM mono-iodoacetic acid was added to the candida-phagocyte suspension, phagocytosis was markedly inhibited; after 60 min only 40% of the candida were ingested (Fig. 1), whereas, without this compound, phagocytosis amounted to 96% at 60 min. Higher concentrations of the drug could not be used because its candidacidal activity, a concentration of 2.5 mM, gave a decrease of 30 to 40% in the number of viable candida after incubation for 1 h at 37°C.

To investigate whether extracellular killing occurs in the supernatant, a supernatant free of candida and phagocytic cells was prepared after 60 min of phagocytosis. Incubation of this supernatant with *C. albicans* for 1 or 2 h at 37°C under rotation showed no decrease in the number of candida units, thus indicating that no candidacidal activity was present (Table 2).

From these results it may be concluded that the decrease in the number of extracellular candida is due solely to ingestion by phagocytic cells.

Effect of the serum concentration on the rate of phagocytosis. Incubation of *C. albicans* and granulocytes or monocytes at 37°C in the presence of serum in various concentrations, as well as without serum, showed that phagocytosis did not occur in the absence of serum and that a high rate of ingestion was reached with 5% serum or more; in the range of 0 and 5%, the rate of phagocytosis was dependent on the concentration (Fig. 2). When heat-inactivated serum (30 min, 56°C) was used rather than fresh serum, the rate of phagocytosis was lower than that found for the similar concentration of fresh serum.

Effect of temperature on the rate of phagocytosis. Phagocytosis proved to be a tempera-

TABLE 2. *Candidacidal activity of supernatant prepared after 60 min of incubation of phagocytes and candida*

Incubation[a] time (min)	Supernatant[b]: Medium	Granulocytes	Monocytes
0	4.3 × 10⁶	4.2 × 10⁶	4.2 × 10⁶
60	4.3 × 10⁶	4.7 × 10⁶	3.6 × 10⁶
120	3.7 × 10⁶	4.0 × 10⁶	3.4 × 10⁶

[a] Incubation of supernatant with 4.3 × 10⁶ candida per ml at 37°C under rotation (4 rpm).
[b] After 60 min of incubation of medium (Hanks balanced salt solution and 10% serum) plus candida, medium plus candida and granulocytes, or medium plus candida and monocytes at 37°C under rotation (4 rpm), cell- and candida-free supernatants were prepared by centrifugation for 10 min at 1,500 × *g* and filtration through a membrane filter (Millipore Corp.; pore size, 0.45 μm).

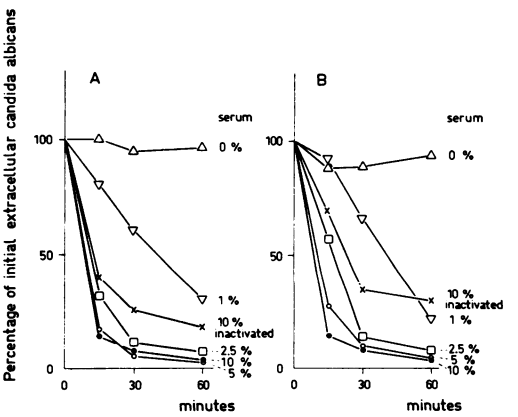

FIG. 2. *Effect of various concentrations of serum and inactivated serum on the phagocytosis of C. albicans by granulocytes (A) and monocytes (B).*

ture-dependent process: incubation at 4°C gave only 15.3% phagocytosis of candida by granulocytes, whereas at 22°C phagocytosis accounted for 87.1%, which was still lower than the ingestion at 37°C, amounting to 96%. The effect of temperature on the rate of phagocytosis by monocytes proved to be the same as for granulocytes with no phagocytosis at 4°C and with reduced phagocytosis at 22°C, i.e., 82.2% compared with 96% at 37°C.

Intracellular killing. Phagocytosis of *C. albicans* by normal granulocytes or monocytes was very fast: more than 80% were ingested within the first 15 min, and only 16% were ingested during the next 45 min (Fig. 1). Thus, the total number of viable candida determined after lysis of the phagocytic cells mainly represented the number of viable intracellular candida and may be considered a good measure of the intracellular killing of the candida. Incubation of candida, granulocytes, and serum at 37°C gave a reduction of the number of viable candida units amounting to 58.8 ± 8.2% (standard deviation) at 60 min, and monocytes gave 50.6 ± 6.6% at 60 min. Incubation of candida with serum but without phagocytes gave no decrease at 60 min (Fig. 3).

Ultrasonic treatment of the samples after lysis to obtain single candida cells led to a similar reduction of the total number of candida colonies, which means that a decrease in the number of viable candida was not due to clumping of the candida within the phagocytic cells, but to intracellular killing (Table 3).

Incubation of candida, phagocytes, and serum with 1 mM phenylbutazone led to a marked decrease in intracellular killing (only 14% at 60 min, compared with 58.8% in the absence of this compound) (Fig. 3), but had no effect on phagocytosis (Fig. 1). This experiment proved that a decrease in the number of viable candida cells determined by the microbiological assay is a reliable measure of the intracellular killing of *C. albicans* by granulocytes and monocytes.

Phagocytosis and intracellular killing by granulocytes and monocytes of patients with a chronic granulomatous disease. The phagocytosis and intracellular killing of *C. albicans* by the granulocytes and monocytes of three patients with chronic granulomatous disease were studied. Phagocytosis was normal in all three cases, amounting to 98.3, 95.4, and 98.1% at 60 min for the granulocytes and 96.8 and 98.7% for the monocytes. Determination of the total number of viable candida after lysis of the cells showed that intracellular killing by both granulocytes and monocytes was impaired at 60 min (Fig. 4).

DISCUSSION

When incubated with normal serum for a relatively short period, *C. albicans* shows bud-

TABLE 3. *Effect of ultrasonic treatment on the number of candida colony-forming units after lysis of the phagocytic cells*

Ultrasonic treatment[a] (s)	Killing index[b]	
	Granulocytes	Monocytes
0	57.3	50.2
10	56.2	47.3
30	63.8	44.9

[a] Treatment was at an amplitude of 5 μm with a 100-W MSE ultrasonic disintegrator with a microprobe.

[b] Expressed as the percentage of decrease of the initial number of colony-forming units after 60 min of incubation of 10^7 candida per ml and 10^7 granulocytes or monocytes per ml with 10% AB serum. Values represent means of three experiments.

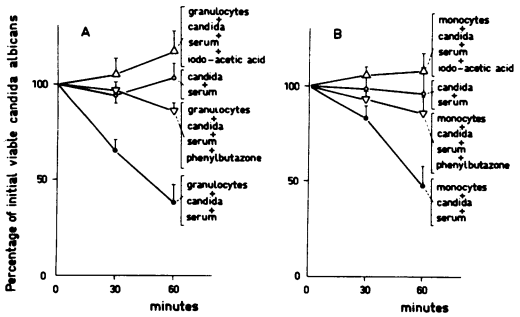

FIG. 3. *Intracellular killing of C. albicans by granulocytes (A) and monocytes (B) in the presence of serum. The effects of mono-iodoacetic acid and phenylbutazone are also shown, as well as the growth curve of candida in the presence of serum without cells.*

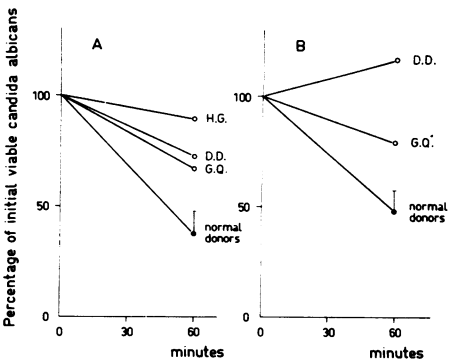

FIG. 4. *Intracellular killing of C. albicans by granulocytes (A) and monocytes (B) from patients with a chronic granulomatous disease.*

ding of the cells and pseudomycelium formation (2, 6), both of which hamper accurate counting of the yeast cells. Overnight incubation at 37°C and reincubation at 37°C leads to a marked drop in the number of candida counted at 60 min. However, when the first incubation is carried out at 30°C for at least 5 days, reincubation of the candida with serum at 37°C gives a constant number of candida for a period of 90 min. Therefore, assessment of phagocytosis and killing on the basis of candida cell counts can be performed over a 60-min period if the candida have been precultured at 30°C for more than 5 days.

The measurement of phagocytosis as a reduction in the number of extracellular candida evaluated by microscopic observation is easy and rapid to perform. Since incubation of candida with serum alone showed no decrease in the number of candida cells within 60 min, it may be concluded that phagocytosis itself is measured without interference from clumping of the candida cells. Phagocytosis of candida by granulocytes and monocytes measured with our method was found to be a rapid process.

Methods described earlier also used suspensions of candida and phagocytic cells, but they measured phagocytosis on the basis of the number of intracellular candida in smears made from suspensions (4, 6) or determined the uptake of candida by glass-attached granulocytes and/or monocytes (6, 7). All of these methods showed that the ingestion of candida is a rapid process.

In hemocytometer counting, it is difficult to distinguish between ingested candida and those attached to phagocytes. However, since incubation with 1 mM mono-iodoacetic acid, which inhibits the ingestion process, gives a decrease of only 35% of the initial number of extracellular candida compared with 96% in the absence of this compound, it may be concluded that a decrease due to attachment alone is only a minor part of the total decrease in the number of extracellular candida. It may therefore be concluded that the decrease in the number of unattached candida in the suspension represents phagocytosis.

The results also show that phagocytosis of *C. albicans* by granulocytes and monocytes is dependent on the concentration of serum in the incubation medium. The fact that heat-inactivated serum is less effective indicates that complement is necessary for optimal opsonization, which is in accordance with the findings of others (6, 7).

The determination of intracellular killing of candida by a microbiological technique, as described here, offers a reliable and rapid method for the evaluation of the functioning of granulocytes and monocytes and provides information about the rate of this process. Ultrasonic treatment of the cell lysates shows that the decrease in the number of colony-forming units is not due to intracellular clumping of candida. Proof that the decrease in the number of colony-forming units is due to intracellular killing is provided by the experiments in which phenylbutazone was added, since this compound gives almost complete inhibition of the intracellular killing of microorganisms (7). Furthermore, this is confirmed by the impaired killing of *C. albicans*, a catalase-positive species, by granulocytes and monocytes of patients suffering from chronic granulomatous disease because phagocytes of such patients cannot kill catalase-positive microorganisms (6).

The microbiological assay for the determination of the rate of intracellular killing in cell suspensions under standard conditions appears to be more reliable than the dye-exclusion, ghost-staining, or radioactive chromium release methods of others (4–7, 9), because it gives direct measurement of viable candida. The staining methods do not indicate the exact time when candida are killed, and intracellularly digested candida can no longer be observed microscopically. In addition, the measurement of intracellular killing by glass-attached granulocytes or monocytes with a dye-exclusion method gives very low values (6, 7) that probably do not reflect the true physiological functioning of the phagocytes. The measurement of the release of a radioisotope by cells is dependent on so many factors that the method is difficult to standardize for determination of the viability of ingested microorganisms.

The present method for the evaluation of the endocytic functions of granulocytes and monocytes, which is rapid, simple, and reliable, has already led to the detection of functional changes in these cells in patients with chronic mucocutaneous candidiasis.

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