JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1991, p. 2013–2023
0095-1137/91/092013-11$02.00/0

Vol. 29, No. 9

Quantitative Analysis of Opsonophagocytosis and of Killing of *Candida albicans* by Human Peripheral Blood Leukocytes by Using Flow Cytometry

EDITH MARTIN AND SUCHARIT BHAKDI*

Institute of Medical Microbiology, University of Mainz, Hochhaus Augustusplatz, D 6500 Mainz, Germany

Received 19 February 1991/Accepted 11 June 1991

We describe a simple, rapid, automated procedure for measuring opsonophagocytosis and killing of *Candida albicans* by human peripheral blood leukocytes. Yeast cells are labelled by allowing uptake and cleavage of membrane-permeable *bis*-carboxyethyl-carboxyfluorescein pentaacetoxymethylester to its membrane-impermeable fluorescent derivative *bis*-carboxyethyl-carboxyfluorescein. The yeast cells are added to cell-rich plasma obtained after dextran sedimentation of erythrocytes. Opsonophagocytosis and killing are quantified by using automated fluorescent cell analysis, and the following parameters can be obtained: (i) relative percentage of phagocytes that participate in opsonophagocytosis, (ii) relative percentage of yeast cells that become associated with phagocytes, and (iii) percentage of killing of *C. albicans*. The first two parameters are obtained through the additional use of a phycoerythrin-conjugated monoclonal antibody that selectively labels monocytes and polymorphonuclear granulocytes in peripheral blood. Killing is assessed by solubilizing blood cells with deoxycholate to liberate yeast cells from the phagocytes. Viable yeast cells retain carboxyfluorescein, but nonviable cells lose the fluorescent marker; thus, the reduction in number of fluorescent particles directly reflects phagocytic killing. Results obtained by the present method correlated excellently with parallel enumerations by colony counting. Test results with seven healthy individuals revealed a marked dissociation between the process of opsonophagocytosis, which was essentially complete after 20 min at 37°C, and killing rates, which were 48% ± 11% and 63% ± 9% (standard deviation) after 1 and 2 h, respectively, when yeast cell-to-phagocyte ratios were in the range of 0.5:1 to 2:1. The described assay is unrivaled in simplicity, rapidity, and reproducibility and generates results for a large number of samples within hours.

Phagocytic killing is the primary mechanism through which the immune system eliminates gram-positive and serum-resistant gram-negative organisms and pathogenic fungi. Deficiencies in phagocyte function, whether primary or secondary, therefore can result in life-threatening infections with microorganisms that otherwise display relatively low virulence or invasive tendency. The mechanisms underlying opsonophagocytosis are known in great detail, and specialized laboratories can today analyze and identify the causes of phagocytic malfunction at a molecular level. These impressive advances are in contrast to, on the other hand, a basic dilemma relating to the nonavailability of a rapid, simple, and reproducible method for simultaneously measuring opsonophagocytosis at the individual cell level and the extent of killing of the target microorganism in a completely autologous system. Colony counting, the "gold standard" for assaying killing, is slow and cumbersome and requires considerable expertise to be reproducible (42). Opsonophagocytosis can be quantified by assessing the numbers of radiolabelled microorganisms that become cell associated or by measurements of uptake of radiolabelled nucleotides by the target organisms (3, 15, 20, 33, 39, 45, 47). However, the necessity of handling radioisotopes mitigates against wide usage of such methods, and, in addition, quantification of killing in these systems is neither easy nor straightforward. Fluorescein-labelled bacteria have been used as targets; in these cases, adherent or ingested cells can be enumerated microscopically or by measuring total cell-associated fluorescence (27, 30, 31, 41). These assays are elegant but do not assess killing, and they are still not simple enough to become routine tests. Differential staining with acridine orange has been described as a means to quantify phagocytic killing (12, 37, 38, 46). However, this approach harbors some danger of artifacts (5), and results cannot be validated by parallel determinations of numbers of CFU.

Today, the technique originally developed by Lehrer (22–25) or modifications thereof (14, 36, 38) still represent the most widely used assays for measuring phagocytic killing of *Candida albicans*. The basis for these methods is that phagocytes can be solubilized by deoxycholate (DOC) to liberate ingested yeast cells, which then stain differentially with conventional dyes (e.g., methylene blue), depending on whether they are viable or killed. This technique is conceptually elegant but necessitates counting in the microscope.

Ideally, a phagocytosis test should meet the following demands. First, it should be easy to perform, requiring no special skills or handling of hazardous material (isotopes). Second, it should provide quantitative data both on the opsonophagocytic function at the individual-cell level and on the extent of killing of the target cells. Third, data acquisition should be rapid and automated. In this communication, we describe a novel phagocytosis assay that fulfills these criteria by using *C. albicans* cells as targets. The test is conceptually related to the original assay of Lehrer (22, 25) but exploits the use of a vital fluorescent marker instead of differential staining with a conventional dye. Opsonophagocytosis of these microorganisms is essentially complete within 20 min in the presence of human plasma, but the killing process is much slower and is incomplete after 2 h.

Principle of the assay. *C. albicans* cells are labelled by incubation in broth containing *bis*-carboxyethyl-carboxy-

* Corresponding author.

fluorescein pentaacetoxymethylester (BCECF-AM), which diffuses into the cells and is cleaved by cytoplasmic esterases to yield the fluorescent, membrane-impermeable product bis-carboxyethyl-carboxyfluorescein (BCECF) that remains trapped in viable cells (4, 8). To assay for killing, labelled yeast cells are incubated with cell-rich plasma (CRP; obtained by dextran sedimentation of erythrocytes). Blood cells are subsequently lysed with DOC to liberate the yeast cells (22, 25), which fall into the following two populations that are quantified by use of a flow cytometer: (i) fluorescent and viable and (ii) nonfluorescent and nonviable.

To assay opsonophagocytosis, phagocytes are labelled with a monoclonal antibody (MAb) and incubations with the yeast cells are terminated by pipetting aliquots into paraformaldehyde solutions (21). Fluorescence-activated cell analyses generate information on the association of yeast cells with the phagocytes.

MATERIALS AND METHODS

Blood was collected in 2.5 IU of heparin per ml in plastic tubes. Cell-free plasma (CFP) was obtained by centrifugation of blood at 1,800 × *g* for 5 min (4°C). To obtain CRP, erythrocytes were sedimented by the addition of 1 volume of 5% dextran 200 (Serva, Heidelberg, Germany) in saline (autoclaved and sterile filtered) and by incubation at a 45° tilt (37°C, 20 to 30 min). CRP (containing 1×10^6 to 3×10^6 polymorphonuclear leukocytes [PMNs] per ml) was collected and divided into two aliquots. One aliquot received phycoerythrin-conjugated MAb My-7 (Coulter Electronics, Krefeld, Germany) at a final antibody concentration of 5 μg/ml. This antibody is directed against CD 13. Incubation for 30 to 60 min on ice led to selective labelling of monocytes and polymorphonuclear granulocytes. The CRP samples were then centrifuged (300 × *g*, 3 min, 4°C), and the cells were resuspended in an identical volume of CFP that was obtained by centrifugation of CRP. The labelled cells could be kept on ice for 4 to 5 h without detectable alteration of opsonophagocytic function.

A *C. albicans* isolate was obtained from the diagnostic laboratory of the Institute of Medical Microbiology, University of Mainz, Mainz, Germany. Culturing was performed in tryptic soy broth (Difco Laboratories, Detroit, Mich.) containing 20 μM BCECF-AM (Calbiochem-Behring Corp., La Jolla, Calif.) for 8 to 12 h at 37°C. Fluorescence was assessed by microscopy and fluorescence-activated cell analysis. The labelled cells were washed three times with sterile saline before use, suspended in saline at a density of 1×10^7 to 5×10^7 cells per ml, and kept on ice.

Each phagocytosis assay comprised three samples, each spiked with an identical number of yeast cells: (i) CFP (control); (ii) unlabelled CRP (for quantitation of killing); (iii) MAb My-7-labelled CRP (for assessment of opsonophagocytosis). Pilot experiments confirmed that the labelled yeast cells remained fully viable, and no differences in growth rate or colony morphology were detected between the labelled cells and unlabelled cells. We also ascertained that My-7-labelled phagocytes exhibited no loss of cell-associated fluorescence during an incubation period of 2 h at 37°C. The major obstacle we encountered was the development of pseudohyphae after approximately 60 min of incubation of yeast cells in plasma, which was accompanied by the gradual formation of aggregates (28). These processes were deleterious for the assay because they led to erratic reductions in numbers of CFU in the controls. The processes were found to be fully inhibitable by the addition of 0.5 μg of amphotericin B (Squibb Pharma, Vienna, Austria) per ml to the samples (final concentration). At this concentration, amphotericin B did not exert any detectable fungicidal activity within the observation period of 2 h as determined by colony counting; the same observation was recently made by Nugent and Couchot (29). Also, the rate of loss of BCECF from viable fluorescent yeast cells was the same in the presence or absence of 0.5 μg of amphotericin B per ml. Unless otherwise stated, amphotericin B was present in all samples at 0.5 μg/ml.

Phagocytosis assays were initiated by the addition of 1 volume of yeast cells to 10 volumes of CFP and CRP in Eppendorf tubes and incubation at 37°C in a Thermomixer 5436 (Eppendorf Inc., Hamburg, Germany) with shaking at 1,100 rpm. Yeast loads ranged from 0.5 to 3 yeast cells per granulocyte.

To study opsonophagocytosis, 50-μl samples were removed from tubes containing My-7-labelled CRP at different time points, pipetted into tubes containing 16 μl of a 2% (wt/wt) paraformaldehyde solution in phosphate-buffered saline (PBS), and kept on ice. A 0.4-ml volume of PBS was added immediately before fluorescence analysis. To quantify killing, 50-μl volumes were removed from tubes containing CFP and unlabelled CRP, and each was admixed with 50 μl of a 25 mM DOC solution (in saline) for 3 min at room temperature. As earlier noted by Lehrer (22, 25), this procedure quantitatively solubilized blood cells so that the remaining particles were represented by viable and nonviable yeast cells. Ten microliters of the detergent-treated samples was diluted 100-fold in saline, and 20 μl was then plated in duplicate for determination of number of CFU. A 50-μl volume of the detergent-treated sample was diluted with 0.4 ml of PBS and analyzed by flow cytometry.

Fluorescence-activated cell analyses were performed by use of a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany) with computer-assisted evaluation of data (FACScan software). Data were acquired by using an instrument status with a linear data mode for FSC and SSC and a logarithmic data mode for Fl1 and Fl2. Compensation of Fl1 − percent Fl2 and Fl2 − percent Fl1 ranged between 1.0 and 1.8% and 30.0 and 35.0%, respectively. The FSC threshold was 52 for measuring opsonophagocytosis. To acquire data for the killing function, the FCS threshold was raised to 180 and a live gate was set around the yeast cells in the control.

RESULTS

Analysis of the association of yeast particles with phagocytes. Figure 1 depicts the results of fluorescence-activated analysis of labelled *C. albicans* (A and B), My-7-labelled CRP (C and D), and labelled CRP spiked with labelled yeast cells at time zero (E and F). The labelled yeast cells were satisfactorily homogeneous with respect to size and fluorescence intensity. In the fluorescence microscope, the cells were observed to present bright cytoplasmic fluorescence that was confined to a relatively small portion of the intracellular compartment. The nonfluorescent areas were presumably occupied by the cell nucleus and organelles. Over 90% of the yeast cells were fluorescent at the commencement of the experiments. After incubation with MAb My 7, peripheral blood cells presented a normal pattern with regard to size distribution and granularity (Fig. 1C). Cells exhibiting phycoerythrin fluorescence were identifiable as a distinct population without any need of washing or lysis of residual, contaminating erythrocytes. Addition of labelled yeast cells

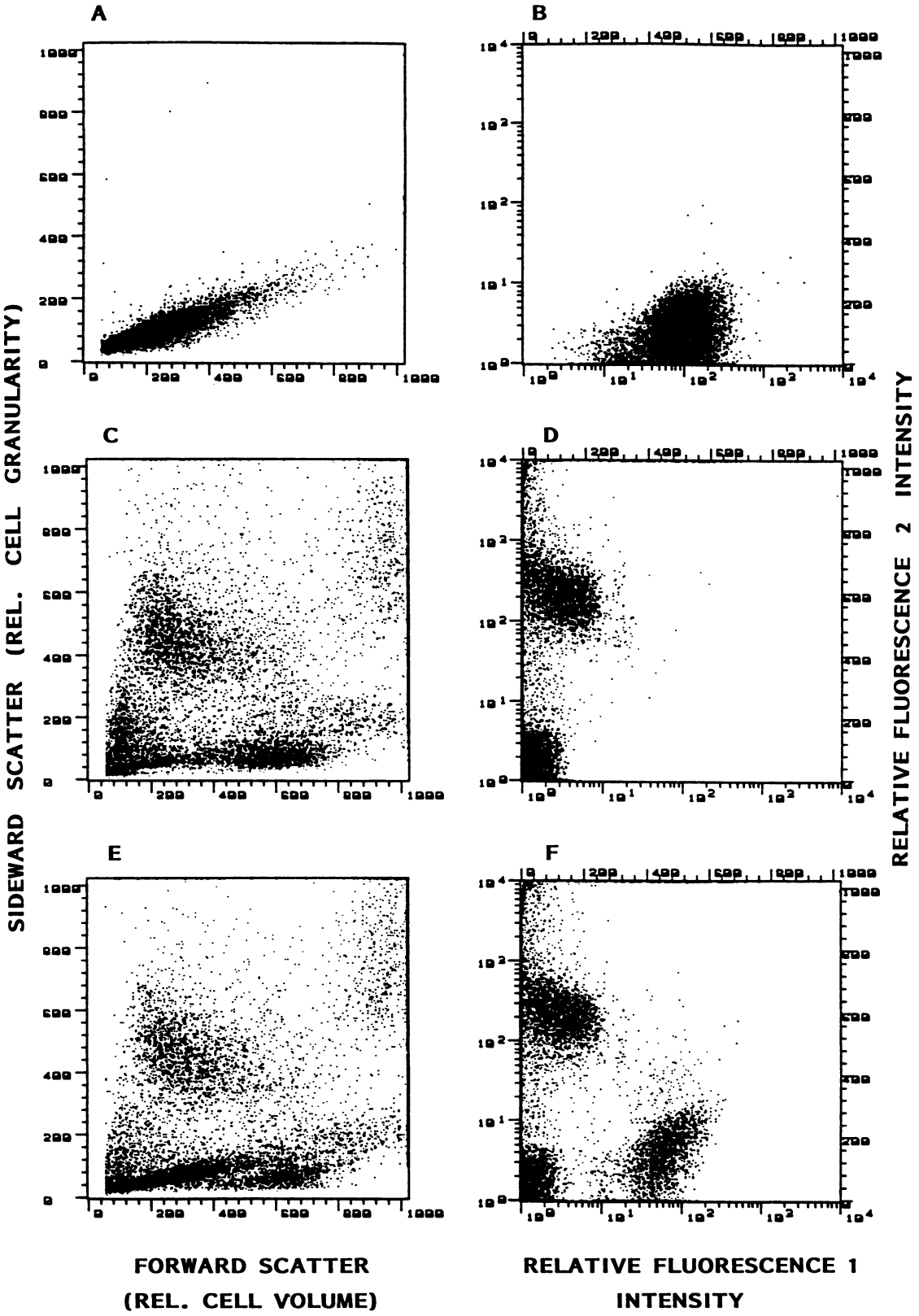


FIG. 1. Flow cytometric analysis of BCECF-labelled *C. albicans* (A and B), CRP after labelling of phagocytes with MAb My-7 (C and D), and an incubation mixture of yeast cells and CRP at time zero (E and F). Panels A, C, and E depict the distribution patterns of the particles analyzed according to forward scatter (relative cell volume) and sideward scatter (relative cell granularity). Panels B, D, and F depict the relative fluorescence intensities: 1, green fluorescence (BCECF); 2, orange fluorescence (phycoerythrin). Fluorescence 1, excitation with a 488-nm argon laser, measurement at 530-nm wavelength; fluorescence 2, same excitation, measurement at 585-nm wavelength.

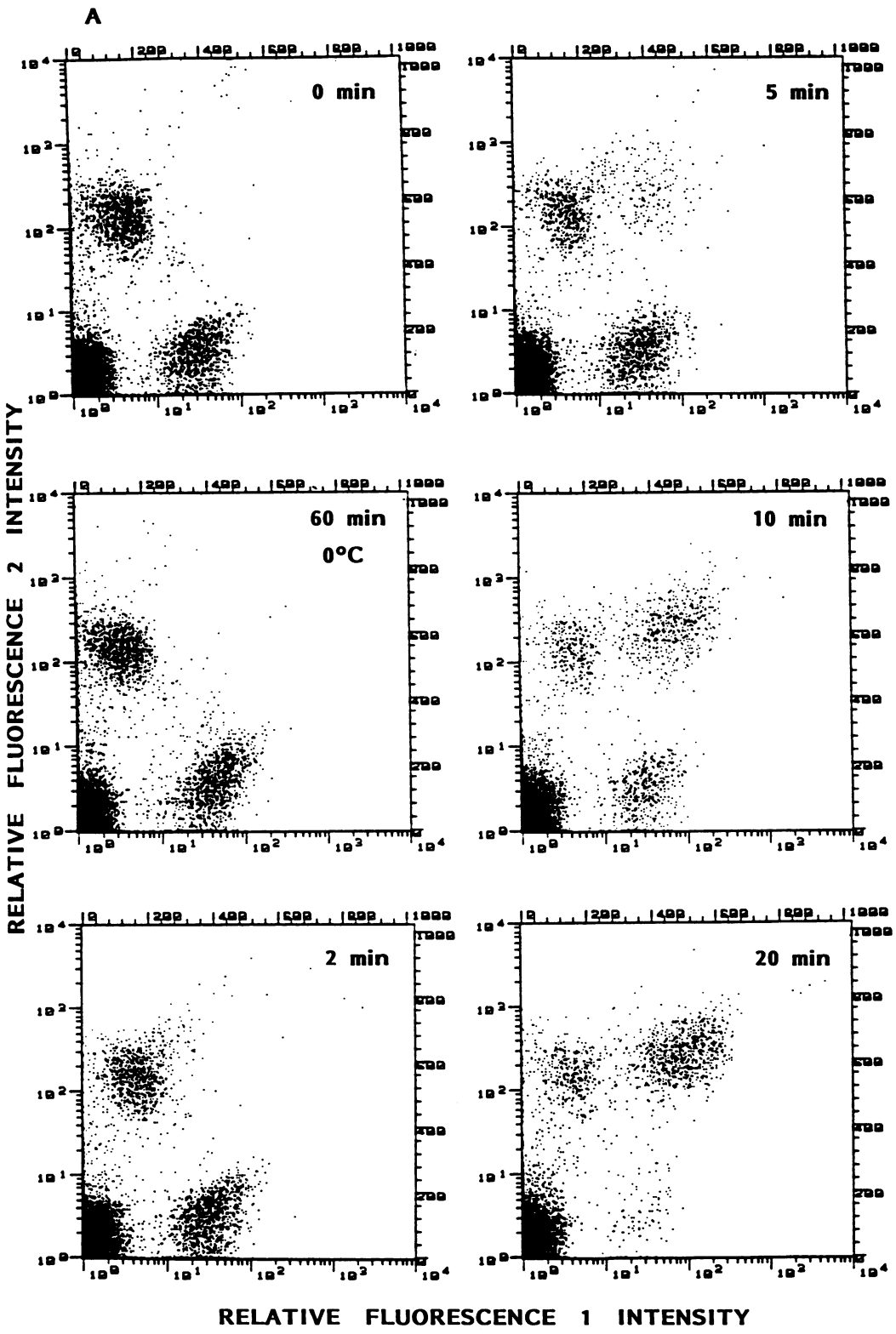


FIG. 2. Flow cytometric analysis of association of BCECF-labelled *C. albicans* (fluorescence 1, green) with blood phagocytes labelled with MAb My-7 (fluorescence 2, orange). The yeast cells were added to CRP at a ratio of approximately 1:1 (yeast cell to phagocyte). A control was kept on ice, and a second sample was incubated at 37°C. At the depicted times, samples were removed for analysis. Ten thousand particles were counted per determination. Note the absence of opsonophagocytosis at 0°C and the rapid association of yeast cells with phagocytes at 37°C that was essentially complete after 20 min.

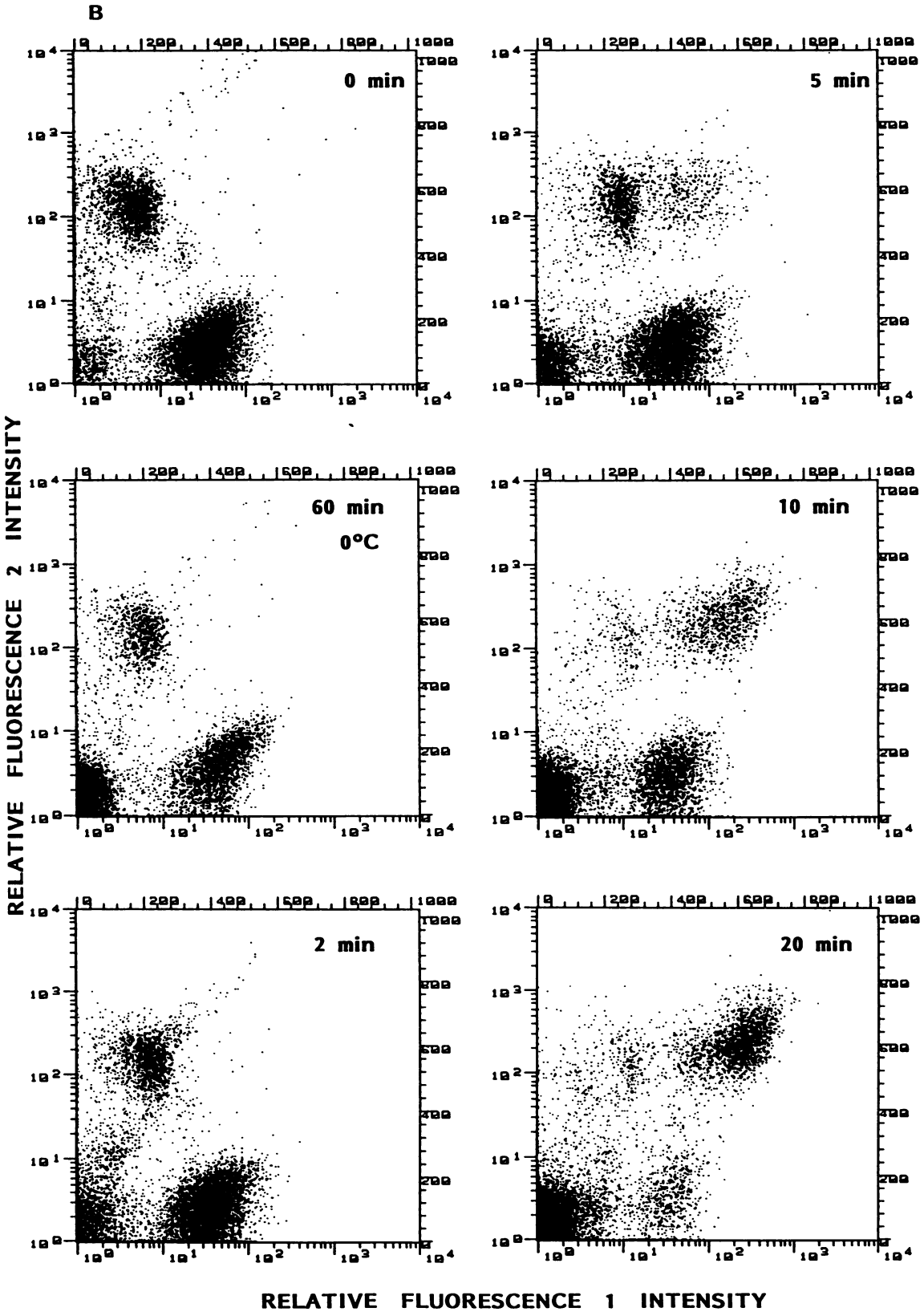
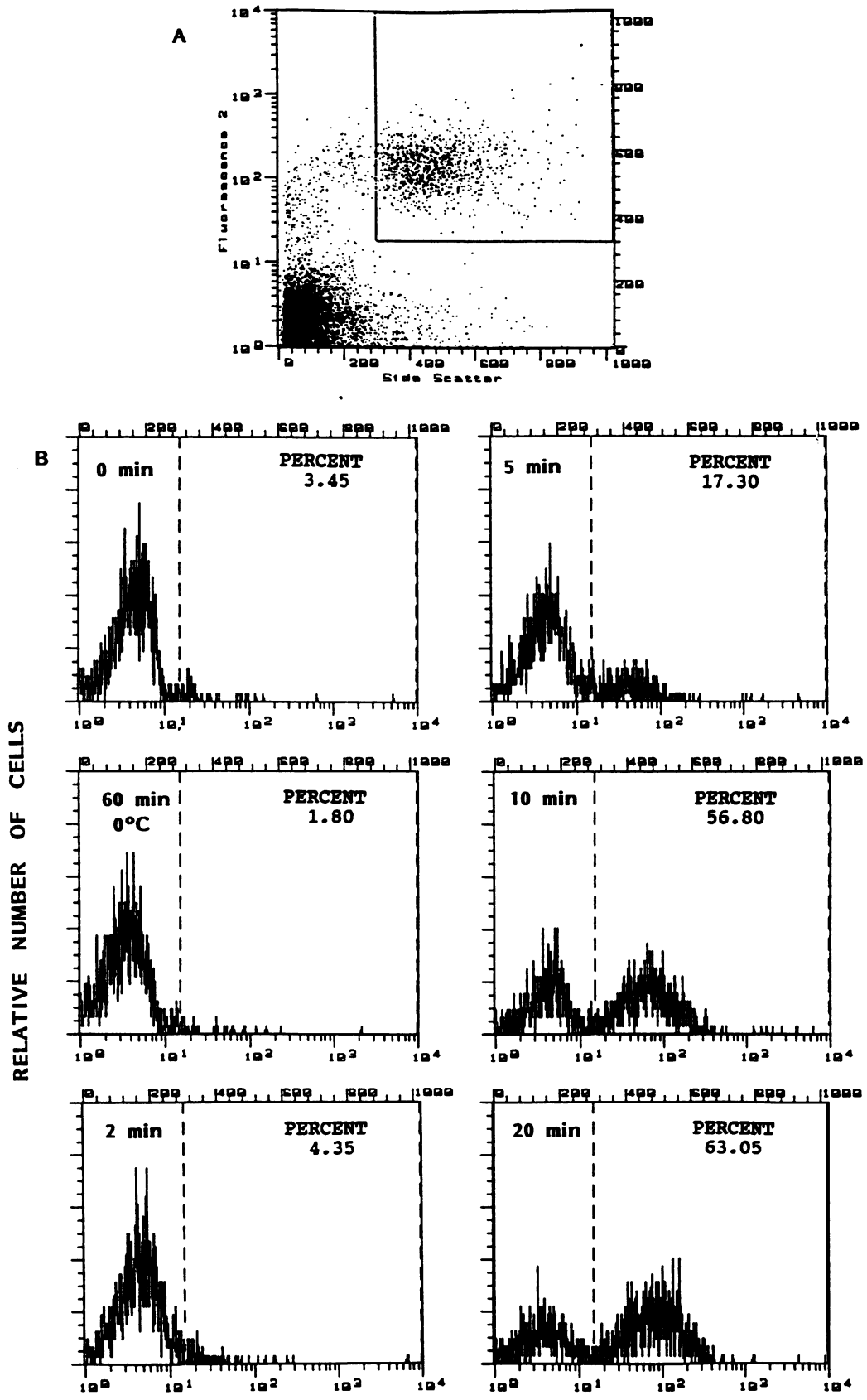


FIG. 3. Flow cytometric analyses of yeast-phagocyte association (as described in the legend to Fig. 2). The yeast cell-to-phagocyte ratio was increased to 3:1. Note the persistence of nonphagocytosing My-7-labelled cells despite the surplus of yeast cells at 20 min.


A
Fluorescence 2
Side Scatter
B
0 min
PERCENT
3.45
5 min
PERCENT
17.30
60 min
0°C
PERCENT
1.80
10 min
PERCENT
56.80
2 min
PERCENT
4.35
20 min
PERCENT
63.05
RELATIVE NUMBER OF CELLS
RELATIVE FLUORESCENCE 1 INTENSITY

to CRP resulted in superpositioning of the above patterns (Fig. 1E), with phagocytes, nonlabelled blood cells, and yeast cells now presenting three easily distinguishable cell populations (Fig. 1F).

Fluorescent yeast cells bound to or phagocytosed by leukocytes caused a shift of the latter from monochromatic (orange) to dual (orange-green) fluorescence. No attempts were undertaken in this study to differentiate extracellular, adherent organisms from ingested particles.

Figures 2 and 3 depict the results of experiments conducted at yeast cell-to-granulocyte ratios of 1:1, evaluated by fluorescence-activated cell analysis. As expected, the shift towards green fluorescence occurred exclusively in the phagocyte cell population. The shift became initially detectable after 5 min of incubation at 37°C, was maximal after 20 min, and remained essentially constant over the following 40 min. Concomitantly, there was a decrease in the number of green yeast particles to virtually nil when PMN-to-yeast cell ratios were 1:1. If required, the relative percentage of yeast cells becoming associated with blood cells could be quantified by observing the reduction of particles in a respective window.

When the yeast cell-to-phagocyte ratio was increased to 3:1, the kinetics of opsonophagocytosis remained essentially the same, but a higher percentage of phagocytes finally bound yeast particles. A small number of nonphagocytosed yeast cells remained detectable, despite the fact that a small percentage of phagocytes was not loaded (Fig. 3). The participation of phagocytes in opsonophagocytosis could be studied at the single-cell level by live-gating the orange, fluorescent particles. In the experiment, 17, 57, and 63% of the phagocytes exhibited a green shift within 5, 10, and 20 min, respectively (Fig. 4). The gating in this experiment was selected to include mainly granulocytes. Specific inclusion or analysis of monocytes is, of course, also feasible.

Of note, at the yeast cell-to-PMN ratio of 1:1, 63% of the PMNs became associated with yeast particles, indicating that yeast cell-phagocyte association occurred randomly. When the yeast load was increased to 3:1, 36, 83, and 85% of the phagocytes exhibited a green shift after 5, 10, and 20 min, respectively (calculated from data of Fig. 3). No association of yeast particles with phagocytes was observed in samples that had been incubated on ice for 60 min (Fig. 2 to 4).

The association with and uptake of yeast cells by phagocytes were readily observable by microscopy. Because the BCECF fluorescence faded rapidly, photographic documentation was not possible, and, as a result, we are limited to written descriptions. At the commencement of the assays, free yeast particles (green), phagocytes (orange), and nonfluorescent cells could be distinguished. After 15 to 20 min at 37°C, no free yeast cells could be detected and most appeared to be located within the phagocytes. In the presence of amphotericin B, there was no formation of pseudohyphae or yeast aggregates in either controls or the CRP samples even after 2 h.

Quantification of killing. Freshly prepared, BCECF-labelled yeast cells withstood treatment with 12.5 mM DOC without detectable alteration of cell-associated fluorescence or viability; the latter was assessed by plating and colony counting. In contrast, blood cells were quantitatively solubilized by the detergent. Therefore, when samples were spiked with yeast cells, only the latter remained detectable after detergent treatment.

When yeast cells were incubated in CFP (controls) for up to 2 h at 37°C, there was only a slight drop in the relative number of fluorescent cells and an appearance of nonfluorescing particles. Treatment of these samples with DOC caused no change in the results (Fig. 5A). In contrast, when samples containing CRP and yeast cells were treated with DOC, there was a progressive decrease in the number of fluorescent particles and a concomitant increase of nonfluorescent cells over time (Fig. 5A). The suspicion that this reflected phagocytic killing, with nonfluorescent particles representing nonviable yeast cells that had been liberated from the detergent-solubilized granulocytes, was confirmed by colony counting. A total of 33 determinations conducted after 1 and 2 h of incubation were performed by using blood from seven healthy individuals. To calculate killing by fluorescence analysis, labelled versus unlabelled particles were enumerated in the CRP samples and compared with the respective controls (yeast cells in CFP). An example is shown in Fig. 5B, wherein 87 and 82% of the yeast cells were fluorescent in the CFP control sample after 60 and 120 min, respectively, compared with 49 and 32% fluorescent particles in the CRP sample. Killing was thus estimated to be $(87 - 49)/87 = 44\%$ and $(82 - 32)/82 = 60\%$ after 1 and 2 h, respectively. The killing estimates derived by fluorescence-activated cell sorter analysis never deviated by more than 20% from determinations obtained by colony countings. The flow cytometric determinations indicated that 48% ± 11% and 62% ± 10% (standard deviation) killing occurred after 60 and 120 min, respectively, at 37°C. The data obtained by colony counting were 54% ± 14% and 76% ± 10%, respectively. Three experiments were conducted in which killing was additionally quantified after 15, 30, and 45 min, and the collective results of all flow cytometric assays are depicted in Fig. 6.

To determine whether killing was dependent on the presence of amphotericin B at the given concentration of 0.5 μg/ml, additional experiments ($n = 3$) were conducted over 60 min in the presence of the fungicidal agent. Killing rates were identical in both cases. Thus, we detected no synergism between amphotericin B and phagocytic killing under the given experimental conditions. The same results were recently reported by Nugent and Couchot (29).

DISCUSSION

Fluorescence-activated cell analysis has opened new frontiers in many areas of immunological research, and a number of publications have appeared on its application to the study of phagocytosis. Since the introduction of the use of fluo-

FIG. 4. (A) Demonstration of live-gating setting for determining the percentage of phagocytes associating with yeast particles. The live-gate is set around the My-7-labelled population of cells (phagocytes), identified on the basis of orange fluorescence (fluorescence 2) and the typical granularity (side scatter). The data are derived from the same sample as described in the legend to Fig. 2. (B) Quantitative analysis of phagocytes participating in opsonophagocytosis of *C. albicans* at a yeast cell-to-phagocyte ratio of 1:1. The percentage of phagocytes exhibiting green fluorescence (fluorescence 1, indicative of association with yeast cells) at the given time points is depicted. The marker (dotted line) defining the commencement of positive green fluorescence was set as shown. Two thousand particles were counted per determination.

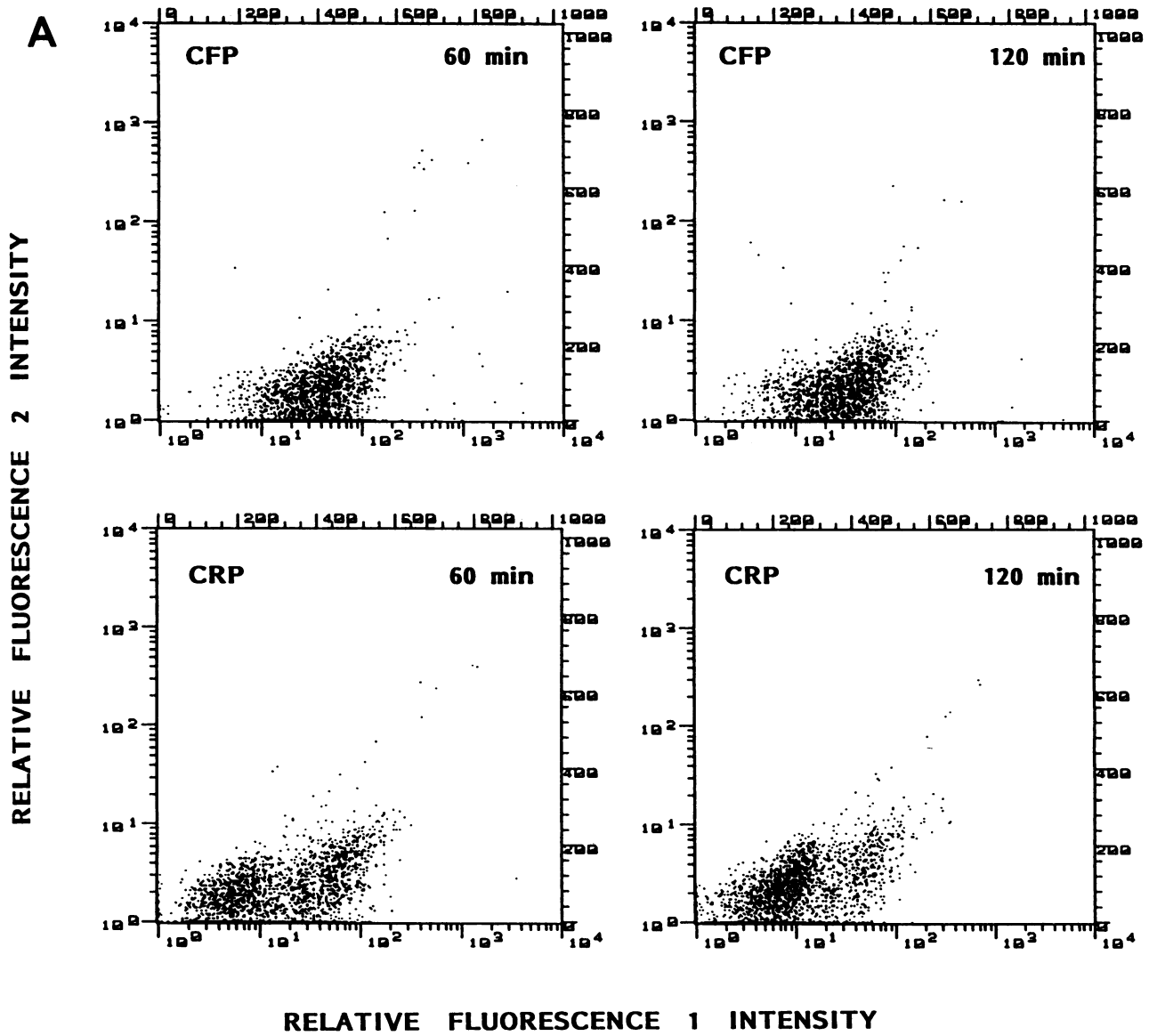
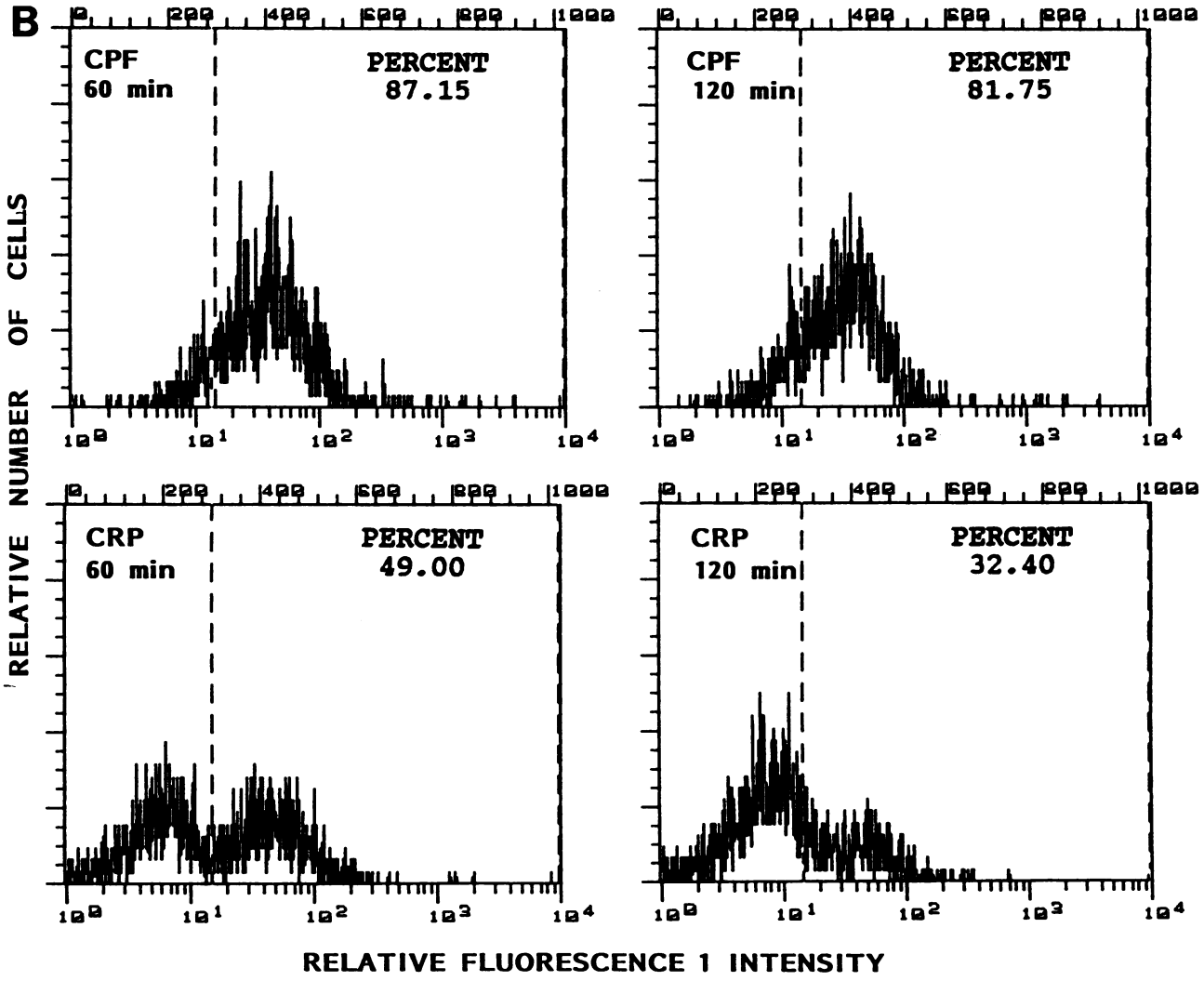


FIG. 5. Assays for killing of *C. albicans*. (A) BCECF-labelled yeast cells were incubated with CFP or CRP for 60 and 120 min. DOC was used to solubilize blood cells and liberate yeast cells from the phagocytes. Flow cytometry was used to quantify the percentage of fluorescent versus nonfluorescent yeast cells. Whereas the majority of yeast cells incubated with CFP, as well as with CRP, at time zero (not shown) were fluorescent, the numbers of nonfluorescent particles increased in the CRP samples over time. (B) The percentage of fluorescent particles was calculated as shown in the histograms. Killing was estimated as the percentage of fluorescent particles in CFP minus the percentage of fluorescent particles in CRP divided by the percentage of fluorescent particles in CFP. Two thousand particles were analyzed in each determination.

rescent microspheres as targets for phagocytosis by Steinkamp et al. (40), this approach has been exploited by several investigators but, of course, does not measure killing (2, 13, 32, 34). In a similar context, Bassoe et al. (1, 2), Cantinieaux et al. (10), and Hasui et al. (16) used flow cytometry to study opsonophagocytosis of *Staphylococcus aureus*, Sveum et al. (41) reported on the uptake of pneumococci by monocytes, and Hed et al. (18) and Bjerknes and Bassoe (6) described flow cytometric analysis of the ingestion of heat-killed yeast particles. These assays also do not measure killing. Most recently, a method has been devised to quantify protein degradation in phagolysosomes by flow cytometry (17).

One attractive method for measuring phagocytosis and killing of *C. albicans* by using flow cytometry was described by Bjerknes (5). The assay was conducted with isolated PMNs and exploited differential staining of dead and viable yeast cells with propidium iodide after their liberation from phagocytes by lysis in water. Buschmann and Winter (9) extended the method to assay killing of baker's yeast. Dérer et al. (12) and Wilson et al. (46) utilized differential staining with acridine orange to assay phagocytosis of yeast cells by isolated PMNs by the use of flow cytometry. Our method offers the advantages that PMNs need not be isolated, that assays are conducted in an autologous system with all blood components from the same donor, that dual labelling of



phagocytes and yeast particles permits detailed kinetic analyses of opsonophagocytosis, and that assessment of killing is very straightforward and harbors virtually no danger of artifacts. In this connection, propidium iodide will also stain nucleic acids liberated from human cells, and staining with acridine orange (12, 37, 38, 46) can also be problematic (for a discussion, see reference 5). Notably, Bjerknes (5) reported distinctly higher killing rates of *C. albicans* (95% in 120 min) with his assay than we have found in the present study.

Our assay demands that three requirements be met. First, the target cells should be readily detectable as single particles by the cytometer. Second, the cells must take up BCECF-AM and retain the stain. Third, killed cells must lose the fluorescent dye. The method should be extendable to any other microorganisms that meet these requirements. Hurst et al. have reported that myeloperoxidase-mediated chlorination can alter the fluorescence properties of fluorescein (19), and this could also contribute to the loss of fluorescence of ingested and killed *C. albicans*.

DOC was used to solubilize blood cells and to liberate yeast cells from the phagocytes. The same principle was exploited 20 years ago by Lehrer (22–25), who developed a quantitative assay for measuring killing of *C. albicans* by peripheral blood cells on the basis of differential staining with methylene blue and evaluation by light microscopy. The extent of killing of *C. albicans* observed by Lehrer was somewhat lower than noted here, i.e., approximately 30% after 60 min of incubation. Leijh et al. (26) utilized the standard colony-counting method and reported killing rates similar to ours. In light of the excellent correlation between evaluations obtained by colony counting and by the fluorescence-activated cell analyzer, we have confidence in the new method that we have presented.

A few studies on the kinetics of cell association and killing of *C. albicans* have been reported. Lehrer (22, 25), Leijh et al. (26), Bjerknes (5), and Solomkin et al. (39) also found that ingestion of *C. albicans* by PMNs was essentially complete within 15 to 20 min. The first two groups reported killing rates of only 30 to 50% after 60 min (22, 25, 26). Their data, therefore, indicated a marked temporal dissociation between the process of ingestion and killing. Notably, diverging results were reported by other investigators who utilized differential staining with acridine orange (38) or propidium iodide (5) to discriminate between dead and viable yeast cells. In those studies, the fungicidal action of human PMNs

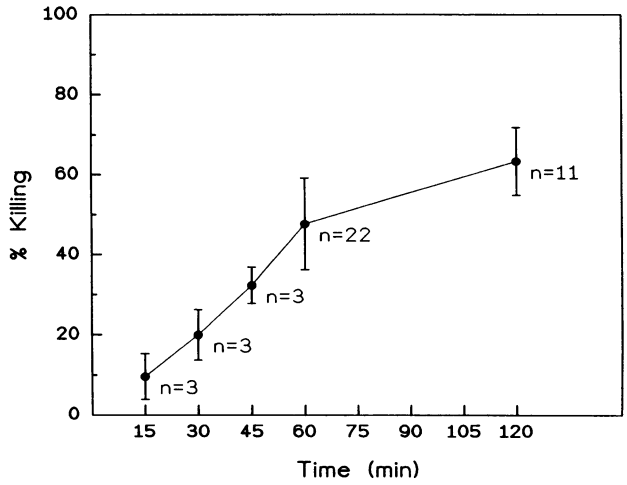


FIG. 6. Rate of killing of *C. albicans* determined by flow cytometry. Plotted values are means ± standard deviations; the number of determinations for each time point is given. The yeast cell-to-phagocyte ratio ranged between 0.5:1 and 2:1.

appeared to be more efficient, and killing rates of 70 to 80% were noted within 60 min. Our data concur with the results of Lehrer (22, 25) and Leijh et al. (26) and do not support the divergent findings.

The relatively slow killing rate of ingested *C. albicans* markedly contrasts with the rapid bactericidal activity of peripheral blood leukocytes. Killing of staphylococci and streptococci is essentially complete (over 90%) within 30 min, and smooth strains of the family *Enterobacteriaceae* are also killed within 60 min (42, 44, 45). In contrast, we never observed more than 75% killing of *C. albicans* even after 120 min. A possible explanation for incomplete killing of *C. albicans* based on phagocyte vacuole heterogeneity has been suggested by Cech and Lehrer (11). Slow killing of *C. albicans* by neutrophils could indicate that blood phagocytes may not efficiently effect their elimination during systemic infections. Possibly, activated macrophages represent another important arm of defense against these organisms (7, 37, 43). Many investigators have used other *Candida* species (e.g., *C. guilliermondii*, *C. pseudotropicalis*, *C. parapsilosis*) as targets in phagocytosis assays (14, 24, 35, 37, 43), because these fungi either exert little cytocidal effect on phagocytes, do not form pseudohyphae, or may be killed via other mechanisms not involving the myeloperoxidase pathway. We elected to use *C. albicans* because of its prominent medical significance, whereby pseudohypha formation and aggregation were suppressed by addition of amphotericin B at a subfungicidal concentration. These effects of amphotericin B have also been demonstrated by Nugent and Couchot (29).

The use of MAb My-7 to label blood phagocytes rendered the study of opsonophagocytosis possible at the single-cell level. This antibody did not affect opsonophagocytic function. However, an effect on killing of *C. albicans* was noted and is currently being investigated.

We did not attempt to differentiate between adherent and ingested yeast cells. If necessary, this might be possible through the use of an appropriate quenching agent (18) or by using a triple label (e.g., specific antibody [32, 41]) to probe the location of the yeast cells. Even when yeast cells were present in surplus, we constantly observed a small population of phagocytes displaying no dual fluorescence. This could reflect the existence of subpopulations of phagocytes with differing opsonophagocytic activities. The possibility that the cells became nonfunctional during the course of the experiments cannot be excluded, although our experimental protocol minimizes manipulation of blood samples to the extreme. It is reiterated that the only processing step that our assay requires is a 20- to 30-min dextran sedimentation of erythrocytes, so that artifactual alterations of leukocyte function are not expected. We have ascertained that the granulocytes present in the CRP do not show enhanced production of superoxide anions. They neither aggregate nor secrete elastase, and they phagocytose *S. aureus* (10 bacteria per granulocyte) within 60 min (data not shown). Finally, since dextran selectively causes erythrocyte sedimentation, the composition and content of other cells in CRP preparations most closely resemble the in vivo situation.

The procedure described in the present study is very simple and generates results within a few hours. A large number of samples can be processed, and this will facilitate investigations into various aspects of phagocytosis. Obvious possibilities include studies on the opsonic requirement for phagocytosis, the identification of cell surface molecules that participate in uptake of microorganisms, the involvement of intracellular machineries in the killing process, the production of antiphagocytic substances by microorganisms, and the possible augmentation of microbicidal phagocyte function by cytokines. In a clinical laboratory setting, our method provides a novel means to screen for primary and secondary defects in phagocyte function, irrespective of whether the cause lies in the humoral or cellular arm of the immune system.

ACKNOWLEDGMENTS

We thank H. Hänel for helpful discussions and M. Hoffman for able secretarial help.

This study was supported by the Deutsche Forschungsgemeinschaft (SFB 311, D9).

REFERENCES

1. **Bassoe, C. F., and C. O. Solberg.** 1984. Phagocytosis of Staphylococcus aureus by human leukocytes. Quantitation by a flow cytometric and a microbiological method. Acta Pathol. Microbiol. Immunol. Scand. Sect. C **92**:43–50.
2. **Bassoe, C. F., J. Svolisk, and O. D. Laerum.** 1980. Quantitation of single cell phagocytic capacity by flow cytometry, p. 170–174. *In* O. D. Laerum, T. Lindmo, and E. Thorud (ed.), Flow Cytometry IV. Universitetsforlaget, Bergen, Norway.
3. **Becker, J., S. W. Carter III, and R. J. Grasso.** 1986. A rapid radiometric assay for measuring phagocytosis of Saccharomyces cerevisiae in macrophage cultures. J. Immunol. Methods **91**:1–10.
4. **Bhakdi, S., S. Greulich, M. Muhly, B. Eberspächer, H. Becker, A. Thiele, and F. Hugo.** 1989. Potent leukocidal action of *Escherichia coli* hemolysin mediated by permeabilization of target cell membranes. J. Exp. Med. **169**:838–854.
5. **Bjerknes, R.** 1984. Flow cytometric assay for combined measurement of phagocytosis and intracellular killing of Candida albicans. J. Immunol. Methods **72**:229–241.
6. **Bjerknes, R., and C. F. Bassoe.** 1983. Human leukocyte phagocytosis of zymosan particles measured by flow cytometry. Acta Pathol. Microbiol. Scand. Sect. C **91**:341–350.
7. **Brummer, E., and D. A. Stevens.** 1989. Candidacidal mechanisms of peritoneal macrophages activated with lymphokines or γ-interferon. J. Med. Microbiol. **28**:173–181.
8. **Bruning, J. W., M. J. Kardol, and R. Arentzen.** 1980. Carboxyfluorescein fluorochromasia assays. I. Non-radioactively labeled cell mediated lympholysis. J. Immunol. Methods **33**:33–44.
9. **Buschmann, H., and M. Winter.** 1989. Assessment of phago-

cytic activity of granulocytes using laser flow cytometry. J. Immunol. Methods **124**:231–234.
10. **Cantinieaux, B., C. Hariga, P. Courtoy, J. Hupin, and P. Fondu.** 1989. Staphylococcus aureus phagocytosis. A new cytofluorometric method using FITC and paraformaldehyde. J. Immunol. Methods **121**:203–208.
11. **Cech, P., and R. I. Lehrer.** 1984. Heterogeneity of human neutrophil phagolysosomes: functional consequences for candidacidal activity. Blood **64**:147–151.
12. **Dérer, M., C. Walker, F. Kristensen, and M. C. Reinhardt.** 1983. A simple and rapid flow cytometric method for routine assessment of baker's yeast uptake by human polymorphonuclear leukocytes. J. Immunol. Methods **61**:359–365.
13. **Dunn, P. A., and H. W. Tyrer.** 1981. Quantitation of neutrophil phagocytosis, using fluorescent latex beads. J. Lab. Clin. Med. **98**:374–381.
14. **El-Maallem, H., and J. Fletcher.** 1976. Defective neutrophil function in chronic granulocytic leukaemia. Br. J. Haematol. **34**:95–103.
15. **Harvey, D. M., K. J. Sheppard, and J. Fletcher.** 1986. A method for measuring rate of neutrophil phagocytosis of Staphylococcus epidermidis or Candida guilliermondii using uptake of tritiated uridine. J. Immunol. Methods **93**:259–264.
16. **Hasui, M., Y. Hirabayashi, and Y. Kobayashi.** 1989. Simultaneous measurement by flow cytometry of phagocytosis and hydrogen peroxide production of neutrophils in whole blood. J. Immunol. Methods **117**:53–58.
17. **Haynes, A. P., J. Fletcher, M. Garnett, and A. Robins.** 1990. A novel flow cytometric method for measuring protein digestion within the phagocytic vacuole of polymorphonuclear neutrophils. J. Immunol. Methods **135**:155–161.
18. **Hed, J., G. Hallden, S. G. O. Johansson, and P. Larsson.** 1987. The use of fluorescence quenching in flow cytofluorometry to measure the attachment and ingestion phases in phagocytosis in peripheral blood without prior cell separation. J. Immunol. Methods **101**:119–125.
19. **Hurst, J. K., J. M. Albrich, T. R. Green, H. Rosen, and S. Klebanoff.** 1984. Myeloperoxidase-dependent fluorescein chlorination by stimulated neutrophils. J. Biol. Chem. **259**:4812–4821.
20. **Husseini, R. H., M. E. Hoadley, J. J. P. Hutchinson, C. W. Penn, and H. Smith.** 1985. Intracellular killing of Candida albicans by human polymorphonuclear leucocytes: comparison of three methods of assessment. J. Immunol. Methods **81**:215–221.
21. **Lanier, L. L., and N. L. Warner.** 1981. Paraformaldehyde fixation of hematopoietic cells for quantitative flow cytometry (FACS) analysis. J. Immunol. Methods **47**:25.
22. **Lehrer, R. I.** 1970. Measurement of candidacidal activity of specific leukocyte types in mixed cell populations. I. Normal, myeloperoxidase-deficient, and chronic granulomatous disease neutrophils. Infect. Immun. **2**:42–47.
23. **Lehrer, R. I.** 1971. Measurement of candidacidal activity of specific leukocyte types in mixed cell populations. II. Normal and chronic granulomatous disease eosinophils. Infect. Immun. **3**:800–802.
24. **Lehrer, R. I.** 1975. The fungicidal mechanisms of human monocytes. I. Evidence for myeloperoxidase-linked and myeloperoxidase-independent candidacidal mechanisms. J. Clin. Invest. **55**:338–346.
25. **Lehrer, R. I., and M. J. Cline.** 1969. Interaction of *Candida albicans* with human leukocytes and serum. J. Bacteriol. **98**: 996–1004.
26. **Leijh, P. C. J., M. T. van den Barselaar, and R. van Furth.** 1977. Kinetics of phagocytosis and intracellular killing of *Candida albicans* by human granulocytes and monocytes. Infect. Immun. **17**:313–318.
27. **Levitz, S. M., D. J. DiBenedetto, and R. D. Diamond.** 1988. A rapid fluorescent assay to distinguish attacked from phagocytized yeast particles. J. Immunol. Methods **101**:37–42.
28. **Louria, D. B., J. K. Smith, R. G. Braton, and M. Buse.** 1972. Anti-Candida factors in serum and their inhibitors. I. Clinical and laboratory observations. J. Infect. Dis. **125**:102–114.
29. **Nugent, K. M., and K. R. Couchot.** 1986. Effects of sublethal concentrations of amphotericin B on Candida albicans. J. Infect. Dis. **154**:665–669.
30. **Oben, J. A., and J. C. Foreman.** 1988. A simple quantitative fluorimetric assay of in vitro phagocytosis in human neutrophils. J. Immunol. Methods **112**:99–103.
31. **Oda, T., and H. Maeda.** 1986. A new simple fluorometric assay for phagocytosis. J. Immunol. Methods **88**:175–183.
32. **Ogle, J. D., J. G. Noel, R. M. Sramkoski, C. K. Ogle, and J. W. Alexander.** 1988. Phagocytosis of opsonized fluorescent microspheres by human neutrophils. A two-color flow cytometric method for the determination of attachment and ingestion. J. Immunol. Methods **115**:17–29.
33. **Peterson, P. K., J. Verhoef, D. Schmeling, and P. G. Quie.** 1977. Kinetics of phagocytosis and bacterial killing by human polymorphonuclear leukocytes and monocytes. J. Infect. Dis. **136**: 502–509.
34. **Rolland, A., G. Merdrignac, J. Gouranton, D. Bourel, R. Le Verge, and B. Genetet.** 1987. Flow cytometric quantitative evaluation of phagocytosis by human mononuclear and polymorphonuclear cells using fluorescent nanoparticles. J. Immunol. Methods **96**:185–193.
35. **Sasada, M., and R. B. Johnston, Jr.** 1980. Macrophage microbicidal activity. Correlation between phagocytosis-associated oxidative metabolism and the killing of Candida by macrophages. J. Exp. Med. **152**:85–98.
36. **Schmid, L., and K. Brune.** 1974. Assessment of phagocytic and antimicrobial activity of human granulocytes. Infect. Immun. **10**:1120–1126.
37. **Schuit, K. E.** 1979. Phagocytosis and intracellular killing of pathogenic yeasts by human monocytes and neutrophils. Infect. Immun. **24**:932–938.
38. **Smith, D. L., and F. Rommel.** 1977. A rapid micro-method for the simultaneous determination of phagocytic-microbicidal activity of human peripheral blood leukocytes in vitro. J. Immunol. Methods **17**:241–247.
39. **Solomkin, J. S., E. L. Mills, G. S. Giebink, R. D. Nelson, R. L. Simmons, and P. G. Quie.** 1978. Phagocytosis of Candida albicans by human leukocytes: opsonic requirements. J. Infect. Dis. **137**:30–37.
40. **Steinkamp, J. A., J. S. Wilson, G. C. Saunders, and C. C. Stewart.** 1982. Phagocytosis flow cytometric quantitation with fluorescent microspheres. Science **215**:64–65.
41. **Sveum, R. J., T. M. Chused, M. M. Frank, and E. J. Brown.** 1986. A quantitative fluorescent method for measurement of bacterial adherence and phagocytosis. J. Immunol. Methods **1986**:257–264.
42. **Van Furth, R., T. L. Van Zwet, and P. C. J. Leijh.** 1977. In vitro determination of phagocytosis and intracellular killing by polymorphonuclear and mononuclear phagocytes. *In* D. M. Weir (ed.), Handbook of experimental immunology, 3rd ed. J. B. Lippincott Co., New York.
43. **Vecchiarelli, A., F. Bistoni, E. Cenci, S. Perito, and A. Cassone.** 1985. In-vitro killing of Candida species by murine immunoeffectors and its relationship to the experimental pathogenicity. J. Med. Vet. Mycol. **23**:377–387.
44. **Vel, W. A. C., F. Namavar, A. Marian, J. J. Verweij, A. N. B. Pubben, and D. M. MacLaren.** 1984. Killing capacity of human polymorphonuclear leukocytes in aerobic and anaerobic conditions. J. Med. Microbiol. **18**:173–180.
45. **Verhoef, J., P. K. Peterson, and P. G. Quie.** 1977. Kinetics of Staphylococcus opsonization, attachment, ingestion and killing by human polymorphonuclear (^{3}H) thymidine labeled bacteria. J. Immunol. Methods **14**:303–311.
46. **Wilson, R. M., A. M. Galvin, R. A. Robins, and W. G. Revees.** 1985. A flow cytometric method for the measurement of phagocytosis by polymorphonuclear leucocytes. J. Immunol. Methods **76**:247–253.
47. **Yamamura, M., J. Boler, and H. Valdimarsson.** 1977. Phagocytosis measured as inhibition of uridine uptake by Candida albicans. J. Immunol. Methods **14**:19–24.