A Flow Cytometric Method for the Analysis of Phagocytosis and Killing by Polymorphonuclear Leukocytes

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INTRODUCTION

Phagocytes represent the first line of defense against invasive microorganisms as they are capable of rapid phagocytosis and killing of bacteria and fungi. A rapid evaluation of both the number and function of the human polymorphonuclear leukocyte (PMN) population could be of fundamental importance in defining the immunological defects of patients with recurrent or persistent infections. Moreover, any technical modifications that could improve the clinical screening of PMNs function would be advantageous for the treatment of many disease states. Flow cytometry can be adopted for routine monitoring of the immune functions of human polymorphonuclear leukocytes (PMNs) in several disease states.[1–4]

In this study, we describe a fast, reliable, and inexpensive method for studying the phagocytosis and killing of *Candida albicans* blastospores by fresh human polymorphonuclear cells, using flow cytometry (FCM). We have modified previously described methods[5] in order to achieve the reproducibility and speed necessary for testing several samples in the diagnostic laboratory. This assay is capable of distinguishing adherent blastospores from those that have been ingested and permits an evaluation of intracellular killing as well. Briefly summarized, the advantages offered by this assay are (*1*) more rapid PMN preparation; (*2*) the staining procedure involves less time, thereby contributing to a faster assay; (*3*) the *C. albicans* to PMN ratio is 2:1, which allows an accurate estimation of truly phagocytic PMN; (*4*) fluorescence-labeled *C. albicans* blastospores can be frozen and used when needed without loss of fluorescence; and (*5*) this assay also measures killing. The technique can be easily

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adapted to monitor the immune reactivity of PMNs against different pathogens, including bacteria and filamentous fungi.

MATERIALS AND METHODS

Blastospores

Candida albicans (C. Albicans CM2 strain) blastospores were grown in Sabouraud broth with 2% dextrose (Difco Laboratories, Detroit, MI) at 37°C for 18–24 h. The blastospores were then washed three times and resuspended in phosphate-buffered saline (PBS). They were then counted with a STKS hemocytometer (Coulter Electronics, Inc., Miami Lakes, FL) and checked for viability by trypan blue exclusion (Sigma Chemical Co., St. Louis, MO). Viability proved to be greater than 90%.

For the killing assays, *C. albicans* blastospores were resuspended in PBS to a final concentration of 10^7 blastospore cells/ml and then labeled with fluorescein isothiocyanate (FITC; Sigma Chemical Co.) (0.1 mg/ml) in a 0.5 M carbonate/bicarbonate buffer (pH 9.5) for 1 h with agitation at room temperature and in a light-protected environment. Following incubation, the blastospores were washed twice in PBS and divided into aliquots of 10^6 blastospores each.

For the phagocytosis assay, *C. albicans* blastospores were ethanol-fixed (70% ethanol for 1 h at room temperature) and washed twice in PBS. They were then labeled with FITC (0.01 mg/ml; Sigma Chemical Co.) in a 0.5 M carbonate/bicarbonate buffer (pH 9.5) for 30 min with agitation, in a light-protected environment. The labeled blastospores were washed and stored in 2×10^6 aliquots at –80°C until use.

Preparation of Human Polymorphonuclear Cells

Human leukocytes were obtained from the heparinized whole blood of healthy donors after lysis of red blood cells by hypotonic shock with ammonium chloride. Total and differential leukocyte counts were made using a Coulter STKS Counter (Coulter Electronics, Inc., Miami Lakes, FL) and the PMN concentration was adjusted to 3×10^6 cells/ml, washed again in PBS, and then divided into three aliquots of 10^6 cells each.

Opsonization and Phagocytosis

Prior to the phagocytosis and cytotoxicity assays, the opsonization of the aliquots of live or ethanol-fixed FITC-labeled *C. albicans* blastospores was achieved by suspension in 200 μl of patient serum for 30 min at 37°C. They were then washed several times in PBS and resuspended in 200 μl of PBS. For the phagocytosis assay, 2×10^6 opsonized ethanol-fixed FITC-*C. albicans* blastospores were incubated in 1×10^6 PMNs for 30 min at 37°C in a water bath with continuous agitation and in a light-

protected environment, to a final volume of 400 μl with a PMN to blastospore cell ratio of 1:2.

After several assays, this ratio proved to be optimal for the discrimination between adherent and ingested blastospores. The samples were centrifuged at 500*g* and resuspended in 1 ml of cold PBS containing 0.02% ethylenediamine tetraacetic acid (EDTA) to stop phagocytosis. Finally, ethidium bromide (EtBr) was added to a concentration of 10 μg/ml. The FCM analysis of the samples was carried out immediately following the addition of EtBr.

FCM Measurement of Phagocytosis

The PMNs were selected by means of forward and side scatter (FSC and SSC) and displayed as a biparametric graph. The energy transfer method was used to provide a distinction between adherent and ingested blastospores.[5,6] This procedure is based on the observation that FITC-labeled *C. albicans* blastospores lose their green fluorescence and acquire red fluorescence after staining with EtBr through the phenomenon of resonance energy transfer.[4] Therefore, internalized *C. albicans* blastospores remain green, whereas adherent and non-phagocytized blastospore cells turn red. The percentage of phagocytizing PMNs was equal to the number of green and double-labeled (green and red) fluorescent PMNs divided by the total number of leukocytes multiplied by 100.

The Killing Assay

The cytotoxicity assay was performed by adding 2×10^6 live FITC-labeled *C. albicans* blastospores to 1×10^6 PMNs from the test sample. Only live FITC-*C. albicans* blastospores were used as controls. Both samples were incubated at 37°C in a water bath with continuous agitation for 2.5 h. The PMNs were then lysed by hypotonic shock. To remove extracellular DNA released from the lysed PMNs, 1 ml of a warm (37°C) DNase solution (2 mg deoxyribonuclease I from bovine pancreas = 4000 Kunitz units dissolved in 100 ml of PBS) was added and the PMNs were incubated for 5 min with periodic agitation. The samples were then repeatedly washed in PBS and incubated with 200 μl of propidium iodide (PI; 100 μg/ml) for 30 min at 4°C in a light-protected environment. The PI penetrates killed blastospore cells and stains DNA, yielding red fluorescence.

FCM Measurement of Intracellular Killing

To analyze the percentage of killed FITC-labeled *C. albicans* blastospores, the green and red fluorescence biparametric graph of the double-labeled blastospores was evaluated. The following formula was adopted to calculate the percentage of killed blastospores in the samples analyzed: % killed blastospores = % double-labeled blastospores of sample – % double-labeled blastospores of control.

Flow Cytometry

The cytometric analysis of phagocytosis and killing was performed using a FACStar cytofluorimeter (Becton Dickinson FACS Systems, San Jose, CA) equipped with a water-cooled 2 W argon ion laser operating at 488 nm, interfaced with a Hewlett Packard 300 series computer (Hewlett-Packard Company, Roseville, CA). Multiparametric data were acquired for 10,000 events and analyzed using FacStarPlus software supported by the PC-LYSYS program (Becton Dickinson, San Jose, CA). Red fluorescence from PI (FL2) was collected through a 620 nm long pass filter, green fluorescence from FITC (FL1) through a 530 nm band pass filter, and finally the fluorescence from EtBr (FL3) was measured through a 630 nm band pass filter.

Data were collected using linear amplifiers for FSC and SSC and logarithmic amplifiers for FL1, FL2, and FL3. Samples were first run using single fluorochrome-stained preparations for color compensation. The FSC threshold was set at 50 to measure the killing function and was raised to 100 to acquire data on phagocytosis.

RESULTS

Fluorescein-labeling of Candida albicans

Homogeneous bright fluorescent staining should also be homogeneous in intensity to obtain correct cytometric readings of *C. albicans* blastospores. For this reason, the fluorescence was checked by fluorescence microscopy for staining uniformity, before reliable cytometric results were obtained. Incubation of ethanol-fixed blastospores with FITC (0.01 mg/ml) for 15 min showed highly heterogeneous staining of the cells, rendering further quantification of *C. albicans* blastospore ingestion by PMNs impossible. Extension of the incubation time to 30 min yielded a more uniform staining and this was the procedure used in all the subsequent tests.

Several concentrations of EtBr were also tested to select an optimal concentration capable of completely extinguishing the green fluorescence, while leaving a low background of red fluorescence. In fact, the green fluorescence did not completely disappear at solutions containing concentrations of 5 or 7 μg/ml and the red fluorescence remained very low. On the contrary, EtBr solutions of 30 or 50 μg/ml produced a strong red fluorescent background. Thus, an intermediate EtBr solution of 10 μg/ml was chosen in the end.

When live *C. albicans* blastospores were used in the killing assay, a different staining procedure was employed. An incubation period of 1 h and a FITC solution of 0.1 mg/ml proved necessary to obtain homogeneous labeling and a suitable stain intensity. In the preliminary trials, FITC was also used at 0.01 mg/ml for 1 h, but *C. albicans* blastospore labeling was not uniform at this concentration. Several trials were also necessary to establish the optimal PI concentration needed to quantify the percentage of killed *C. albicans* blastospores. When 100 μg/ml of PI were used at 4°C for 30 min, upon microscopic observation, the ethanol-fixed FITC-labeled blastospores appeared to be completely stained red inside with an outer green fluorescence. Concentrations of 50 and 200 μg/ml produced a red fluorescence that was ei-

ther too low or too high, with respect to the green fluorescence of the external blastospores.

Phagocytosis of FITC-labeled C. albicans *by PMNs*

The analysis of PMN phagocytosis according to cytometric readings is visualized in correlated double-parameter contour plots. Four distinct PMN subsets were distinguishable on the basis of the fluorescence emitted. FIGURE 1 illustrates typical trial results: region 1 (4%) contains PMNs with adherent blastospore particles only, region 2 (12%) shows PMNs with ingested and adherent FITC-labeled *C. albicans* blastospores, whereas PMNs with no interactions with particles appear in region 3

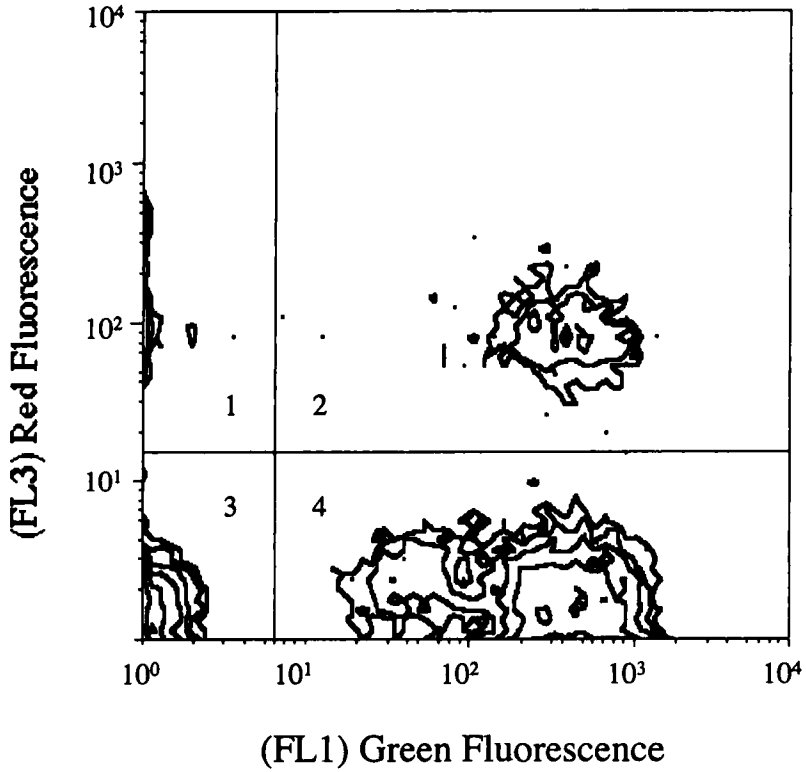

FIGURE 1. Contour plot of green (FL1) and red (FL3) fluorescence of PMNs incubated with ethanol-fixed fluorescein-labeled *C. albicans* blastospores at 37°C after the addition of EtBr. In this experiment, 4% (region 1) were PMNs with only membrane-bound blastospores, 55% (region 4) were PMNs with ingested FITC-labeled *C. albicans*, 12% (region 2) were PMNs with both ingested and adherent FITC-labeled *C. albicans* blastospores, and 27% (region 3) were PMNs without any interactions with blastospore cells.

TABLE 1. Phagocytosis and Killing Function of PMNs

	% PMN (Mean ± SD; $N = 15$)
PMNs/FITC-labeled *C. albicans* Fluorescence/Interaction[a]	
Adherence	9±6%
Adherence and ingestion	15±10%
Ingestion	50±14%
No interaction	26±13%
PMN Killing Function[b]	
	% Killed FITC-labeled *C. albicans*
	33±19

[a]Percentage of phagocytes with adherent and/or ingested FITC-labeled *C. albicans* blastospores after incubation with opsonized FITC-labeled *C. albicans* blastospores at 37°C for 30 min with continuous agitation.

[b]Killing function of PMNs expressed as percentage of killed FITC-labeled *C. albicans* blastospores after incubation with opsonized FITC-labeled *C. albicans* at 37°C for 2.5 h with continuous agitation.

(27%), and PMNs with only ingested FITC-labeled *C. albicans* blastospores are found in region 4 (55%).

TABLE 1 provides a summary of the analysis of phagocytosis and adherence conducted on blood samples obtained from 15 healthy donors. The table reports the means and standard deviations of the percentages of PMNs that ingested FITC-labeled *C. albicans* blastospores, as well as those with adherent and phagocytized FITC-labeled *C. albicans* blastospores.

Killing of FITC-labeled C. albicans *by PMNs*

The cytometric analysis of the percentage of blastospores killed by PMNs is represented in a two-color biparametric graph: green fluorescence and red fluorescence (FIG. 2). Dead FITC-labeled *C. albicans* blastospores are visible in region 2 among the double-labeled cells. In fact, the killed blastospores proved to be red inside due to PI penetration of the cell and green outside due to FITC. FIGURE 2 (A and B) illustrates a representative killing assay. FIGURE 2 (A) shows the fluorescence of live *C. albicans* blastospore before the killing assay. The percentage of killed blastospores proved to be 30% and was calculated by comparing the results of the analysis of region 2 of FIGURE 2(A) and (B). Residual live FITC-labeled *C. albicans* blastospores are visible in region 4 of FIGURE 2(B). Moreover, dead FITC-labeled *C. albicans* blastospores were easily discriminated from leukocyte debris by cytogram simultaneously evaluating their different FSC and SSC properties. The means and standard deviations for the percentages of dead FITC-labeled *C. albicans* blastospores following incubation with PMNs from the peripheral blood samples collected from the 15 healthy donors were 33±19.

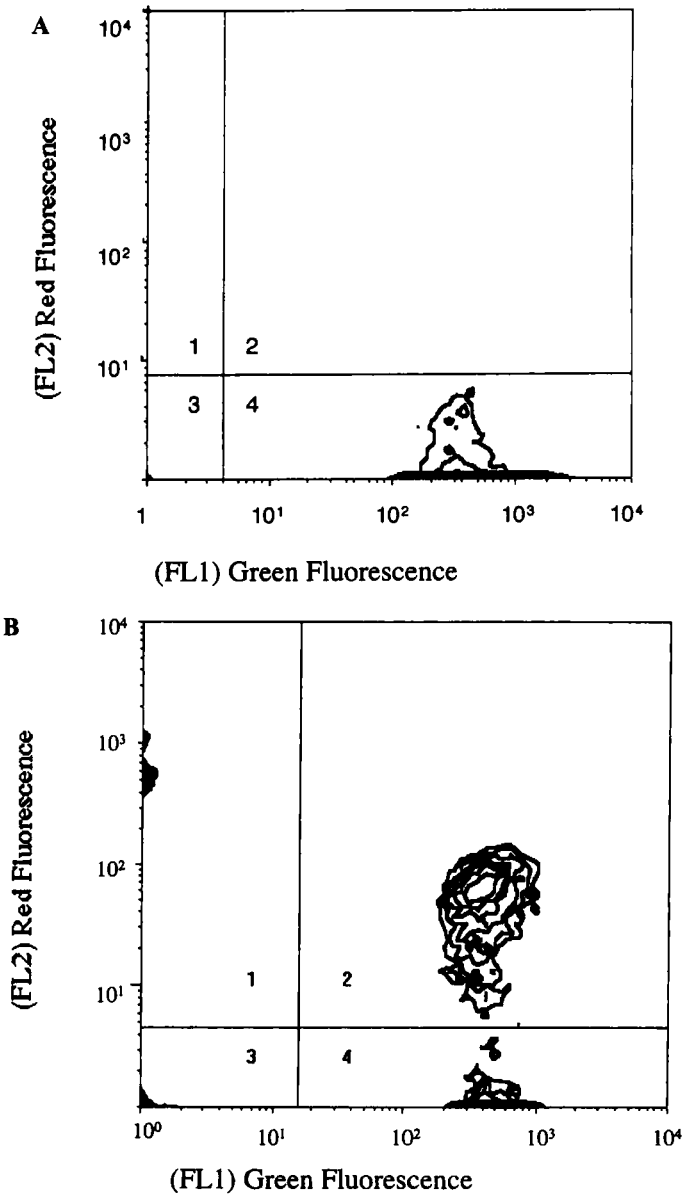

FIGURE 2. (**A**) Contour plot of green (FL1) and red (FL2) fluorescence of control FITC-labeled *C. albicans*. The percentage of living blastospore cells was 98% (region 4). (**B**) Contour plot of FL1 and FL2 fluorescence of killed FITC-labeled *C. albicans* blastospores after incubation at 37°C for 90 min with PMNs. The percentage of killed blastospore cells was 30% (region 2).

DISCUSSION

We have described a quick and reliable method for the analysis of phagocytosis and killing of *Candida albicans* blastospores by human PMNs using flow cytometry. This assay offers several advantages compared to previous microscopic and/or cytometric techniques. First of all, it confirms that it is possible to utilize unseparated PMNs, which can be easily distinguishable from the leukocyte population by selection on the basis of their FSC and SSC properties. This reduces the time normally required for the preparation of specimens for routine assays because in this case, only the lysis of red blood cells is required for the preparation of the phagocytosis and killing assays.

In addition, unlike microscopic or microbiological techniques, this method is less time-consuming and more precise: it allows for the detection of about 10,000 *C. albicans* blastospores or phagocytes in less than 2 min and with a yield of accurate and reproducible data.

The reason for the selection of *C. albicans* blastospores as a marker of phagocytosis and killing lies not only in the prevalence of this pathogen among opportunistic agents, but also in the practical advantages it provides. *C. albicans* blastospores present good flexibility *in vitro:* they are easily grown in culture broth; they can be easily opsonized by human serum; they can be labeled with FITC in a shorter time period than *A. fumigatus* conidia; they do not lose fluorescence after freezing at –70°C; and, finally, they can be used for both phagocytosis and killing assays. We now know that this assay can be easily adapted to evaluate the phagocytosis and the killing of *A. fumigatus* conidia by macrophages (Taramelli and Saresella, unpublished report). Therefore, we believe that it may be possible to adapt it to other fungi as well, provided that they can be consistently and quickly labeled with FITC.

One of the primary technical problems encountered in this study was the discrimination between adherent and internalized blastospores. This distinction is necessary to avoid overestimating the phagocytosis function of PMNs. Ethidium bromide, which transforms FITC-green fluorescence into red fluorescence[5] was used for this purpose. This phenomenon can be explained as an energy transfer between the two fluorochromes. In this case, EtBr is the donor, while FITC is the electron acceptor.[7] However, EtBr does not enter the living cells, and ingested FITC-labeled *C. albicans* blastospores thus retain their green fluorescence, whereas extracellular blastospores turn red. To further optimize the assay and reduce the number of non-ingested particles, a low phagocyte to blastospore ratio (1:2) was used. This ratio allows a better estimation of ingested versus membrane-bound yeasts, because it minimizes the crowding effect due to an excess of yeasts.

These results are a variation of what has been reported by others.[1,5,6] However, a careful examination of the data shows that the total number of phagocytic PMNs is quite similar among different reports, whereas the number of non-phagocytic cells or cells with ingested and adherent yeasts varies considerably.[5] This confirms a strong interference of free yeasts in the assay.

The possibility of using frozen FITC-labeled *C. albicans* blastospores without finding substantial changes in fluorescence intensity made the phagocytosis assay faster because it was possible to maintain frozen aliquots of FITC-labeled *C. albicans* for quite some time and to have the blastospores readily available for the assay.

This is particularly advantageous for routine assays, when several PMN samples have to be checked at one time. Furthermore, the use of the same preparation of FITC-labeled *C. albicans* blastospores reduces variability when follow-up monitoring of the recovery or the loss of PMN function for a individual patient is required. It was not possible to utilize frozen FITC-labeled *C. albicans* for the killing assay, because the percentage of killed blastospore cells in the control group samples increased, especially after thawing. Therefore, live *C. albicans* blastospores had to be labeled with FITC immediately prior to testing. Thanks to the brief incubation time required, the method was fairly rapid. The distinction between live and killed *C. albicans* blastospores was made possible by the use of PI, which does not penetrate the plasma membrane of living cells.[8] Thus, live *C. albicans* blastospores do not stain with PI, however, killed blastospores turn red.[6] As PI binds to DNA, it is very important that external DNA is destroyed by means of DNase treatment before PI is added.[8] Elimination of residual DNA from PMNs and the subsequent gate designed around the blastospores to eliminate debris, allow for a reliable calculation of the percentage of killed FITC-labeled *C. albicans* blastospores. Killed FITC-labeled *C. albicans* blastospores appear as double-labeled and emit both red and green fluorescence, thereby rendering them unmistakably distinct upon the cytometric reading in a biparametric graph showing two fluorescences in the common region.

A simple quantitative cytometric assay for the combined kinetic study of PMN phagocytosis and the intracellular killing of *C. albicans* blastospores has been presented here. The method may be useful for rapid automatic screening of phagocyte function in infectious diseases, hematological disorders, and in cases of immunosuppression.

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