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Quantitative Analysis of Phagocytosis and Killing of *Cryptococcus neoformans* by Human Peripheral Blood Mononuclear Cells by Flow Cytometry

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Monocytes may represent an important defense mechanism in disseminated cryptococcosis. We have developed a flow cytometric method to study the interaction of *Cryptococcus neoformans* with monocytes. For phagocytosis, *C. neoformans* was labelled with fluorescein isothiocynate (FITC). Monocytes were identified on the flow cytometer by labelling with anti-CD14-*R*-phycoerythrin. Discrimination between attached cells (association) and internalized cells (uptake) was made by quenching FITC-labelled *C. neoformans* with trypan blue. Only internalized cells kept their FITC fluorescence after quenching. For comparison under the microscope, specific staining of the cell wall of *C. neoformans* with Uvitex was used. Internalized *C. neoformans* cells were not stained, as Uvitex was occluded from phagocytes. To assay killing, *C. neoformans* was labelled with 0.2 mM 2′-7[1]-*bis*(2-carboxyethyl)-5-carboxyfluorescein acetoxymethylester. After phagocytosis of labelled cells by monocytes, blood cells were lysed with 25 mM deoxycholate. Viable yeast cells retained the fluorescence, but nonviable cells lost it. Quantitative counts of viable cells on Sabouraud dextrose agar were performed for comparison. The change in the relative fluorescence of green within the monocyte region was used to quantitate association, uptake, and killing of *C. neoformans* by monocytes on the flow cytometer. The flow cytometry methods showed that 18% ± 2%, 35% ± 14%, 50% ± 11%, 51% ± 6% of monocytes had become associated with *C. neoformans* after 0, 30, 60, and 120 min, respectively. After 2 h of phagocytosis time, 30% of *C. neoformans*-associated monocytes had taken up the cells, and killing rates of 23% ± 17%, 22% ± 9%, and 40% ± 13% were obtained with effector-to-target cell ratios of 1:1, 10:1, and 50:1, respectively. Results with the flow cytometry methods compared favorably with those by the conventional methods used, but the flow cytometry methods are simpler, rapid, more reproducible, and objective.

Cryptococcosis, defined as disease caused by the encapsulated yeast-like fungus *Cryptococcus neoformans*, is a major cause of morbidity and mortality in individuals with impaired immunity, particularly AIDS patients (14). While an adequate cell-mediated immune response appears to be essential for controlling infections caused by *C. neoformans*, it remains unclear which cell type(s) is ultimately responsible for mediating antifungal activity. Mononuclear phagocytes are presumed to play a critical role in host defenses against cryptococcosis (5, 8, 14, 21, 23), whereas in cryptococcemia, polymorphonuclear neutrophils (PMN) and monocytes are likely crucial defense cells (5, 8, 21).

Presently, however, there are no rapid, simple, and reproducible methods available for simultaneous measurement of association (attached cells) and uptake (internalized cells) at the individual cell level or for killing of *C. neoformans* by phagocytes. Such methods would allow an evaluation of the primary mechanism of phagocytic killing through which the immune system eliminates the fungi. Several methods that allow differentiation between microorganisms simply attached and those internalized by phagocytes have been described. Basic staining and light microscopy have been used but have the major disadvantage of being cumbersome, and interpretation of the results is subjective (5, 11). The electron microscope is useful to show internalization, but serial sections are required to determine that the whole organism is enclosed within a vacuole membrane. Chemiluminescence is emitted when phagocytes engulf bacteria, and this has been employed to quantify uptake (4). Internalization can also be quantified by uptake of radiolabelled microorganisms (6, 7, 22, 26) except that wide use is prevented by the handling of radioisotopes. The gold standard for assaying killing, colony counting, is not easily reproducible (5, 16, 19, 20, 23). It requires the tedious and time-consuming counting of viable cells. The method of solubilizing phagocytes to release ingested cells and then staining with conventional dyes is an attractive alternative, but the microscope is still required (18).

We describe here simple, rapid, and reproducible assays for measuring phagocytosis (association and uptake) and killing of *C. neoformans* by human monocytes by flow cytometry. In the phagocytosis assay, trypan blue was used as a quencher of fluorescein isothiocynate (FITC)-labelled cryptococci. This enabled us to distinguish yeast cells that had been taken up by monocytes from those that were simply attached. The flow cytometry assay for measuring killing of *C. neoformans* by human monocytes was based on the ability of *C. neoformans* to take up the dye 2′-7[1]-*bis*(2-carboxyethyl)-5-carboxyfluorescein acetoxymethylester (BCECF-AM) and retain the hydrolyzed form 2′-7[1]-*bis*(2-carboxyethyl)-5-carboxyfluorescein (BCECF). Viable cells retained the dye, but killed cells lost the dye and

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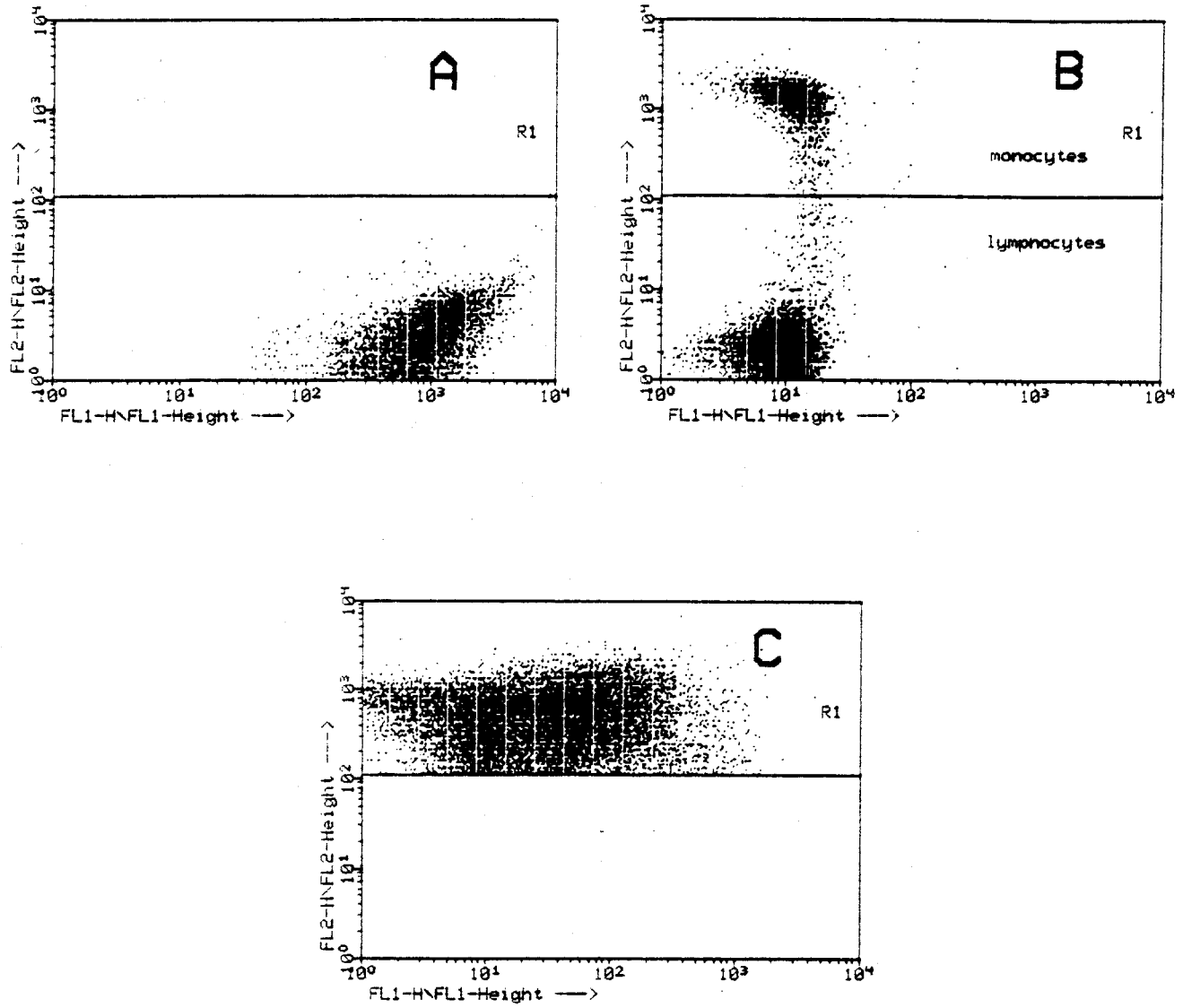

FIG. 1. Flow cytometric analysis of FITC-labelled *C. neoformans* (10^7) (A), PBMC (10^6) after labelling with anti-CD14-PE (B), and a mixture of *C. neoformans* and PBMC (E:T, 2:1) at 2 h (C). The distribution patterns of the fluorescence show particles with FL2 (red) against FL1 (green). *C. neoformans* cells are seen as a distinct population (A), and labelled monocytes are seen as the population above the line (B). Incubation of *C. neoformans* with monocytes resulted in a shift in the distribution of fluorescence (C). A total of 5,000 particles were counted for each determination.

thus their fluorescence (18). These methods were compared with the available standard techniques.

MATERIALS AND METHODS

Strains. Encapsulated *C. neoformans* NIH 37 serotype A used for all experiments was obtained from the National Institutes of Health (NIH, Bethesda, Md.). NIH 37 is a clinical isolate obtained from cerebrospinal fluid by K. J. Kwon-Chung. Most clinical isolates are predominantly of the A serotype (8). NIH 37 was maintained on Sabouraud dextrose agar (Merck, Darmstadt, Germany) at 4°C.

Isolation of PBMC. Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats from healthy donors. Centrifugation on a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden) was performed as described by Böyum (3). The mononuclear cells layer was harvested and suspended at 5×10^7 ml^{-1} in RPMI 1640 (Gibco BRL, Gaithersburg, Md.) containing 0.05% human AB serum.

Sera. Sera from 30 healthy Dutch individuals were pooled and stored in small aliquots at −70°C until use; this pool is referred to as normal human serum (NHS). In some experiments, NHS was heated at 56°C for 30 min to inactivate complement components (5, 9, 12); this is referred to as heat-inactivated (HI) NHS. Rabbits were given three weekly intravenous injections of antigen consisting of *C. neoformans* NIH 37 in 0.01 M phosphate-buffered saline (PBS) with 0.3% Formalin. The rabbits were bled, and the immune sera were collected. Specific immunoglobulin G (IgG) was purified from rabbit immune serum by protein G chromatography (Pharmacia). A concentration of 200 μg of IgG per ml was used in the study.

Determination of association of *C. neoformans* with monocytes by flow cytometry. For use in the phagocytosis assay, *C. neoformans* cells were heat killed (56°C for 30 min), and the concentration was adjusted to 10^8 ml^{-1}. The yeast cells were labelled with FITC (Sigma Chemical Co., St. Louis, Mo.) at 0.5 mg ml^{-1} in PBS at 22°C for 10 min. Initial experiments gave low association between nonopsonized *C. neoformans* and monocytes. Preopsonization of *C. neoformans* with various concentrations of NHS showed that 10% NHS gave optimal association with monocytes. The FITC-labelled yeast cells were then opsonized with either 10% NHS or 10% HI NHS for 30 min at 37°C. Opsonized *C. neoformans* (100 μl; 10^8 ml^{-1}) was incubated with 100 μl of PBMC (5×10^7 ml^{-1}) in a water bath (37°C) for the different phagocytosis times. Phagocytosis was stopped by adding 1 ml of ice-cold PBS to the suspension. Half of the suspension was used for association experiments, and the other half was used for the quenching assay to determine uptake (see below).

C. neoformans and monocytes are the same size, which makes it difficult to distinguish free FITC-labelled *C. neoformans* cells from *C. neoformans* cells

associated with monocytes in the flow cytometer. In order to distinguish monocytes from *C. neoformans* as well as from lymphocytes, monocytes were labelled with anti-CD14 phycoerythrin (PE)-labelled monoclonal antibody. The *C. neoformans*-PBMC suspension was centrifuged, and 5 μl of monoclonal anti-CD14-PE (10 μg ml^{-1}) (Becton Dickinson, Mountain View, Calif.) was added to the pellet, mixed gently, and incubated at 4°C on ice for 30 min. The pellet was then resuspended in PBS, and the suspension was fixed in 1% paraformaldehyde solution (Polysciences Inc., Warrington, Pa.) in PBS (13).

The association of FITC-labelled yeast cells (FL1) to anti-CD14-labelled monocytes (FL2) was analyzed with a FACScan flow cytometer (Becton Dickinson) with computer-assisted evaluation of data (LYSYS II).

Determination of uptake by flow cytometry. Uptake of *C. neoformans* by monocytes by flow cytometry was determined by a modified method of Van Amersfoort et al. (24). An equal volume of trypan blue (200 μg/ml) (Merck) was added to each test suspension and incubated for 10 min. At 200 μg ml^{-1}, optimal quenching of *C. neoformans* was achieved. Unbound trypan blue was then removed by centrifugation. Monocytes were labelled as previously described. By flow cytometry, percent uptake was determined as 100 × [(FL1 after quenching)/(FL1 without quenching)].

Fluorescence microscopy. For comparison with association observed by flow cytometry, microscopy slides were prepared from the supernatant before paraformaldehyde fixation in association experiments for the flow cytometry studies (see above). Slides were analyzed under a fluorescence microscope (Leitz Inc, Rockleigh, N.J.). Monocytes with one or more yeast cells were counted as associated cells. To compare the results on uptake, the method of Uvitex 2B staining originally described by Levitz (16) was employed. Briefly, after phagocytosis (as above), samples were centrifuged, and the pellet was treated with 10 μl of 1% Uvitex 2B (Reinehr & Rembold, Kandern, Germany). Uvitex specifically stains the cell membranes of fungi (16). The pellet was then resuspended in PBS and centrifuged over a Ficoll-Paque gradient, the interface with monocytes associated with *C. neoformans* was collected and cytospun, and the slides were fixed with methanol. Yeast cells that were internalized were not stained, as Uvitex is occluded from phagocytes. Excitation of Uvitex 2B by UV light gave a blue fluorescence. Noninternalized yeast cells stained blue, but internalized cells retained FITC green fluorescence.

Determination of killing of *C. neoformans* by flow cytometry. The method of Martin and Bhakdi used to study killing of *Candida albicans* by leukocytes (18) was extended to study killing of *C. neoformans* by monocytes. *C. neoformans* was transferred from stock culture and grown overnight at 37°C in Sabouraud liquid medium (Merck). Cells were incubated with 20 μl of 0.2 mM BCECF-AM (Molecular Probes Inc, Eugene, Oreg.) per ml. Viable cells retained the fluorescent dye, whereas dead cells lost it. *C. neoformans* (100 μl) was incubated with 100 μl of PBMC to achieve three effector-to-target cell ratios (E:T), 1:1, 10:1, and 50:1. After the incubation period, blood cells were solubilized by adding 1 ml of 25 mM deoxycholate (DOC) (Merck) per ml of suspension as described by Lehrer (13a). The blood cells were lysed by DOC to release internalized cells. By flow cytometry, killing was quantified as the difference in green fluorescence (FL1) of *C. neoformans* phagocytosed by monocytes at 37°C and that of control samples kept on ice.

Determination of killing of *C. neoformans* by colony counts. Colony counts were made to determine the number of viable *C. neoformans* cells remaining after phagocytosis to compare cell death with killing observed after flow cytometry. A known volume was taken from the same test suspension used on the flow cytometer to assay killing (above), serially diluted, and plated on Sabouraud dextrose agar. After 72 h, the colonies on the plates were counted. Only plates with 50 to 200 colonies were included. Percent killing was calculated as 1 − [(viable cells in test)/(viable cells in control [yeast cells alone])] × 100.

RESULTS

Association of *C. neoformans* with monocytes. In order to determine the association of *C. neoformans* with and its uptake by mononuclear cells, monocytes were labelled with anti-CD14-PE. This allowed monocytes to be distinguished from lymphocytes and from *C. neoformans*. Figure 1 shows the results of flow cytometric analysis of FITC-labelled *C. neoformans*, anti-CD14-labelled monocytes, and nonlabelled lymphocytes, and an incubation mixture of yeast cells and monocytes after 2 h of incubation. Prior to the experiment, the yeast cells were observed to be homogeneously fluorescent. The FITC-labelled yeast cells and the PE-labelled monocytes were seen as distinct populations on the flow cytometer (Fig. 1A and B). Association of *C. neoformans* with monocytes could be observed as a change in the distribution pattern of green fluorescence (FL1) in the gated region of monocytes. This FL1 was used for calculations on association, uptake, and killing of *C. neoformans*.

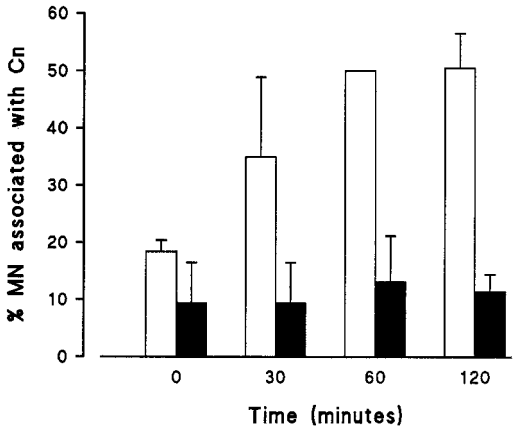

FIG. 2. Determination of the percentage monocytes (MN) associated with *C. neoformans* (Cn) over time by flow cytometry. *C. neoformans* was opsonized with either NHS or HI NHS, and an E:T ratio of 1:2 was used. Each bar is the average of three measurements ± standard deviation. Each determination was performed in duplicate. Note the low association observed with HI NHS compared with NHS. Optimal association is seen after 60 min.

To study the kinetics of association of *C. neoformans* to monocytes, phagocytosis was allowed to proceed for 30, 60, or 120 min. Increasing phagocytosis time resulted in enhanced association of NHS-opsonized *C. neoformans* with monocytes over time, reaching a maximum after 1 h (50% ± 11%) (Fig. 2). The association of *C. neoformans* opsonized with HI NHS remained low during all phagocytosis times studied. In subsequent experiments, a phagocytosis time of 2 h was used in order to ensure optimal association. Association of FITC-labelled *C. neoformans* with monocytes was readily observable by fluorescence microscopy. On average, 3 to 4 cryptococci were associated with 1 monocyte when an E:T ratio of 1:2 was used.

Uptake of *C. neoformans* by monocytes. In order to differentiate between extracellularly adhering *C. neoformans* cells and those that had been taken up, we used trypan blue as a quenching agent for the FITC-labelled *C. neoformans. C. neoformans* alone was effectively quenched with trypan blue (96.6% ± 1.5%, $n = 5$) (Fig. 3A) and also when *C. neoformans* cells were associated with monocytes (90.36% ± 7.9%, $n = 5$) (Fig. 3B). The green fluorescence of *C. neoformans* cells that have been taken up by monocytes will not be removed by quenching, as trypan blue is excluded from monocytes. Uptake was therefore determined as the difference in the percentage of monocytes associated with FITC-labelled *C. neoformans* (FL1) before and after quenching. Figure 4A shows a comparison of the flow cytometry method with microscopy to determine the uptake of *C. neoformans* by monocytes after 0 and 2 h of phagocytosis times. The pattern of uptake was similar for both methods, although a large difference (>10%) was seen in the absolute number of monocytes that had taken up *C. neoformans*. Hardly any uptake was seen at 0 h. It was possible to apply the flow cytometry method as described to the study of the influence of opsonins (NHS, HI NHS, IgG, and specific IgG) on monocyte association and uptake of *C. neoformans*. Specific IgG (200 μg/ml) enhanced both association with and uptake by monocytes, as shown by flow cytometry (Fig. 4B).

Quantification of killing. In order to study killing of viable *C. neoformans* by monocytes, we used dye that is capable of permeating the membrane, BCECF-AM, which on entering the yeast cells was cleaved to a form that cannot permeate the membrane, BCECF. The labelled cells were found to be homogeneous in size and fluorescence intensity prior to use in

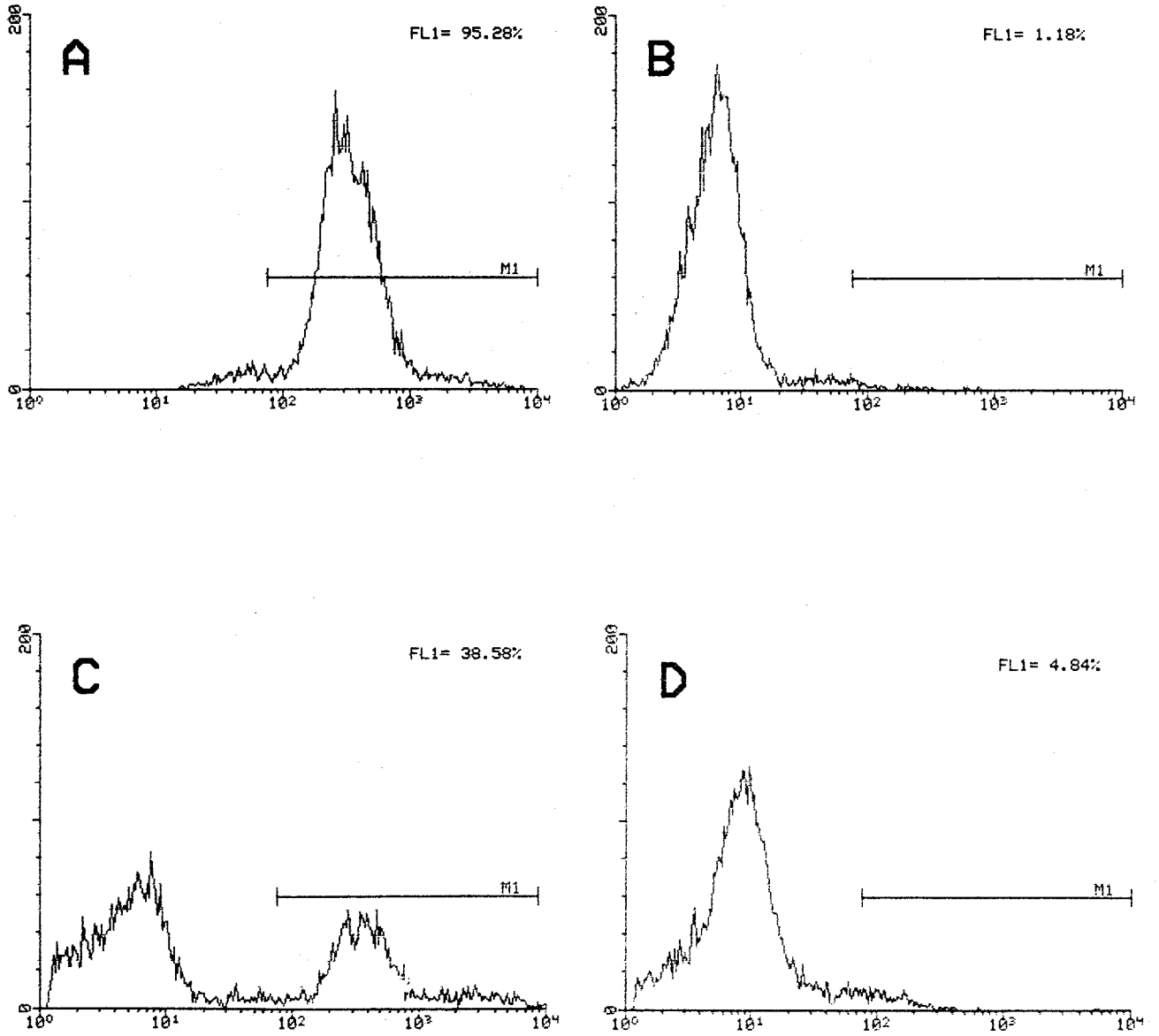

FIG. 3. Efficiency of the trypan blue quenching technique with *C. neoformans* alone (A and B) and *C. neoformans* in association with monocytes (C and D) as assessed by flow cytometry. The marker (M1) defines the area of green fluorescence exhibited by the FITC-labelled yeast cells. (A and C) Green fluorescence of *C. neoformans* alone and *C. neoformans* in association with monocytes, respectively, before quenching. (B and D) Green fluorescence of *C. neoformans* alone and *C. neoformans* in association with monocytes, respectively, after quenching with trypan blue. Trypan blue effectively quenched *C. neoformans* alone (96% ± 2%, $n = 5$) and when associated with monocytes (90.36% ± 7.9%, $n = 5$). The graphs show the results of single experiments (5,000 particles per determination) that are representative of five experiments. The high quenching efficiency makes it possible to study uptake of *C. neoformans* by monocytes with high reliability.

experiments, and approximately 92% of the cells were fluorescent at the start of each experiment. DOC treatment of the supernatant to lyse blood cells did not alter the viability of the yeast cells, as determined by colony counting (data not shown). The fluorescence of the cells was also not affected. The change in fluorescence pattern in DOC-treated samples therefore indicated loss of viability of *C. neoformans* (Fig. 5). The gold standard to assay killing was used parallel to the flow cytometry assay, and the two methods were compared. Both methods yielded similar results. Figure 6 shows the results of killing of NHS-opsonized *C. neoformans* by monocytes after 3 h of phagocytosis. An increase in the E:T ratio resulted in higher killing, as assessed by both assays. With flow cytometry, killing rates of 23% ± 17%, 22% ± 9%, and 40% ± 13% were obtained with E:T ratios of 1:1, 10:1, and 50:1, respectively. The percent killing of *C. neoformans* by monocytes as assessed by flow cytometry and colony counting for any particular experiment never deviated by more than 10%.

DISCUSSION

The conditions under which phagocytic uptake versus attachment alone takes place appear to be important determinants of the activation process of phagocytes. They determine the microbicidal capacities of these cells, as there is evidence that incompletely phagocytosed microorganisms are not killed as readily as those that are completely phagocytosed (15). Methods for the measurement of phagocytosis should there-

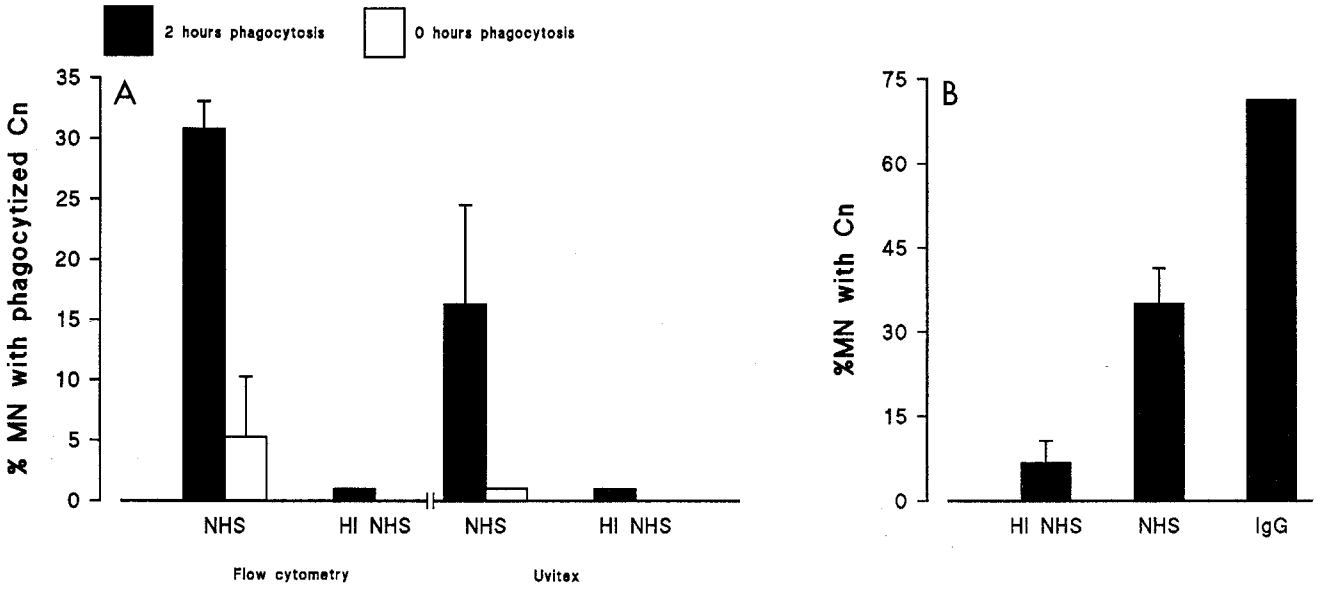

FIG. 4. (A) Comparison of microscopy and flow cytometry methods to study the uptake of *C. neoformans* (Cn) by monocytes (MN). *C. neoformans* opsonized with 10% HI NHS or NHS was incubated with PBMC (E:T, 1:2) for 0 and 2 h. Higher uptake of *C. neoformans* opsonized with NHS was seen by both methods at 2 h of phagocytosis time, but almost no uptake was seen with HI NHS. Note the similar pattern with both assays. The bars represent the mean ± standard deviation of three experiments performed in duplicate. (B) Influence of opsonin on percent uptake of *C. neoformans* by monocytes. *C. neoformans* was opsonized with either 10% HI NHS, NHS, IgG (data did not differ from those with HI NHS and are therefore not shown), or specific IgG (200 μg/ml) and incubated with monocytes (E:T, 1:2) for 2 h. Association and uptake were then determined by the flow cytometry method. Percent uptake is the number of cryptococci taken up by monocytes, expressed as a percentage of the total number of cryptococci associated with monocytes. Specific IgG enhanced both association and uptake. The bars represent the mean ± standard deviation of three experiments performed in duplicate.

fore be able to discriminate between adherence and uptake and sufficiently sensitive to detect this difference in the early stages of phagocytosis. Furthermore, they should be objective and, for the clinical setting, easy to perform.

Few studies on the kinetics of cell association, uptake, and killing of *C. neoformans* by monocytes have been reported (9, 14, 18, 21). These studies were performed with staining methods and counting under the microscope (association and uptake) or colony counting (killing). Microscopic methods to determine association and uptake, however, are cumbersome to perform and subjective. In addition, they require preparative procedures that may influence test results. For example, in the Uvitex method, the samples have to be centrifuged over Ficoll-Paque, the interface with monocytes associated with *C. neoformans* must be collected, washed, and cytospun, and the slides must be fixed with methanol. This method is cumbersome, and the preparatory steps are such that cells may be lost. This may account for the low absolute numbers of monocytes

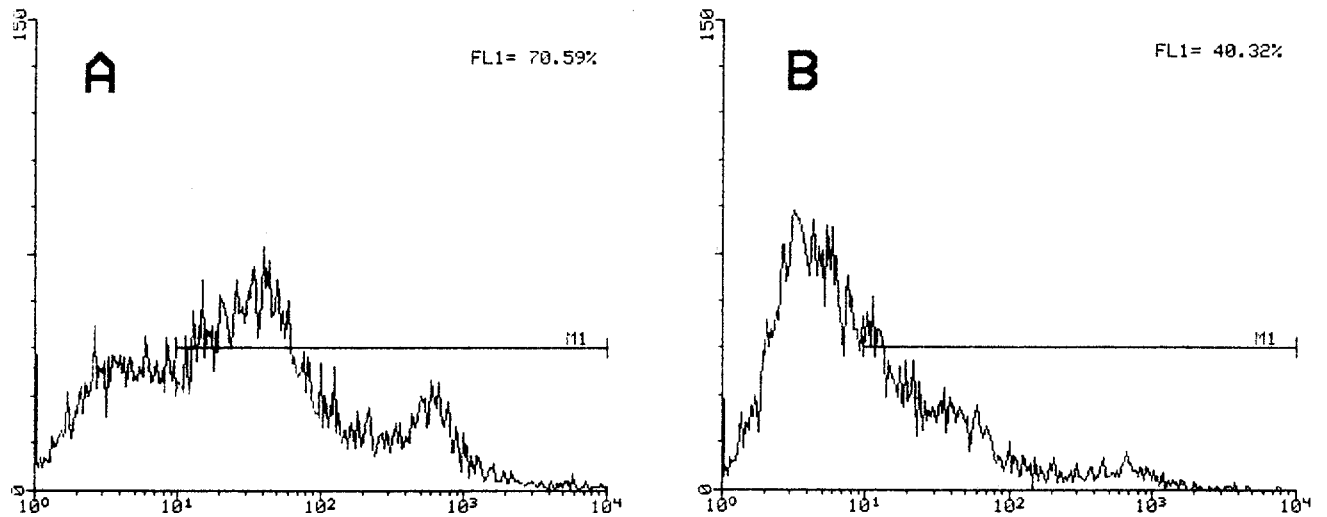

FIG. 5. Assay for killing of *C. neoformans* (Cn) by monocytes (MN) by flow cytometry. M1 defines the area of green fluorescence of BCECF-labelled *C. neoformans*. After incubation with PBMC, the loss in green fluorescence indicated a loss in viability (confirmed by colony counting). Killing was estimated as the difference in green fluorescence of *C. neoformans* incubated with monocytes at 37°C and that of control samples kept at 4°C. The histogram shows 30% killing of *C. neoformans* by monocytes in a single experiment. A total of 5,000 particles were counted per determination.

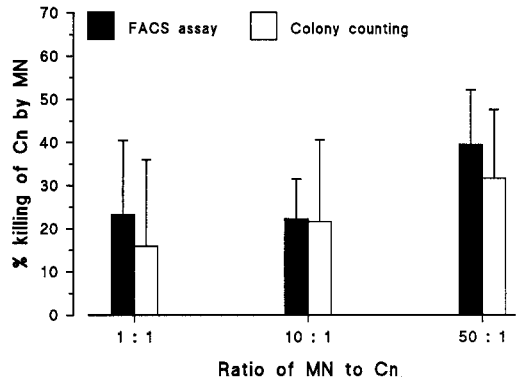

FIG. 6. Comparison of killing of NHS-opsonized *C. neoformans* (Cn) by monocytes (MN) after 3 h of phagocytosis as determined by flow cytometry and colony-counting methods. Three E:T ratios were used, 1:1, 10:1, and 50:1. The 50:1 ratio gave the highest killing. The percent killing assessed by the two methods was comparable. The bars represent the mean ± standard deviation of three experiments performed in triplicate.

that have taken up *C. neoformans* that are found when the Uvitex method is used in comparison to those seen by the flow cytometry method under the same experimental conditions (Fig. 4A).

Colony counting is also a slow and cumbersome assay which requires considerable expertise to be reproducible. In killing assays, phagocytes have to be lysed, and viable organisms are determined by colony counts. Killing is said to occur if fewer colonies are found at the end of the experiment than in controls containing cryptococci alone. A major problem with this type of assay is the high detection limit. A substantial number of the cryptococci have to be killed, or, as a result of the growth of *C. neoformans* during incubation, no differences are found between the control and the sample. This is why high phagocyte-microorganism ratios are often used, in order to observe killing in a colony-counting assay.

This paper describes flow cytometry assays for the determination of association, uptake, and killing of *C. neoformans* by monocytes. The preparative procedure for the flow cytometry determinations is straightforward, requiring only the addition of the quenching agent and thereafter washing and labelling of the monocytes for the uptake assay. It minimizes the time-consuming and tedious counting of cells under the microscope. The results obtained are more reliable, as higher numbers of cells (5,000) were counted per determination than with the microscope method (100). Rapid measurements of parameters such as total uptake and percent phagocytic cells can be done, and there is no bias in the interpretation of results. In addition, the flow cytometry method allows the kinetics of the phagocytic process to be monitored, including the early phases of the process.

We compared the flow cytometry methods developed for association, uptake, and killing with the classical methods. The flow cytometry methods for association and uptake as well as that for killing give results comparable to those of the classical methods (Fig. 4A and 6). A limitation of the flow cytometry killing method is that killing has to be determined within 3 h, because the replication time of *C. neoformans* is 2 to 3 h (data not shown). Growth of *C. neoformans* diminishes the extent of BCECF labelling of the cells, and therefore killing after 16 h cannot be determined by this method.

Previous results on the association, uptake, and killing of *C. neoformans* by monocytes have been reported (5, 17, 25, 27). Levitz et al. used microscopy to look at the association of NHS-opsonized *C. neoformans* with monocytes and observed a binding index (number of associated yeast cells per 100 monocytes) of 98 after 30 min of phagocytosis time (17). They used an E:T ratio of 0.4:1. Diamond et al. (5) and Vecchiarelli et al. (25) evaluated phagocytic uptake of *C. neoformans* by monocytes by staining methods and observed an uptake of 61% (E:T, 1:1) and 62% (E:T, 1:10) respectively. In our study with the flow cytometry methods, only 50% of the monocytes became associated with *C. neoformans*. Of those monocytes that were associated with *C. neoformans*, about 30% had taken up *C. neoformans* after 2 h (Fig. 4A). We, however, are determining a different parameter, i.e., the percentage of monocytes associated with *C. neoformans* or monocytes which have taken up the cells, whereas the others counted the number of *C. neoformans* cells per 100 monocytes. Moreover, we used a mixed PBMC population, which contained more lymphocytes than monocytes, instead of the adherent monocytes used by Levitz and Farell (16).

The capsular polysaccharide of *C. neoformans* is known to be an important determinant in the phagocytosis of *C. neoformans* (12, 16), and the differences between our observations and those of other investigators could be due to different capsule sizes of the strains used. The low association of NHS-opsonized *C. neoformans* with monocytes seen in Levitz's study (17) and our study is, however, in contrast to that found in experiments with NHS-opsonized *Candida albicans*, which showed that association was essentially complete within 20 min, after which no free yeast cells were detectable (1, 18). The association and therefore the uptake of *C. neoformans* by monocytes appears to be a slow and inefficient process compared with that by *Candida albicans*. The low association and uptake of *C. neoformans* by PBMC could however be enhanced by specific IgG (200 $\mu g\ ml^{-1}$) (Fig. 3B). This is in agreement with previous reports on the role of specific antibody in the phagocytosis of *C. neoformans* (9, 11, 20).

The first documentation of killing of *C. neoformans* by monocytes employed the technique of colony counting. Killing ranged from 22 to 76% within 1 to 2 h in the presence of NHS (5). Washburn et al. found 58% ± 4% and 75% ± 3% killing of *C. neoformans* by monocytes from normal subjects and AIDS patients, respectively (27). In another study, killing of 12 strains of encapsulated *C. neoformans* (E:T, 1:1) ranged from 14.9 to 63.5% (19). Our results with flow cytometry are in the same range: 22 to 40% killing with E:T ratios of 1:1, 10:1, and 50:1. They demonstrate the low efficiency of killing of *C. neoformans* by monocytes.

In conclusion, the procedures described in this study allow investigations of various aspects of phagocytosis and killing of *C. neoformans*. By using anti-CD14-PE to stain monocytes, it was possible to study phagocytosis and killing at the single-cell level without having to separate monocytes from lymphocytes. Given the importance of *C. neoformans* as an opportunistic pathogen in AIDS patients, the new flow cytometry method that we have described is attractive to study phagocyte function in terms of elimination of *C. neoformans* in a clinical or laboratory setting.

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