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Phagocytosis of Bacteria by Human Leukocytes Measured by Flow Cytometry (41722)

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Abstract. A new method has been developed for the evaluation of the phagocytic activity of human leukocytes using fluorescently labeled bacteria and flow cytometry. By simultaneous measurement of cellular light scatter and fluorescence, extracellular bacteria, phagocytes, and nonphagocytes could be discriminated and quantified. All leukocytes assumed to be capable of phagocytosis were phagocytosing, and about 90% of these cells were polymorphonuclear neutrophilic granulocytes. Within 15 min 85% of the bacteria were phagocytosed and each phagocyte contained an average of 15–20 bacteria. The phagocytic capacity of the leukocytes from healthy individuals showed minor interindividual and day-to-day variations. This method facilitates a rapid and accurate *in vitro* evaluation of the phagocytic activity of human leukocytes.

Ingestion of bacteria by phagocytic cells represents a cornerstone in host defence against infection, and several *in vitro* methods are available for the measurement of this function (for review see (1)). Usually, these methods estimate the phagocytic capacity of large cell populations, and limited information is obtained on the activity of each cell. So far, identification of separate phagocytic cells and estimation of the number of microorganisms ingested by each cell have required time-consuming microscopy. In a preliminary report (2), two of us have described a method for rapid and automatic detection of phagocytosing leukocytes using flow cytometry (FCM). The method is based on the phagocytosis of bacteria labeled with fluorescein-isothiocyanate (FITC) and discrimination of phagocytes and nonphagocytes by differences in narrow angle light scatter and fluorescence intensity. Similar approaches have been used by others (3–8).

Materials and Methods. *Leukocytes.* Ten milliliters heparinized (10 U/ml) human blood from each of 85 healthy individuals was mixed with 3 ml 6% dextran (mol wt 70,000) (Pharmacia, Uppsala, Sweden) and allowed to sediment for 1 hr at 20°C. After centrifugation of the leukocyte-rich plasma (400*g*, 5 min) the red cells were lysed with 0.8% NH_4Cl. The leukocytes were then washed in 0.85% NaCl containing 2% bovine serum albumin (Sigma Chemicals, St. Louis) and resuspended in Hanks' balanced salt solution (HBSS) containing 2% bovine serum albumin to make a concentration of 10^7 nonlymphocytes/ml. Total leukocyte counts were made in a Coulter Counter Model B (Coulter Electronics, Harpenden, England) and differential counts by FCM measurements of acridine orange-stained cells (9), see below.

Serum. Pooled fresh, human serum from eight healthy individuals was stored in 1-ml aliquots at −80°C. Immediately before use, 1 ml freshly thawed serum was diluted with 3 ml HBSS.

Bacteria. Staphylococcus aureus (Strain 209, obtained from the National Collection of Type Cultures, Colindale, London 1958) was cultured overnight in Penassay broth (Difco, Surrey, England), washed twice in 0.85% NaCl, killed by exposure to 60°C for 1 hr, and labeled with FITC, without adding gelatin, according to the method of Gelfand *et al.* (10). All preparative solutions were filtered through 0.2-μm-pore-size filters to avoid contamination. The bacteria were pelleted by centrifugation, resuspended in HBSS, and sonicated for 30 sec at 2.5 A. For adjustment of the concentration of bacteria known volumes of bacteria and leukocyte suspensions were added to 6 ml 0.85% NaCl containing 0.02% EDTA. The relative numbers of bacteria and leukocytes were then measured by FCM (see below). In later experiments the numbers of bacteria were calculated from known numbers of leukocytes in the mixed suspensions and these relative counts. If not

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otherwise stated, the bacteria concentration was adjusted to 10^9 per milliliter in HBSS, aliquots stored at −80°C, and thawed at room temperature immediately before each experiment.

Phagocytosis. One-half milliliter of the leukocyte suspension, 0.1 ml of the bacteria suspension, and 0.4 ml of diluted serum were mixed in 12 × 75-mm disposable plastic tubes. Under standardized procedures this provided 20 bacteria per nonlymphocyte and a final concentration of 10% serum. The tubes were incubated at 37°C for 15 min with an end-over-end rotation in order to promote contact between bacteria and leukocytes. Phagocytosis was terminated by the addition at 4°C of 6 ml 0.9% NaCl, containing 0.02% EDTA. The suspensions were then subjected to FCM.

Flow cytometry. A Cytofluorograf Ortho 50H interfaced to a Model 2150 Computer (Ortho Diagnostic Instruments, Westwood, Mass.) with an excitation wavelength at 488 nm was used. For phagocytosis assay, FITC fluorescence was measured at 515–575 nm and forward angle light scatter at 488 nm. The pulse area values were used throughout. Leukocytes and bacteria were discriminated by fluorescence and light scatter. For differential counting of acridine orange stained leukocytes, according to Melamed *et al.* (9), green fluorescence was measured at 515–575 nm and red fluorescence at 600–650 nm.

Morphology. Cytological smears of leukocyte and bacteria suspensions were prepared before and after flow sorting and stained by May–Grünwald–Giemsa. To inhibit cell lysis, the cells were sorted into drops of 0.85% NaCl containing 2% bovine serum albumin layered on slides. Sorted bacteria and leukocytes were examined by combined light and fluorescence microscopy using an Orthoplan microscope equipped with a Ploemopak Fluorescence Vertical Illuminator (Leitz, Wetzlar, West Germany).

Statistical methods. Significance of correlation was determined by Student's *t* test.

Results. The extracellular bacteria were clearly discriminated from phagocytes and nonphagocytes by simultaneous measurement of light scatter and fluorescence (Fig. 1). The leukocytes caused a more pronounced light scatter, mainly because of larger cell size. The majority of these cells exhibited a high fluorescence most likely due to ingestion of bacteria.

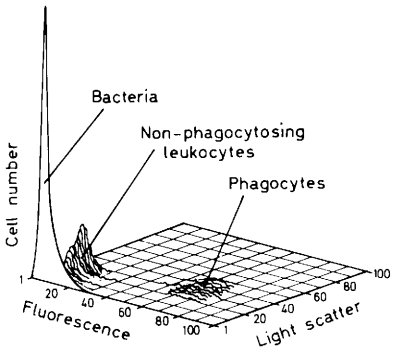

FIG. 1. Three-dimensional computer drawing of bacteria, phagocytes, and nonphagocytes following incubation of leukocytes and bacteria and combined measurement of fluorescence and narrow-angle light scatter. The vertical axis represents the number of cells counted.

When phagocytosis was inhibited by dilution with EDTA solution, more than 98% of the leukocytes were localized in region 2, i.e., to the left of the extracellular bacteria along the fluorescence axis (Fig. 2A). Following phagocytosis, however, leukocytes were also found within region 3 of the cytogram (Fig. 2B). The cell count within regions 1–3 amounted to more than 97% of the total cell count.

By means of electronic windows, extracellular bacteria, phagocytes, and nonphagocytes were sorted out and examined by light and fluorescence microscopy. More than 99% pure suspensions of bacteria, phagocytes, and nonphagocytes were obtained. No fluorescence was observed in the separated nonphagocytes, which consisted of 98% mononuclear cells.

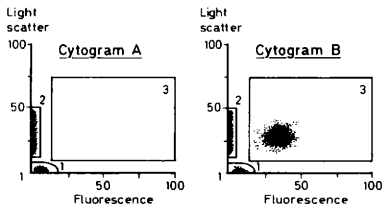

FIG. 2. Cytograms of leukocytes and bacteria before (A) and after (B) phagocytosis. Each dot represents one cell. The numbers of cells in the selected regions 1, 2, or 3 can be counted as separate populations and represent bacteria, nonphagocytes, and phagocytes, respectively.

The original suspension of leukocytes from healthy individuals, stained by acridine orange and analyzed by FCM contained 68.1 ± 12.1% PMNLs, 25.5 ± 12.2% lymphocytes, and 5.2 ± 6.6% monocytes (mean ± SD). Only 4.3 ± 3.9% of the cells were registered as doublets or larger cell aggregates. The fraction of phagocytosing leukocytes, i.e., the fraction of leukocytes within region 3 (Fig. 2B), amounted to 70.0 ± 12.4%. The proportion of doublets or larger phagocyte aggregates was 3.5 ± 3.0%. A linear correlation ($r = 0.8056$, $P < 0.001$ slope = 0.8825) was observed between the percentage of nonlymphocytes measured by acridine orange staining and the percentage of phagocytosing leukocytes (Fig. 3). This indicates that all leukocytes assumed to be capable of phagocytosis were actually phagocytosing. Since only 5.2 ± 6.6% of the total leukocyte count were monocytes about 93% of the phagocytosing leukocytes were PMNLs.

The fluorescence distribution of the extracellular bacteria was unimodal, i.e., exhibiting only one peak, and roughly symmetrical (Figs. 1 and 4) indicating limited aggregation of bacteria. On 60 consecutive FCM determinations of the bacteria concentration, the coefficient of variation was 7.7%. On twofold dilutions of the suspensions, the numbers of bacteria measured by FCM correlated well ($r = 0.9878$, $P < 0.001$) with the estimated numbers based on dilutions of the original bacteria suspension.

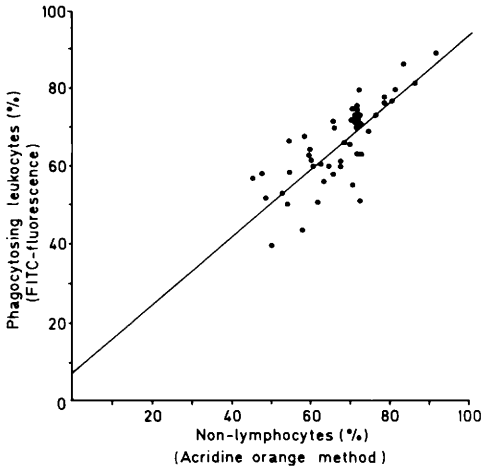

FIG. 3. Correlation between the proportion of phagocytosing leukocytes and nonlymphocytes, i.e., leukocytes capable of phagocytosis. Phagocyte counts were obtained by flow cytometry after uptake of FITC-labeled bacteria and nonlymphocyte counts after acridine orange staining. Each dot represents one individual leukocyte suspension.

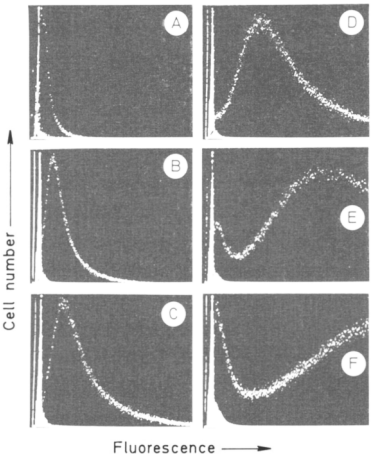

FIG. 4. Effect of bacteria concentration on phagocyte fluorescence. The solid histogram represents the bacteria and the dotted lines the phagocytes. The initial ratios of bacteria to nonlymphocytes were: (A) 1:1, (B) 5:1, (C) 10:1, (D) 20:1, (E) 40:1, and (F) 60:1.

When constant numbers of leukocytes were incubated with increasing numbers of bacteria, the bacteria-to-nonlymphocyte ratio ranging from 1:1 to 60:1, the fluorescence of the phagocytes increased (Fig. 4). At the same time the peak fluorescence of the bacteria was unaltered. However, at a ratio of 40:1 or higher coincidences between single bacteria and between bacteria and nonphagocytes was prominent (Figs. 4E and F). At very high bacteria concentrations only part of the phagocyte populations could be visualized simultaneously with the bacteria (Figs. 4E and F). For further experiments, therefore, a ratio of bacteria to nonlymphocytes of 20;1 was chosen.

In leukocyte suspensions from 25 healthy individuals, 1–15 contaminating erythrocytes per 10 PMNLs did neither influence the fractions of nonphagocytosing leukocytes, nor the fluorescence of the phagocytes. In the sorted populations less than 1% erythrocytes were seen among the phagocytes and nonphagocytes (results not shown).

After termination of phagocytosis, the relative numbers of extracellular bacteria and phagocytes were obtained from regions 1 and

3 of the cytogram (Fig. 2B). Based on these measurements the loss of bacteria from the suspensions was calculated. Leukocytes from healthy individuals engulfed approximately 85% of all bacteria within 15 min. Each phagocyte then contained between 15 and 20 bacteria (Table I). Only minor differences in the average number of bacteria ingested by phagocytes from different individuals on different days were observed. The FCM estimates of the numbers of phagocytosed bacteria correlated to microscopical counts of bacteria in phagocytes sorted by FCM (Fig. 5).

Discussion. Discrimination of the cell types in a phagocytic system remains a prerequisite for the measurement of phagocytosis by FCM. In the present study, the suspensions contained phagocytes, nonphagocytes, and fluorescent-labeled bacteria. When cells from each of these populations were separated by flow sorting and examined by combined light and fluorescence microscopy, more than 99% pure populations of either bacteria, phagocytes, or nonphagocytes were observed. No fluorescence was observed in the nonphagocyte population. This demonstrates a high discrimination capacity of our FCM method.

A precise quantitation of cells in the suspensions is also necessary for the FCM measurement of phagocytosis. In the present study a linear correlation was observed between the fractions of phagocytosing leukocytes and the fractions of nonlymphocytes determined by acridine orange staining. Furthermore, the FCM bacteria counts were reduced in parallel to the dilutions of the bacteria suspensions. Finally, during phagocytosis the numbers of extracellular bacteria were reduced conversely in proportion of the numbers of phagocyte-associated bacteria. Accordingly, the numbers of extracellular bacteria, phagocytes, and nonphagocytes seemed to reflect the actual numbers of cells in the suspensions.

TABLE I. NUMBER OF BACTERIA PER PHAGOCYTE FOLLOWING INCUBATION OF 20 BACTERIA PER PHAGOCYTE

Day	Individual no.	Bacteria per phagocyte (mean ± SD)
1	1–7	17.4 ± 0.6 (16.3–18.2)[a]
2	8–11	16.5 ± 1.1 (15.0–17.4)
3	12–18	17.4 ± 1.6 (14.8–19.3)
4	19–28	17.6 ± 0.8 (16.5–19.1)
5	29–37	17.5 ± 1.3 (15.1–18.6)

Note. The phagocytes were obtained from the blood of 37 healthy individuals on each of five consecutive days. The measurements were performed by flow cytometry.

[a] Range in parentheses.

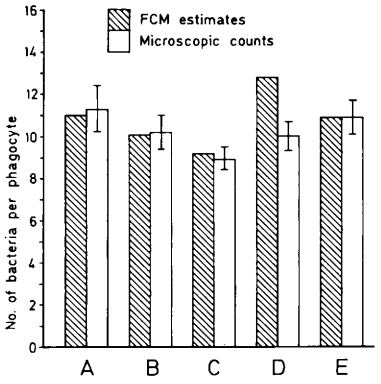

FIG. 5. Correspondence between flow cytometric estimates and microscopic counts of bacteria associated with phagocytes. Phagocytes were sorted by flow cytometry and the numbers of bacteria per phagocyte counted by combined light and fluorescence microscopy. Leukocyte suspensions from five healthy individuals were used, and values are expressed as means ± SEM after the counting of 30 leukocytes.

Aggregation of leukocytes would result in underestimation of phagocytic capacity of the leukocytes. On the other hand, agglutination of bacteria, or coincidence of extracellular bacteria and leukocytes in the laser beam, would result in overestimation of the number of bacteria per phagocyte. In our study, however, the number of leukocyte aggregates, measured by acridine orange staining, were about the same as the numbers of phagocyte aggregates measured in our FCM phagocytosis assay. The fluorescence histograms of bacteria did not change following addition of serum and leukocytes, indicating limited agglutination of bacteria. Furthermore, coincidence between two or more bacteria, or between bacteria and leukocytes, was significant only at very high bacteria concentrations and not at the concentrations recommended in the present FCM method. Accordingly, aggregation of leukocytes, agglutination of bacteria, and coincidence between bacteria, or bacteria and leukocytes, did not interfere significantly with our test results. Finally, as demonstrated in our study, the small numbers of erythrocytes

present in the phagocytic suspension did not influence the measurement.

Phagocyte fluorescence increased with increasing bacteria concentrations, and phagocyte fluorescence may be used as an indirect measure of the number of phagocyte-associated bacteria (2, 6). However, we have observed that the fluorescence of FITC-labeled bacteria was altered when they were associated with phagocytes (manuscript in preparation). This has also been observed by workers using other methods (11). As in our study, however, the problem of altered phagocyte fluorescence can be overcome when phagocytosis is expressed by means of the loss of extracellular bacteria.

FCM offers a rapid and precise technique for the measurement of phagocyte uptake of fluorescent particles. Since up to 5000 cells may be counted and differentiated per second, the results can be obtained almost immediately, and multiple parameters can be measured in a single determination. The percentage of phagocytosing leukocytes and the fluorescence distribution of the phagocytes and the extracellular bacteria add important dimensions to the common measurement of total particle uptake. All these parameters should be considered since they measure the levels of individual cell functions as well as the average function of the phagocyte population.

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