

Phagocytosis, Intracellular pH, and Cell Volume in the Multifunctional Analysis of Granulocytes by Flow Cytometry¹

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Phagocytosis of *Escherichia coli* K12 strain bacteria was used to measure by flow cytometry the functional activities of human granulocytes in whole blood or buffy coat preparations. In a first measurement, the increase in electric cell volume and acridine orange (AO) green and red fluorescence were used to quantify the degree of phagocytosis. In a second measurement, the intracellular pH and esterase activity of each cell were determined with 1,4-diacetoxy-2,3-dicyanobenzene to obtain information on the metabolic activities during phagocytosis and degradation of bacteria. The DNA of dead cells was simultaneously counterstained with propidium iodide in both assays. The volume, the AO green and red fluorescence, the internal

pH, and esterase activity were automatically averaged for all granulocytes or lymphocytes of a measurement. The calculated mean values were transferred into the self-learning database of the DIAGNOS1-program system. The functional granulocyte parameters of normal healthy individuals can be used as reference values for the automated diagnosis of abnormal granulocytes in various infectious disease states. The assays require 1 ml of heparinized whole blood and the results are available within 1 hour.

Key terms: Neutrophils, phagocytosis, cytoplasmic hydrogen-ion concentration, cell volume

Granulocytes play an important role in the defense of the organism through phagocytosis and degradation of bacteria, viruses, or fungi and through endocytosis of immune complexes. The number and activity of granulocyte change during severe infection (1,2,3), and sepsis (6,32), but also after major surgery (9,40), polytrauma, and thermal injury (5,21).

Activated granulocytes cause endothelial damage by the release of proteinases and oxygen radicals in the adult respiratory distress syndrome (41) and during hemodialysis (18).

Analysis of granulocyte activation encounters difficulties in research and clinical laboratories because of the lack of fast, sensitive, and reliable in vitro methods for measurement of granulocyte functions.

Microbiological methods require plating and culture of bacteria surviving after phagocytosis on agar plates (15). Microscopic methods for the quantification of phagocytosis (27) are time-consuming. In cuvette assays, only the total amount of phagocytosis but not the activity of single cells or granulocyte subpopulations can be measured. Chemiluminescence (1), the clearance of [¹⁴C]-

labelled bacteria from suspension (4), the incorporation of [³H]-uridine into extracellular bacteria (26), the uptake of fluorescent latex beads, heat-killed bacteria coupled with FITC (23), latexbeads coupled to fluorogenic substrates (33), or phagocytosis of oil particles containing Oil Red O (7) are determined in such assays with suspensions of isolated granulocytes. The isolation of granulocytes by ficoll-hypaque or dextran sedimentation has been shown to induce spontaneous depolarization of the transmembrane potential (28), increased binding of C3b-coated microspheres, and morphological alteration of the cells (24). Even buffy coat preparation increases the expression of C3b-receptors if centrifugation or storage are performed at a temperature above 28°C (10).

The purpose of this study was to develop sensitive methods for the study of alterations of granulocyte function. Gentle isolation of buffy coat cells from heparinized blood and incubation with viable bacteria in autologous

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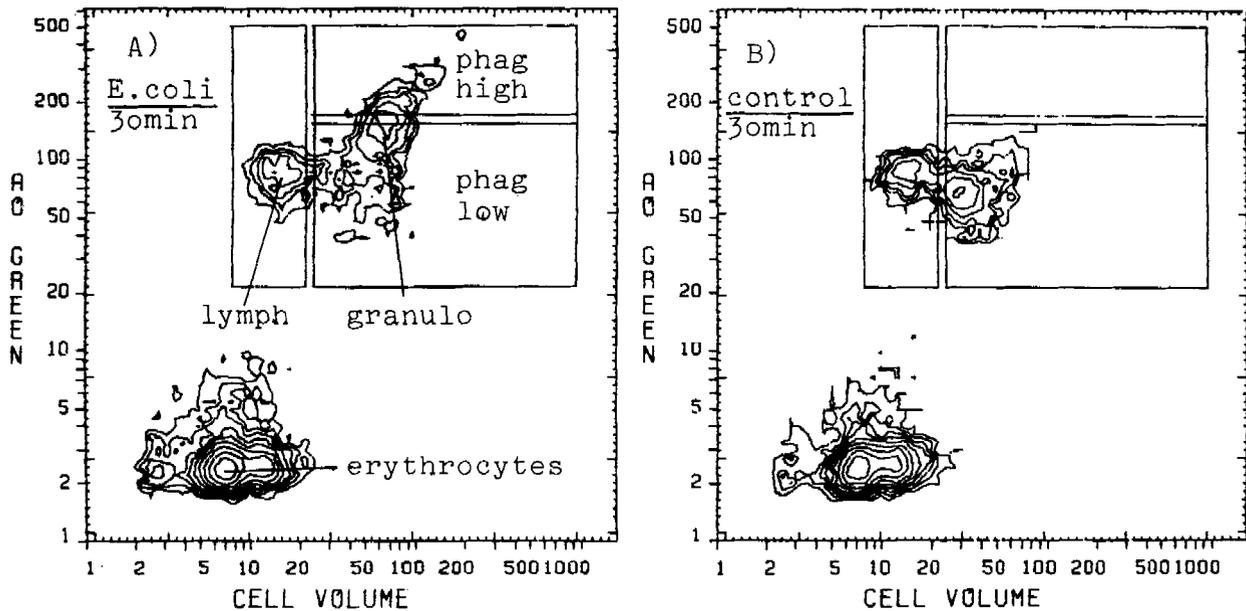


FIG 1. Cell volume vs. acridine orange green fluorescence of buffy coat cells after 30 min incubation at 37°C with (A) and without (B) *E. coli* bacteria. The AO green fluorescence as a measure of cellular DNA increased in granulocytes during incubation with bacteria (A), while the location of the lymphocyte and erythrocyte cell clusters was not altered. The graphs are standardized to the maximum logarithmic

channel contents (100%). Contour lines were drawn in linear steps of 10% downwards. The histograms contain 8,378 and 7,189 cells with 443 and 324 cells in the maximum channel. The cells were simultaneously stained with acridine orange and propidium iodide as shown in FIG. 2. The flow cytometric data were obtained in a simultaneous cell volume, green and red fluorescence list mode measurement.

plasma were followed by functional staining of the cells with fluorescent dyes so as to remain as closely as possible to the physiological environment of the granulocytes. The fluorescences and cell volume of single cells were measured by multiparameter flow cytometry. The phagocytosis of bacteria was quantified by measuring the cell volume and the acridine orange (AO) DNA/RNA staining (25,34) of granulocytes. The esterase activity and the intracellular pH of the granulocytes were determined with 1,4-diacetoxy-2,3-dicyano-benzene (ADB) (38).

MATERIALS AND METHODS

Preparation of Human Leukocytes

Buffy coat leukocytes were prepared from heparinized (10 U/ml) venous blood obtained from 24 healthy donors (age 24 to 45 years). The leukocytes were enriched at the erythrocyte/plasma interface by centrifugation of the blood at 200g and 4°C for 10 min. The buffy coat cells were carefully removed with a 100- μ l disposable tip pipette and resuspended in the supernatant plasma to give a final concentration of 3.5×10^7 leukocytes/ml. The extracellular pH of the plasma, which rose to pH 7.80 by loss of CO₂, was readjusted to pH 7.40 by addition of 10 μ l 1 N HCl per milliliter plasma.

Preparation of *E. coli* K12

Escherichia coli K12 (Sigma, Taufkirchen, FRG) were cultured in RPMI 1640 (Gibco, Karlsruhe, FRG) for two days at 37°C in air with 5% CO₂. The bacteria of a 75-ml polycarbonate culture flask were washed twice in 50 ml HEPES buffered saline (HBS) (0.15 M NaCl, 5 mM HEPES, pH 7.35; Serva, Heidelberg, FRG) and resuspended in HBS to give a final concentration of 7×10^9 bacteria/ml. Bacteria (7×10^7 bacteria/ml) gave a photometric extinction of 0.100 at 350 nm in 1-cm cuvettes.

Incubation of Leukocytes with Bacteria

Buffy coat leukocytes (50 μ l) suspended in autologous plasma were incubated with 5 μ l of *E. coli* in HBS at 37°C in a shaking waterbath. Leukocyte suspension (50 μ l) was incubated with 5 μ l HBS as a control. Aliquots of 10 μ l were taken from the assay at given time intervals and diluted in 1 ml of ice-cold HBS to stop phagocytosis. The cells were stored on ice and were measured within a maximum delay of 2 h. Stable flow cytometric results were obtained during this time.

Cell Staining

Phagocytosis and degradation of bacterial DNA were measured by incubation of 250 μ l of the cell sample for 15 min at 0°C with 5 μ l of a dye cocktail containing 0.4

mg/ml of acridine orange (Sigma) and 2 mg/ml of propidium iodide (PI) (Sigma) in dimethylformamide (DMF) (Merck, Darmstadt, FRG). Propidium iodide was used to counterstain the DNA of dead cells. Fluorescence was excited between 400 and 500 nm. AO green fluorescence (F1) was collected between 500 and 530 nm, and AO red fluorescence and PI red fluorescence (F2) between 550 and 700 nm.

Intracellular pH and esterase activity were measured with ADB (Paesel, Frankfurt, FRG) (38). ADB is the membrane-permeable ester of the pH-sensitive dye 2,3-dicyano-hydroxyquinone (DCH). DCH is trapped intracellularly following cleavage of ADB by intracellular esterases. The esterase activity was determined from the total fluorescence of the cell. The intracellular pH of each cell was calculated from the ratio of blue to green fluorescence according to a calibration curve in the computer program. The cell sample (250 μ l) was incubated with 5 μ l of dye cocktail consisting of 1 mg/ml of ADB and 2 mg/ml PI in DMF for 15 min at 20°C. The DNA of dead cells was counterstained by the PI in the cocktail. The fluorescence was excited between 340 and 400 nm. DCH blue fluorescence (F1) was collected between 418 and 440 nm, and DCH green fluorescence and PI red fluorescence (F2) between 500 and 700 nm.

Flow Cytometric Measurements

The electrical cell volume and two fluorescences of each cell were measured with a Fluvo-Metricell flow cytometer (17) (HEKA-Elektronik, Lambrecht/Pfalz, FRG). A minimum of 2,000 leukocytes was measured per sample. Fluorescence was excited with light from a HBO 100 W/2 high pressure mercury arc lamp (Osram, Augsburg, FRG). The electrical cell volume was measured in a cylindrical orifice of 80 μ m diameter and 80 μ m length with hydrodynamic focusing of the cells through the center of the orifice at an electrical current of 0.15 mA. The instrument was calibrated with porous, NH₂-group bearing particles (Paesel, Frankfurt, FRG) with a diameter of 5 μ m prestained either with fluorescein isothiocyanate (FITC) or DCH. The electrical cell volume and fluorescence pulses were amplified by 2.5 decade logarithmic amplifiers. The maximum amplitude of each signal was digitized by 128-step analog-to-digital converters. The measured data were stored for each cell in list-mode on magnetic tape.

Data Evaluation

The list-mode data were evaluated by FORTRAN computer programs (35-37) on an INTERDATA 7/32 computer (768 kByte core memory, 200 MByte disk, Perkin Elmer, Oceanport, NJ). The data were displayed in a three-dimensional cube and further processed. In a first step dead cells, which show reduced accumulation of AO or DCH but readily take up PI with resulting nuclear staining, were separated by computer analysis on the basis of their high red fluorescence from vital DCH or AO stained cells that fluoresced blue or green. The cell

volume versus DNA histograms of all dead cells was then plotted. In a second step the cell volume of the vital cells was displayed with the cellular DCH fluorescence as a measure of esterase activity and with the ratio of blue to green fluorescence of the vital cells, which is a measure of intracellular pH. Furthermore the cell volume was plotted against the green AO fluorescence of vital cells as a measure of cellular DNA and against their ratio of AO red/green fluorescence. Granulocytes and lymphocytes were well separated by cell volume or by the ratio of AO red fluorescence to green fluorescence. Monocytes showed a small cell cluster with intermediate AO red fluorescence between granulocytes and lymphocytes (Fig. 2B) as this has been described earlier by others (20). Standardized windows were used for all evaluations. Mean values and standard deviations of fluorescences, cell volume, and internal pH of lymphocytes and granulocytes were calculated and automatically transferred to a database using the newly developed DIAGNOS1 program system (39).

RESULTS

The granulocyte cell volume increased markedly upon incubation with bacteria in autologous plasma, while the volume of lymphocytes remained unchanged (Fig. 1). A ratio of 20 *E. coli* K12 bacteria per granulocyte induced a maximal cell volume increase in granulocytes as determined in an initial series of experiments (data not shown). This bacteria/granulocyte ratio was used in all subsequent experiments.

The mean cell volume of viable granulocytes increased to 152.6% of control values after 30 min of incubation with bacteria and was stable for a further 60 min of incubation (Fig. 1, Table 1). Aggregation of granulocytes was not significant, which could be deduced from the volume versus fluorescence histogram.

Granulocytes stained with AO showed approximately the same F1 (500 to 530 nm) green fluorescence as lymphocytes but higher F2 (above 550 nm) red fluorescence (Fig. 2B). The AO green fluorescence of the granulocytes increased to 134.2% after 30 min and to 156.9% of the control values after 90 min of incubation (Table 1; Figs. 1A, 2A). AO red fluorescence increased only moderately. As a consequence of this, the F1/F2 ratio was increased. Direct staining of the *E. coli* bacterial suspension with AO showed a majority of nearly 90% of green staining bacteria and a minority of red staining bacteria, which probably correspond to dead bacteria. Quenching of the AO fluorescence of extracellular bacteria with 0.05% trypan blue did not alter the difference of green fluorescence as compared to the control values indicating that after 30 min most of the bacteria were ingested and not attached to the outer granulocyte surface.

The granulocytes with the highest increase in cell volume also showed the highest increase in AO fluorescence (Fig. 1). The increase of granulocyte cell volume and AO green fluorescence was frequently heterogeneous after 30 min of incubation but was rather homogeneous after 90 min of incubation. For the analysis of

Table 1
Flow Cytometric Parameters of Phagocytosing Granulocytes

Granulocyte parameter	Time of incubation with <i>E. coli</i> K12 ^a		
	0 min	30 min (% of 0 min)	90 min (% of 0 min)
Cell volume	354 ± 10 μm ³	152.6 ± 4.1 (<i>P</i> < 0.001)*	162.1 ± 4.8 (<i>P</i> < 0.001)
AO green fluorescence	1.088 ± 0.058 arb. fl. units ^b	134.2 ± 5.8 (<i>P</i> < 0.001)	156.9 ± 6.6 (<i>P</i> < 0.001)
Internal pH	7.55 ± 0.03	-0.07 ± 0.02 (<i>P</i> = 0.002)	-0.04 ± 0.02 (<i>P</i> = 0.019)

^aMeans ± standard error (S.E.M.). The mean cell volume and the mean AO green fluorescence of 30 and 90 min are expressed as the percentage ratio of the measured values of cell assays with and without bacteria. The internal pH of 30 and 90 min is expressed as the difference between cell assays with and without bacteria (n = 24).

^bArb. fl. units, arbitrary fluorescence units.

*Probability of equality of means between paired assays with and without bacteria (t-test).

Table 2
Flow Cytometric Parameters of Lymphocytes

Lymphocyte parameter	Time of incubation with <i>E. coli</i> K12 ^a		
	0 min	30 min (% of 0 min)	90 min (% of 0 min)
Cell volume	156 ± 3 μm ³	101.4 ± 1.1 (<i>P</i> = 0.200)*	99.6 ± 0.8 (<i>P</i> = 0.695)
AO green fluorescence	1.123 ± 0.061 arb. fl. units	94.5 ± 1.9 (<i>P</i> = 0.007)	101.6 ± 2.4 (<i>P</i> = 0.504)
Internal pH	7.63 ± 0.04	-0.01 ± 0.01 (<i>P</i> = 0.471)	-0.01 ± 0.01 (<i>P</i> = 0.331)

^aMeans ± standard error (S.E.M.). The mean cell volume and the mean AO green fluorescence of 30 and 90 min are expressed as the percentage ratio of the measured values of cell assays with and without bacteria. The internal pH of 30 and 90 min is expressed as the difference between cell assays with and without bacteria (n = 24).

*Probability of equality of means between paired assays with and without bacteria (t-test).

heterogeneity of the phagocytic response a low and high phagocytosis field was defined for database analysis (Fig. 1A). There were, however, less than 5% of granulocytes that did not show a significant shift of AO green fluorescence in the presence of bacteria as compared to the control samples without bacteria.

Lymphocytes showed a decrease of AO green fluorescence to 94.5% of the controls after 30 min of incubation with bacteria but no significant changes after 90 min of incubation or of AO red fluorescence (Table 2).

Staining of the blood samples with AO alone gave a cell cluster of low AO staining between the erythrocyte and leukocyte cell clusters in the green versus red fluorescence diagram. Simultaneous staining with PI shifted this cell cluster to a nonoverlapping area (Fig. 2) indicating that these cells were dead cells. This was used for the simultaneous quantitation of dead and vital cells and for their clear separation.

Also in the ADB measurement, vital nucleated cells were well separated from dead cells, erythrocytes, and debris (Fig. 3A,B). Granulocytes stained more brightly with ADB than did lymphocytes (Fig. 3C). When the

fluorescence per unit cell volume was calculated, it was, however, similar for lymphocytes and granulocytes. Thus a comparable intracellular concentration of DCH was reached in both cell types. The DCH fluorescence of granulocytes decreased upon incubation with bacteria while that of lymphocytes remained constant.

The internal pH of granulocytes was calculated from the F1 to F2 ratio of the intracellular DCH fluorescence. It was similar in normal peripheral blood granulocytes and lymphocytes with a tendency for lower pH values in granulocytes (Fig. 3D; Tables 1, 2). The internal pH of granulocytes was 7.55 before incubation and decreased significantly by 0.07 units (Table 1) after 30 min of incubation with *E. coli* bacteria. It did not change upon further incubation with bacteria. The internal pH of lymphocytes was 7.64 prior to incubation. It did not change significantly during the incubation period (Table 2). There was no heterogeneity of intracellular pH in lymphocytes and granulocytes upon incubation with bacteria.

The percentage of dead cells, as determined by the uptake of propidium iodide, never exceeded 15% of the

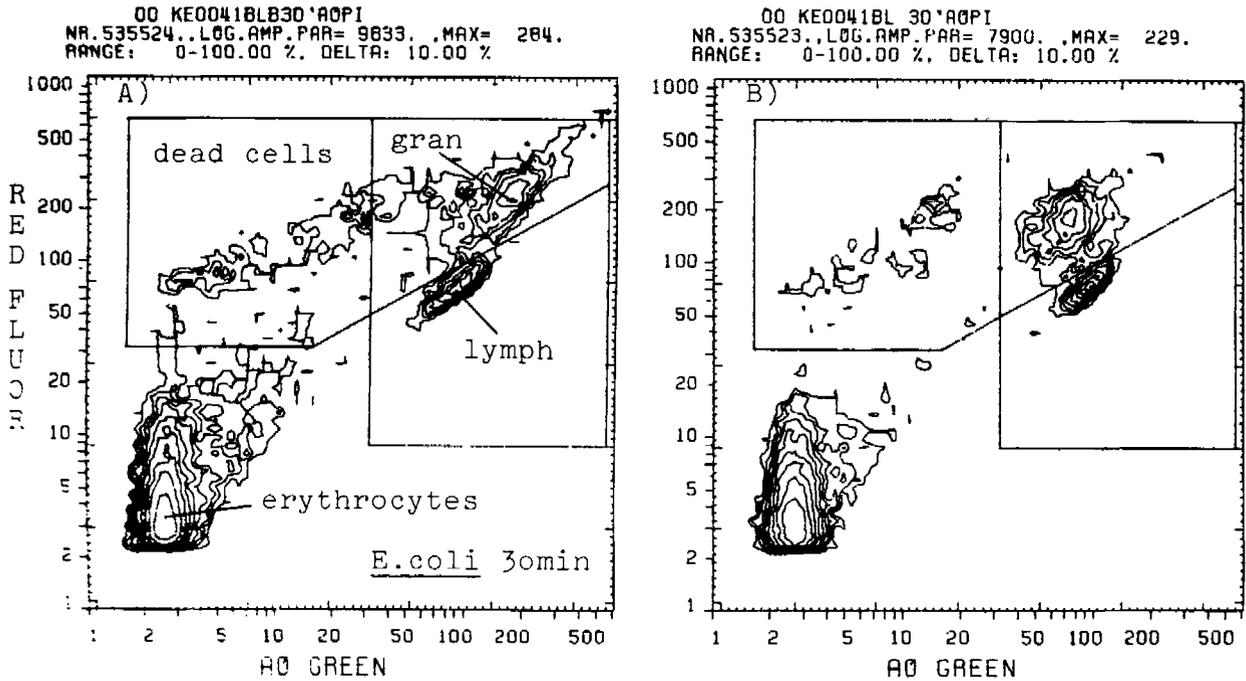


FIG. 2. Green vs. red fluorescence of acridine orange (8 $\mu\text{g/ml}$) and propidium iodide (20 $\mu\text{g/ml}$) stained buffy coat lymphocytes and granulocytes after 30 min incubation at 37°C with (A) and without (B) *E. coli* bacteria. Acridine orange green fluorescence corresponds to cellular DNA. The RNA and granule content are stained red. Propidium

iodide stains the DNA of dead cells red. The phagocytizing granulocytes have increased in green (DNA) and red (RNA) fluorescence. The position of lymphocytes, erythrocytes, and dead cells is not altered. Histogram display and standardization as in Figure 1. The maximum channel contents were 284 and 229 cells.

total leukocytes at the beginning of the incubation (Fig. 3A,B). There was no significant variation of the amount of dead cells during the incubation with bacteria.

DISCUSSION

The most significant changes of granulocyte functional parameters during phagocytosis of *E. coli* bacteria in this study concern cell swelling, increase of AO green fluorescence, and decrease of intracellular pH.

Two phases of swelling are known after stimulation of granulocytes with chemotaxins. The cell volume of granulocytes increases by 6% 30 sec after stimulation with C5a (19). There is a transient ruffling and a biphasic change of the right angle light scatter at the same time. The changes of light scattering are accompanied by a polymerization of actin and are in part dependent on the availability of intracellular calcium (31).

The prolonged phase of cell swelling is maximal after 10 to 20 min. It is energy-dependent and inhibited by deoxyglucose (14). No extracellular divalent cations are required during this phase (14). Swelling occurs at 37°C but not at 4°C (18). Swelling is furthermore associated with an increase of the internal $^3\text{H}_2\text{O}$ space of the cell (12), which is mediated via an amiloride-sensitive Na^+/H^+ -antiport. This antiport is activated by an acidification of the cell below an internal pH of 7.2 as a result of

the respiratory burst and leads to simultaneous alkalization and swelling of the cell (11).

The increase of granulocyte volume in the present study starts approximately 3 to 5 min after the onset of incubation. Maximal swelling is reached after 30 min and is stable for a further 60 min. This corresponds to the well-known prolonged swelling of granulocytes. The maximal increase of electrical cell volume of granulocytes that has been reported after stimulation with soluble stimuli is 21.7% or 22.6% after stimulation with N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), or phorbol 12-myristate 13-acetate (PMA), respectively (12). The high volume increase of 52.6% (Table 1) in our assay as compared to the volume increase following stimulation with soluble stimuli is probably due to the ingestion of numerous bacteria.

Acridine orange is a metachromatic dye that fluoresces green when intercalating into double-stranded nucleic acids and fluoresces red as a result of dye-dye interaction when complexing with single-stranded or denatured nucleic acids (8). The specificity for DNA and RNA is dependent on the presence of cations and the concentration of the dye (34). The dye also accumulates in lysosomes when used as a vital stain, with resulting granular red fluorescence. AO has been used in flow cytometry as a stain for differential leukocyte counts

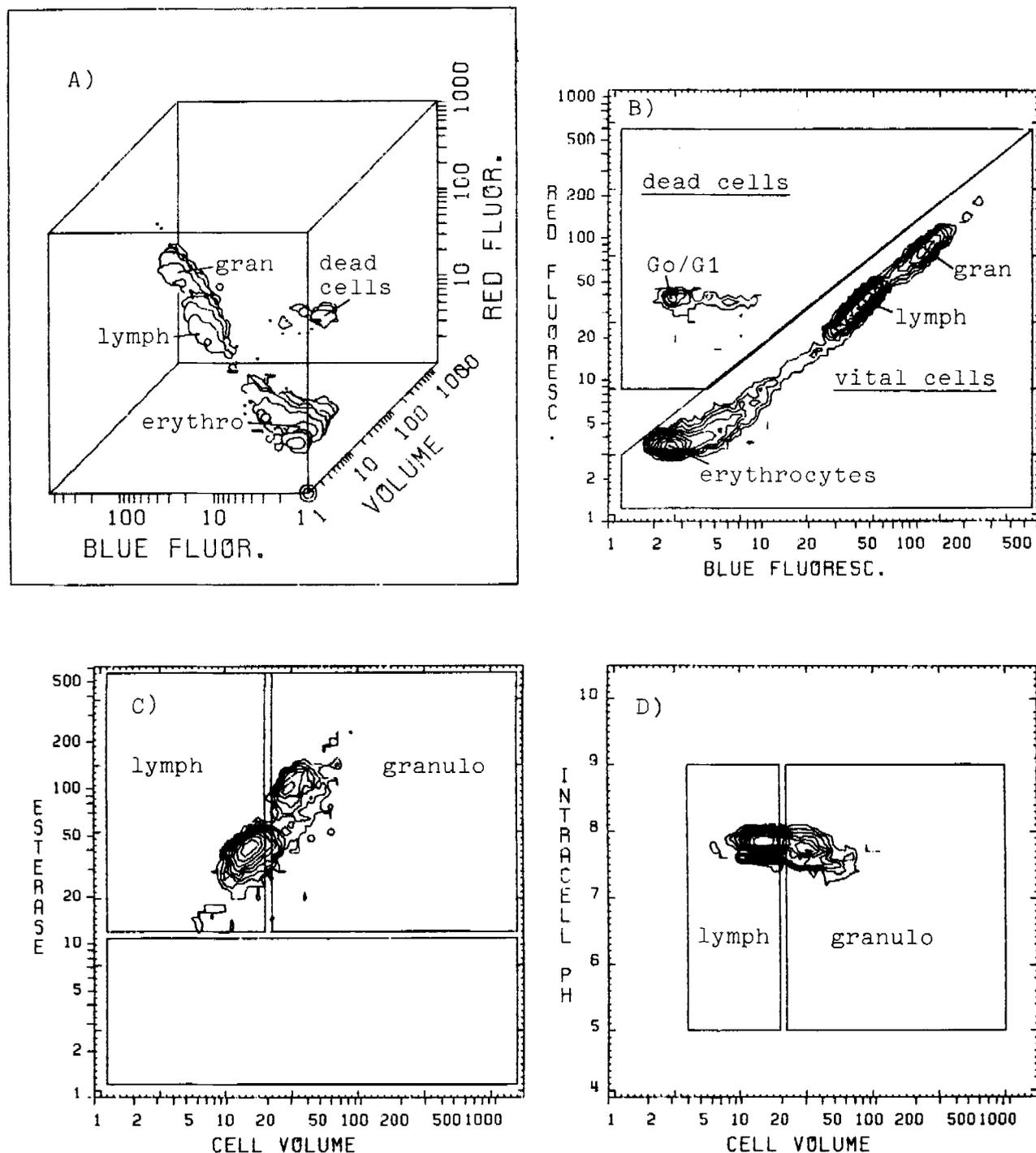


FIG. 3. Simultaneous cell volume, blue and green plus red fluorescence of ADB and PI stained buffy coat cells. The vital lymphocytes and granulocytes as well as the erythrocytes and dead cells can be distinguished (A). The cloud display was further evaluated by projecting the contents of the cube onto the front plane to obtain the two-parameter fluorescence histogram (B). The vital and dead cells are now quantitatively separated (B) and can be independently evaluated. Cell volume vs. esterase activity (C) and intracellular pH (D) of the viable cells. The granulocytes have a higher esterase activity than the

lymphocytes, and their intracellular pH is slightly lower as in lymphocytes. The cloud display was obtained by standardizing the cube content to the maximum logarithmic channel content (100%) and by plotting contour lines around channels with logarithmic cell contents of 10% of the maximum. The contour lines contour channels with a content of two cells. More than 95% of all cells are enclosed within the clouds of the cube display (A). Display and standardization of histograms (B-D) are as in Figure 1. The graphs (A-D) contain 5,980 cells with 514, 354, 253, and 299 cells in the maximum channel.

because the simultaneous measurement of AO green and AO red fluorescence allows discrimination of granulocytes from lymphocytes by the higher red fluorescence of granulocytes (20).

Vitally stained yeast, spermatozoa, and bacteria fluoresce green when viable, and red when dead. This has been used for a coverslip method for the measurement of phagocytosis and intracellular killing of bacteria and fungi by leukocytes (13,25) and also to measure the percentage of granulocytes that have ingested yeast by an increase in AO red fluorescence (42).

AO green fluorescence in our assay increased significantly over a period of 90 min in granulocytes that had phagocytosed bacteria (Figs. 1, 2). Red fluorescence increased only moderately until 90 min. This could be due to the fact that vital bacteria are only slowly digested. The lack of increase in red fluorescence may also be the result of the low concentration of the dye (8 $\mu\text{g}/\text{ml}$) we used in contrast to others (100 $\mu\text{g}/\text{ml}$) (25). This may not have been enough to get stacking and red fluorescence of AO on digested DNA. It was not possible to correlate precisely the number of ingested bacteria with the increase of the granulocyte AO green fluorescence because the bacteria taken from a stationary culture were heterogeneous by size and AO fluorescence. Furthermore microscopic counting of AO-stained intracellular bacteria was difficult, as a large amount of bacteria was ingested after 30 and 90 min of incubation.

The intracellular pH of vital cells can be determined by several fluorescent pH indicators such as 6-carboxyfluorescein (6-CF), 2',7'-bis-carboxy-ethyl-5 (6)-carboxyfluorescein (BCECF), 4-methylumbelliferone (4-MU), and ADB. 6-CF, BCECF, and 4-MU indicate pH changes by intensity changes of fluorescent light that is critical in cellular work because dye leakage or changes in cell volume may be interpreted as intracellular pH changes. To overcome this problem, dual wavelength laser excitation is necessary, which is complicated. ADB has the advantage that intracellular pH changes are indicated by changes of the color of emitted fluorescent light, which makes the intracellular pH measurement independent of dye leakage and cell volume changes. Intracellular pH changes can be measured by single light source excitation and measurement of fluorescence in two fluorescence channels (38). Such measurements can be performed in all flow cytometers with UV excitation. The fluorescence of DCH is strongly pH-dependent and thus gives a better resolution of pH than BCECF or 4-methyl-umbelliferone (22). The fluorescence ratio is stable for at least 30 min despite some leakage of the dye.

The internal pH of granulocytes kept at an external pH of 7.40 had a mean value of 7.55 as measured with ADB in our assay. The measurement of intracellular pH in cells with multiple different compartments like granulocytes depends on the intracellular distribution of the respective dye. Furthermore the separation and purification of the cells as well as the external pH and the composition of the media used for the storage of the cells have an influence on the intracellular pH. Thus differ-

ent values ranging from 7.15 to 7.40 have been calculated from the fluorescence intensity of different fluorescein derivatives in cuvette assays for the intracellular pH of neutrophils (11,30). Similar differences have been measured in a human leukemic T-cell line that gave an internal pH of 7.25 as measured with BCECF and gave an internal pH of 7.39 with ADB (22).

The internal pH of granulocytes decreased by 0.07 pH units (Table 1) during the phagocytosis of bacteria as determined with ADB in the described assay. The fact that the cytoplasmic pH is nearly unchanged in spite of the activation of the respiratory burst of phagocytosis suggests efficient regulation of the internal pH by cells obtained from healthy individuals.

Several mechanisms of pH regulation are known in the granulocyte. Upon experimental external acid loading, pH recovery is probably due to an exchange of internal H^+ for external Na^+ . An exchange of internal Cl^- for external HCO_3^- seems to be mainly responsible for pH_i recovery from alkalization (30).

Changes of the intracellular pH of the neutrophil can be induced by stimulation with the same soluble stimulants that produce cell swelling. Stimulation with the tumor-promoting phorbol ester PMA causes cytoplasmic acidification followed by alkalization. The acidification seems to be linked to the activation of the respiratory burst (11). The alkalization is probably mediated by an amiloride-sensitive Na^+/H^+ antiport.

Stimulation of granulocytes with the chemotactic peptide FMLP leads to a rise from a resting pH_i of 7.25 to a new steady state pH of 7.75 after 10 min (29). This alkalization, mediated by an exchange of external Na^+ for internal H^+ , is positively correlated with the chemotactic response of the cells.

Our assay differs from many other protocols by the lack of separation procedures, the use of viable unstained bacteria, and incubation in 100% autologous plasma. Furthermore the incubation was rather long and a saturating excess of bacteria over leukocytes was used. Thus toxic effects of separation media or lysis agents were avoided, and phagocytosis was not restricted by lack of opsonization, by low number of bacteria, or by short incubation time. This is probably the reason that less than 5% of the granulocytes did not show a significant change of AO green fluorescence in the presence of bacteria.

Monocytes, which are also capable of phagocytosis of bacteria, are distinguishable as a small overlapping cluster on the red fluorescence axis between lymphocytes and granulocytes, usually slightly above the lymphocyte-granulocyte separation line drawn in Figure 2. Monocyte activity was, however, not analyzed separately due to considerable overlap of the monocyte cluster with lymphocytes and monocytes, which became apparent during the separate measurement of ficoll-hypaque separated mononuclear and polymorphonuclear cells (data not shown). In the ADB/PI assay, monocytes could not be distinguished from granulocytes based on cell volume, esterase activity, or intracellular pH. Mono-

cytes are usually a small part of the peripheral blood nucleated cells (2–10%). They behave qualitatively similarly to granulocytes. Otherwise they would have become apparent as a discernible cell cluster. The overall granulocyte results are, therefore, not substantially influenced by the monocyte contribution.

Lymphocytes were not influenced by incubation with bacteria as measured by cell volume, AO red fluorescence, esterase activity, internal pH, and propidium iodide exclusion. Only a 5.5% decrease of AO green fluorescence occurred after 30 min of incubation with bacteria. Thus lymphocytes, besides fluorescent calibration particles, may serve as a biological control for stable measurement conditions of the flow cytometer.

Our study has shown that flow cytometric methods allow the measurement of phagocytosis by granulocytes directly after the incubation of buffy coat cells in whole plasma with bacteria. The measurement of multiple functional parameters during phagocytosis is of value for the characterization of the interaction of phagocytes, microorganisms, and soluble modulators of granulocyte function, as well as for the detection of changes of granulocyte function in normal individuals or post-traumatic and infected patients.

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