Size Distribution, Electronic Recognition, and Counting of Human Blood Monocytes

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During a study on the separation of human blood monocytes from lymphocytes, a method was developed to recognize and count monocytes by electronic means. Lightscattering (Cytograf, Bio/Physics), and changes in electrical resistance (Channelizer, Coulter) were used to size mononuclear leukocytes directly in cell suspensions. Both methods revealed a size distribution profile in which two populations of mononuclear leukocytes could be distinguished. The largest cells were virtually eliminated after phagocytosis of iron particles. We confirmed that these cells were monocytes by three different criteria: the intracellular lysozyme activity, the number of phagocytes, and the percentage of cells with kidney-shaped nuclei. The highly significant correlations we found showed that monocytes could be recognized and counted by electronic sizing. For this method, purified mononuclear leukocyte preparations had to be used, since the presence of erythrocytes, platelets, and polymorphonuclear cells interfered. Statistical analysis revealed that electronic sizing permitted discrimination of differences in monocyte content of 4.5%, with a probability of 95%. It was calculated that this sensitivity of electronic monocyte counting was about three times higher than the sensitivity of microscopic methods. Since 100,000 cells can be sized within a few seconds, not only the efficiency of the preparation but also minor changes in the size of monocytes and lymphocytes introduced during the isolation can be followed.

Since investigations on the actual role of monocytes in immunologic systems are in progress and almost all of these studies are performed with mononuclear leukocyte suspensions, there is a considerable need for a reliable and simple method to discriminate between monocytes and lymphocytes in mononuclear leukocyte suspensions and in purified monocyte preparations.

Several techniques have been described for the quantitation of monocytes in mononuclear blood leukocyte preparations. The majority of these methods use cytochemical staining based on the activity of lysosomal enzymes, such as peroxidases, esterases, and lysozyme. Other methods are based on the phagocytosis of particles which can be visualized by light or electron microscopy. Almost all of these methods involve fixation of the cells followed by a rather laborious staining procedure. In addition, most of the methods that use phagocytosis cannot discriminate between ingested and adherent particles; this may lead to an overestimation of the number of phagocytic cells.

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Moreover, the reliability of the methods based on microscopic differentiation of cells is rather limited, since there is not only a statistical error due to the small number of cells that can be visually counted but also a considerable variation introduced by the staining procedure and the subjective recognition of monocytes. Therefore we have investigated the possibility of recognizing and counting monocytes electronically in suspensions of mononuclear blood leukocytes. We now describe a method based on electronic discrimination of cells of different sizes, which enables recognition of monocytes as a distinct large cell population in the size distribution profile of mononuclear leukocytes. We have found that the number of monocytes estimated either by morphology or by phagocytosis is identical with the number of large cells in the mononuclear leukocyte suspensions, within the experimental error of microscopic estimations. Using the activity of lysozyme as a sensitive enzymatic marker for phagocytes, we have proved that the cells which are electronically recognized as large cells in mononuclear leukocyte preparations are identical with monocytes. Since the electronic counting of monocytes has an overall reproducibility of $2^{\circ}$, we are able to use this method for the estimation of the contribution of staining and subjective recognition of monocytes to the variability of two independent counting chamber methods. The least significant difference that can be estimated after differentiation of 200 cells appears to be about 15%, for both microscopic methods, which is about three times the least significant difference that can be determined by electronic monocyte counting.

Since it has recently been reported that the reactivity of lymphocytes in vitro may be enhanced considerably by the presence of 1%–10% monocytes, it is clear that only the accuracy of electronic monocyte counting will allow a further improvement of the quantitative aspects of this type of cellular interaction.

**MATERIALS AND METHODS**

Trypsin (1:250) was obtained from Gibco, Grand Island, N.Y., ethidium bromide from Calbiochem, Los Angeles, Calif., and acridine orange (1B 307) from Chromagesellschaft Schmid & Co., Stuttgart, B.R.D. Human γG, Cohn fraction II,11 was prepared in the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service. Carbonyl iron powder (code 1-1-63.763) was obtained from General Aniline and Film Corporation, Linden, N.J., Micrococcus lysodeikticus and lysozyme from Worthington Biochem. Corp., Freehold, N.J.

**Preparation of Cells**

Mononuclear cells were prepared as described before.12 In brief, mononuclear leukocytes were isolated from diluted blood on a Ficoll-Isopaque layer of 1.077 g/ml (at 25°C) as described by Böyum.10 Contaminating erythrocytes were lysed with isotonic NH_{4}Cl solution. The final preparation contained less than 2% granulocytes and less than 5% erythrocytes.

**Preparation of Chicken Red Cell Nuclei for Phagocytosis Studies**

Suspensions of chicken red cell nuclei were prepared according to Ten Veen and Feltkamp.13 The suspension was diluted with phosphate-buffered saline, filtered through a plug of nylon wool to remove aggregates, and centrifuged (2000 g, 10 min). The pellet was resuspended into 1 ml of a 10% (w/v) solution of human γ-globulin in Tris (260 mM)-boric acid (35 mM)-disodium EDTA (20 mM) buffer, pH 9.1, and incubated for 30 min at 25°C.14 The nuclei were then washed four times with 1 mg/100 ml ethidium bromide in phosphate-buffered saline and four
times in 1 mg/100 ml acridine orange in phosphate-buffered saline (concentration of nuclei 5 × 10^7/ml, wash volume about 10 ml).

Finally, the nuclei were washed three times with phosphate-buffered saline, and a suspension of 8 × 10^8 nuclei/ml in phosphate-buffered saline was prepared (counted electronically with a Coulter Counter). Such suspensions could be stored at 4°C for 1 wk without any detectable change in morphology and agglutination with rabbit anti-human-yG serum.

Quantitation of Phagocytes

Mononuclear blood leukocytes were suspended in Ringer’s solution (a mixture of 9 parts 125 mM NaCl, 5 mM KCl, 1.2 mM MgSO₄, 35 mM Tris-HCl, pH 7.4 at 37°C, 1 mM phosphate, 5.5 mM glucose, and 1 part of human AB serum) to a final concentration of 5 × 10^6/ml; 0.3 ml of the cell suspension was mixed with 0.07 ml nuclei suspension (8 × 10^8/ml) and incubated for 30 min at 37°C in a shaking waterbath. In order to detach nuclei adhering to the cell surface, 1 ml 0.5% (w/v) trypsin solution was added, and the incubation was continued for 10 min. The cells and nuclei were separated by layering the total mixture on 2 ml of Ficoll-Isoopaque solution with a density of 1.081 g/ml at 4°C in a 10-ml reagent tube. After centrifugation (10 min at 2200 g and 0°C), the cells had sedimented at the interface of the suspension medium and the gradient material, while the free nuclei were found at the bottom of the tube. The cells were harvested and resuspended in 10 ml of phosphate-buffered saline, supplemented with 10%, AB serum. After centrifugation (10 min, 400 g, 25°C), the cells were resuspended in 0.1 ml Ringer’s solution.

The cell suspension was viewed with a fluorescence microscope (Ortholux II fitted with optics according to Ploem, Leitz, Wetzlar, B.R.D.). All cells showed faint green fluorescence due to acridine orange that had leaked from the nuclei during the previous incubation (when only ethidium bromide was used for the staining of the nuclei, only the phagocytic cells were recognizable by fluorescence). The phagocytic cells were easily distinguishable from the lymphocytes since the former contained one to five bright orange fluorescent nuclei. Two hundred cells were counted in each preparation. The test conditions specified above, were found to be optimal by four different criteria: (1) a concentration of 10 nuclei/ml induced optimal stimulation of the oxygen consumption of the monocytes as tested with an oxygen electrode according to Weening et al.,15 (2) the number of recognizable phagocytes reached a plateau after 15 min of incubation; (3) density analysis of a mononuclear leukocyte suspension before and after submission of the phagocytosis test revealed a random cell loss of 1000;10 (4) the separation of cells and nuclei became worse when the number of cells and nuclei applied to the gradient was increased above the amount used in the described test.

Determination of Lysozyme Activity

Lysozyme activity was measured by following the decrease in light absorbance (λ = 540 nm) of a suspension of M. lysodeikticus (16.6 mg/100 ml in sodium phosphate buffer, 0.67 M, pH 6.2) induced by the addition of cell lysate. The cell lysate was prepared by freezing and thawing a cell suspension in the presence of 0.2% Triton X-100. Activity was expressed as μg lysozyme per 10⁶ cells. In this system, 1 μg lysozyme caused a decrease in absorbance of 0.270/min, at 540 nm and 25°C. For cell preparations with a low monocyte content, the amount of lysate used was adapted in such a way that a decrease in absorbance of at least 0.020 per 10 min could be recorded (full scale 0.20 A).

Determination of Size Distributions

Light scattering.17 A sample of 0.1 ml of a mononuclear leukocyte suspension (5 × 10⁶ cells/ml) was diluted with 5 ml particle-free phosphate-buffered saline (filtered through a 0.8-μm Gelman filter). Two milliliters of this cell suspension was introduced into a Cytograf, model 6300A, supplemented with a pulse height analyzer (distribution analyzer, model 2100, Bio/Physics, Mahopac, N.Y.). The light scattering caused by the cells was measured by the decrease of the axial light intensity of the red laser (“absorbance” signal).17 Optimal conditions were as follows: the gain was set at “high,” the threshold at 16, and the angle at 46. The scatter
pulses were grouped according to pulse height in the 100-channel pulse-height analyzer. A total count of 30,000 was accumulated, and the content of each channel was displayed on the oscilloscope. The percentage of large cells was computed in the following way: the lower threshold of the distribution analyzer was set at the minimum between the two peaks in the size distribution profile (Fig. 1A); the total count between this threshold and channel 60 was integrated and expressed as percentage of total count between channels 8 and 60. Size distribution curves were obtained by expressing the content of each channel as a percentage of the total count accumulated and by plotting these values against the channel numbers. The channels higher than number 60 contained less than 1%, of the total count and were therefore ignored.

**Coulter counter.** A sample of 0.2 ml of a mononuclear leukocyte suspension, containing $5 \times 10^6$ cells/ml, was diluted with 20 ml of particle-free phosphate-buffered saline. This dilution was introduced into a Coulter Counter, model ZF, supplemented with a pulse height analyzer (Channelizer model C-1000). Optimal conditions were as follows: a manometer with a 100-μm orifice, the attenuator at 2, the aperture at 16, and the threshold at 10. The lower threshold of the Channelizer was set at 10 and the window width at 100. Size distribution curves were obtained by plotting the count of each channel (as a percentage of the total count) against the channel number. In order to determine the percentage of large cells, the lower threshold switch of the Channelizer was set at the minimum between the two peaks in the size distribution profile (see Fig. 1B). The total count between this channel and 99 was integrated and expressed as percentage of the total count between channels 0 and 99.

## RESULTS

### Size Distribution of Mononuclear Blood Leukocytes

Figure 1A shows the size distribution of mononuclear leukocytes, as determined with the Cytograf. Similarly, the sizing of the mononuclear leukocytes from another donor was performed with the Coulter Counter (Fig. 1B). In both
Correlation Between the Number of Monocytes Estimated by Morphology and by Size

In 21 mononuclear leukocyte preparations with various proportions of monocytes, differential counting was performed after nuclear staining with Türk's Blue solution. Large cells with kidney-shaped nuclei were counted as monocytes. Two hundred cells were differentiated in every sample. The same preparations were analyzed with the Cytograf. The results (Fig. 2) show a highly significant correlation \( (p < 0.0005) \) between the number of monocytes estimated by morphology and the number of large cells as calculated with the method of least squares \( (r = 0.97) \). In fact, the results of both methods could not be discriminated from each other, since no significant difference was found between the percentage of monocytes determined morphologically and the percentage of large mononuclear leukocytes counted electronically in the same preparation \( (t \text{ test for paired observations, } 0.3 > p > 0.20; \text{ this large } p \text{ value indicated that the assumption that the two methods gave different results was highly unlikely}) \).

Correlation Between the Number of Monocytes Estimated by Phagocytosis and by Size

In 17 mononuclear leukocyte preparations with different proportions of monocytes, the percentage of phagocytic cells was estimated by the chicken red blood cell nuclei technique. Size distribution profiles of the same cell suspensions were obtained with the Coulter Counter, and the percentage of large cells was determined. The results (Fig. 3) show a highly significant correlation.
Fig. 3. Correlation between the amount of monocytes estimated by phagocytosis and by electronic sizing. Abscissa: percentage of phagocytes estimated with fluorescent chicken RBC nuclei; ordinate: percentage of large mononuclear leukocytes estimated with a Coulter Counter (conditions under Materials and Methods). The line drawn in this figure is the 45° line. The dotted lines indicate the boundaries of the least significant differences ($\alpha = 0.025$, one sided) when 200 elements are differentiated microscopically.

(\rho < 0.0005) between the percentage of phagocytes and the number of large mononuclear leukocytes ($r = 0.96$). In fact, again both methods gave identical results, since no significant difference was found between the percentage of mononuclear phagocytes and the percentage of large mononuclear leukocytes determined in the same preparations ($t$ test for paired observations, $p > 0.4$).

**Lysozyme Activity in Small and Large Mononuclear Leukocytes**

The highly significant correlation found between the number of large cells and the number of monocytes estimated by both chamber counting methods does not imply that indeed all large cells are monocytes. It can be calculated, for example, that a value of 5% monocytes, found by microscope differentiation of 200 cells, may vary from 2% to 9%, merely on the basis of the statistical error.\^\textsuperscript{18} This confidence interval may be easily doubled by the uncertainty introduced by the staining and recognition of monocytes. Since all methods so far described for the recognition and quantitation of monocytes are afflicted with the same statistical error, no further improvement of identification of the large cells could be expected from these methods. Therefore, we correlated the number of large cells with the lysozyme activity of the mononuclear cell populations. Lysozyme is commonly used as a specific marker enzyme for phagocytic leukocytes.\^\textsuperscript{46} The reproducibility of the spectrophotometric determination of the lysozyme activity allows detection of less than 1.5% of the activity present in pure mononuclear phagocytes. Therefore, we investigated whether the activity of this enzyme in mononuclear leukocyte preparations correlated with the proportion of large cells. From a suspension of mononuclear leukocytes, a fraction enriched in large cells can be prepared on a discontinuous density gradient.\^\textsuperscript{12} Lysozyme activity was determined in the fractions enriched with large mononuclear cells, as well as in the original mononuclear leukocyte suspensions. The percentage of large mononuclear cells in each fraction was estimated with the Coulter Counter.

From these data, the lysozyme activity of pure large and pure small mononuclear leukocytes of each preparation was calculated (two equations with two unknowns). In leukocytes from a series of 11 healthy volunteers, the lysozyme activity in the large mononuclear leukocytes was found to be $1.30 \pm 0.15 \mu g$ lysozyme per $10^6$ cells (SEM), whereas less than $0.02 \mu g$ per $10^6$ cells was found
in the fraction of small mononuclear cells. These experiments definitely confirmed that large cells, as discriminated from lymphocytes by electronic sizing, were identical with the phagocytes in mononuclear cell preparations.

Reproducibility and Sensitivity of the Electronic Estimation of the Monocyte Content

A monocyte-enriched and a monocyte-depleted mononuclear leukocyte suspension were prepared by separation on the basis of density. After morphological differentiation, 11 different mixtures were prepared containing increasing proportions of monocytes. The monocyte content of each mixture was calculated from the mixing ratio, and the percentage of large mononuclear leukocytes was determined with the Coulter counter. The correlation between the calculated (x) and the Coulter counter-derived (y) monocyte content was obtained by the method of least squares ($y = 0.9542 x + 0.3353$, $SE_r = 2.07$, $r = 0.9973$ (Fig. 4). The “residual standard error” in the electronic estimation ($y$) ($S_r[1 - r^2]^{1/2}$, $S_r$ the standard error in $y$ and $r$ the correlation coefficient) was found to be 2.0%, (all percentages mentioned are absolute values). This finding indicated that the method of electronic sizing could discriminate between differences in monocyte content of more than 4.5%, with a probability of 95%. We want to stress that the electronic sizing was performed directly on mononuclear leukocyte suspensions. By morphological procedures, a similar discrimination could be reached theoretically by counting 200-500 cells after specific staining or labeling of the monocytes. However, it should be mentioned that, in the latter procedures, staining and monocyte recognition may introduce further uncertainties.

An impression of the total variability in the estimation of monocyte contents by microscopic methods could be calculated from the data depicted in Figs. 2 and 3. The “residual standard error” of the estimations, based on morphology and phagocytosis, was found to be 7% and 8%, respectively. This indicated that, by these methods, differences of 14% and 16%, respectively, could be recognized, with a probability of 95%. It was evident from these figures that electronic sizing greatly improved the reproducibility and, therefore, the sensitivity of monocyte counting in mononuclear leukocyte suspensions. No significant differences were found in the proportions of monocytes as determined by either
Cellular Contaminants Interfering With Sizing of Mononuclear Leukocytes

In order to elucidate under which conditions erythrocytes and granulocytes contaminating the mononuclear leukocyte preparations would interfere with the recognition of monocytes, we compared the size distribution profiles of these cells with the Coulter Counter. Suspensions were prepared which contained 79% monocytes and 88% granulocytes (estimated microscopically after staining with Türk’s Blue solution). The size distributions are shown in Fig. 5.

Although the mean size of monocytes and granulocytes was significantly different, discrimination of both cell types on the basis of size was not possible because of a considerable overlap of the size distribution profiles. However, it should be mentioned that the large difference in density between granulocytes and monocytes allowed the preparation of suspensions which contained less than 5% of the other cell types.

In the size distribution profile of whole blood, the presence of leukocytes is completely masked by the overwhelming number of erythrocytes. Mononuclear leukocytes obtained in a one-step procedure, as described by Bøyum, usually are still contaminated with erythrocytes (Fig. 6). The number of erythrocytes can be reduced drastically by specific lysis with isotonic ammonium chloride at 0°C. The mean size of lymphocytes is hardly influenced by this treatment.
Under the described conditions, the size distributions of thrombocytes and erythrocytes are almost identical (not shown here). Therefore, thrombocytes are the third kind of cell that may interfere with the proportion of the size distribution of mononuclear leukocytes when they are present in overwhelming numbers. This disturbance is caused by coincidental passage of thrombocytes through the detector and by the normal distribution of the size of thrombocytes. The presence of thrombocytes may be prevented by starting with defibrinated blood. When citrate or other anticoagulants are used, the thrombocytes can effectively be removed by aggregation with ADP, as described before.20

A significant correlation ($p < 0.01$, correlation coefficient = 0.98, $n = 4$) was found between the channel numbers indicating the mean size of erythrocytes, lymphocytes, granulocytes, and monocytes, and the mean volumes reported in the literature.21 However, the data obtained by light scattering with the Cytograf suggested a larger size for granulocytes than for monocytes. Since both signals (scatter and absorbance) of the Cytograf gave similar results, it was probable that cellular geometry and contents, in addition to cell volume, affected this type of sizing.

DISCUSSION

Size distribution profiles of mononuclear leukocyte suspensions, obtained by electronic counting, consistently indicated the existence of two distinct populations of cells. The population of the larger-sized cells was shown to be composed mainly of phagocytes on the basis of the capacity to ingest iron particles. This conclusion was supported by the highly significant correlation that was found between the number of large mononuclear cells—as determined by electronic sizing—and the number of monocytes counted on the basis of nuclear morphology and phagocytosis. Since the reliability of methods based on microscopic quantitation of cells is rather limited, the identification of large mononuclear leukocytes as monocytes was further improved by correlating the lysozyme activity and the number of large mononuclear leukocytes. It was found that the lysozyme activity was completely associated with the large mononuclear cell population in 11 different cell preparations. In our experi-
ments the lysozyme activity could not be due to neutrophil contamination, since these cells had been virtually eliminated by density centrifugation.

Lysozyme activity as such cannot be used for the quantitation of phagocytes since there is an individual variation in the cellular content of this enzyme, but its intracellular presence is a generally accepted criterion for phagocytes. Both the intracellular lysozyme activity and the number of large cells can be determined with a reproducibility of 2%. Therefore, we have confirmed by these experiments, with the highest available accuracy, that the monocytes present in mononuclear cell preparations can be recognized electronically as a distinct population of large cells.

Not only discrimination of monocytes and lymphocytes, but also electronic sizing of the lymphocytes contaminating monocyte preparations is important, since we have found recently (not shown here) that there is a tremendous difference in the immune reactivity in vitro of large and small lymphocytes.

Electronic sizing is superior to any microscope sizing method since it is directly performed on cell suspensions. Therefore, all three dimensions of the cells equally contribute to the analysis, and no artifacts are introduced by smearing, drying, fixing, staining, or slicing of cells. Moreover, the statistical significance of electronic sizing is better than any morphological method since this analysis can be based on the sizing of 100,000 cells.

In conclusion, we have shown that counting and recognition of monocytes can easily be performed with electronic sizing equipment, provided pure mononuclear leukocyte suspensions are used. The use of electronic sizing for the determination of monocyte contents greatly improves the reproducibility and therefore the sensitivity of the estimations.

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10. Blyum A: Isolation of mononuclear cells


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