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Characterization of human monocyte subpopulations by flow cytometry

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Summary. Human monocytes separated from peripheral blood by Ficoll-Hypaque and by adherence to serum-coated dishes show a bimodal volume distribution measured with a fluorescence-activated cell sorter. In the first peak of size distribution histogram of living mononuclear cells, lymphocytes and small monocytes were characterized by latex phagocytosis and non-specific esterase staining, whereas in the second peak the large monocytes dominated. The percentage of esterase stained small monocytes was lower than that of the large ones. Parallel to these data, the rate of the FDA hydrolysis of the small monocytes was lower than that of the large ones. The majority of the large monocytes reacted with sensitized sheep red blood cells (sSRBC) while only the minority of the small monocytes bound sSRBC. Scatchard plots on the binding of fluorescein isothiocyanate (FITC)-labelled human monoclonal IgG1 to the two subpopulations indicated similar association constants, $K = 1{\cdot}2 \pm 0{\cdot}3 \times 10^5$ M^{-1}. The number of Fc receptors was significantly different for the small ($3{\cdot}3 \pm 0{\cdot}6 \times 10^5$) and the large monocytes ($10 \pm 1 \times 10^5$).

INTRODUCTION

Macrophages from different compartments are known to differ considerably with respect to various functions (Kávai, Laczkó & Csaba, 1979; Neumann & Sorg, 1981; Tzehoval, de Baetselier, Feldman & Segal, 1981). Monocytes isolated by counterflow centrifugation have formed a bimodal volume distribution. The large monocytes are predominantly Fc receptor positive and active in antibody-dependent cell-mediated cytotoxicity (ADCC) assays (Norris, Norris, Sanderson & Kohler, 1979). Volume analysis of human monocytes with a multiparameter cell sorter has revealed three distinct populations with different chemotactic capabilities (Arenson, Epstein & Seeger, 1980).

In the present study purified human monocytes were separated and analysed in a fluorescence-activated cell sorter (FACS III) in order to investigate the heterogeneity of monocytes at population level measuring the distribution of cells according to their size, esterase activity and the number and association constant of Fc receptors. Two distinct subpopulations of monocytes could be identified according to their size. Regarding the hydrolysis of fluorescein diacetate, the large monocytes showed higher non-specific esterase activity. The cell surface density of the Fc receptors of large monocytes was also higher than that of the small ones, but the association constant (K) between the fluorescein-labelled human IgG1 and Fc receptors was approximately equal in both monocyte populations.

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MATERIALS AND METHODS

Mononuclear cell suspension

Mononuclear cell suspensions were isolated from

heparinized blood samples of thirty-four healthy human donors (100 ml each) over Ficoll-Hypaque (Weston, Duskin & Hecht, 1975). Approximately $2–3 \times 10^6$ mononuclear cells/ml were incubated in medium 199 (Flow) supplemented with 10% FCS and buffered with 20 mM HEPES (pH 7·2) and Hanks's salts in serum-coated dishes in a CO_2 incubator at 37° for 60 min (Kumagai, Itoh, Hinuma & Tada, 1979). After removing completely the non-adherent cells, the adherent cells were removed from the dishes by incubation with PBS containing 0·2% ethylenediamine tetraacetic acid (EDTA) and 5% foetal calf serum (FCS) at 4° for 15 min. After washing the cells were resuspended in medium 199 and counted. Trypan blue exclusion test and non-specific esterase staining (Manconi, Marrosu, Pagki, Correale & Zaccheo, 1979) were performed to determine percentages of viable (90%–95%) and phagocytic cells ($93 \pm 5\%$).

Latex phagocytosis by monocytes

Monocytes ($1–5 \times 10^5$) in 0·5 ml medium were incubated with 25 μl 0·2% latex suspension (1·091 μm in diameter polystyrene beads, Difco) at 37° for 60 min and then examined microscopically. The percentage of monocytes ingesting five or more latex particles was obtained by counting at least 200 cells. Non-viable cells and lymphocytes were not counted.

Uptake of sensitized sheep red blood cells by monocytes

Monocytes ($1–5 \times 10^5$) in 0·3 ml medium were incubated with 0·2 ml 2% suspension of SRBC sensitized with a subagglutinating dilution of an IgG fraction isolated on Sephadex G-200 from a rabbit anti-SRBC serum at 37° for 30 min (Kávai, Lukács, Sonkoly, Jókay, Berndt & Szegedi, 1980). The percentage of cells attaching or ingesting three or more sensitized SRBC (sSRBC, EA) was determined microscopically. Non-viable cells and lymphocytes were excluded.

Preparation of IgG and F(ab')$_2$

Human IgG myeloma protein was a gift from Dr É. Rajnavölgyi (Department of Immunology, Lóránd Eötvös University, Göd, Hungary). IgG1 myeloma protein was purified with Na_2SO_4 fractionation followed by DEAE cellulose chromatography using 0·01 M phosphate buffer (pH 8) as eluent. F(ab'2 fragments of IgG prepared by 18 h digestion with pepsin, were fractionated by gel-chromatography on Sephadex G-100 as described by An & White (1979). The contamination of the F(ab')$_2$ by native IgG was judged to be less than 5% by electrophoresis.

Fluorescein diacetate (FDA) staining

The cells were incubated with FDA (Koch-Light) and fluorescein was liberated by intracellular esterases. FDA was dissolved in aceton of spectroscopic grade and stored at 5°. The reaction was initiated by the injection of 10 μl of FDA (10^{-4} M) into 1 ml of 2×10^6 cells/ml suspension, at 25°. The fluorescence intensity distribution histograms of cells were recorded with a flow cytometer after different FDA hydrolysis times.

Labelling of IgG1 and F(ab')$_2$ with fluorescein isothiocyanate

The monoclonal IgG1 and the F(ab')$_2$ (20 mg/ml) were depleted of gross aggregates by centrifugation at 10 000 ***g*** for 20 min. Protein concentrations of the supernatants were determined spectrophotometrically using an optical density, $A_{280}^{1\%}$, 14 and also by the Folin method with human IgG as a standard. The proteins were incubated for 1 h at room temperature with FITC isomer I (ICN) in 0·1 M NaCl buffered with 0·05 M carbonate, pH 9 (15 μg FITC/mg protein) (de Petris, 1978). The unreacted FITC was removed by gel chromatography. The fluorescein:protein molar ratios (F:P)of FITC conjugates, determined spectrophotometrically (de Petris, 1978), were within the range of 2:3. Aliquots of conjugates were stored below −20°. After thawing the aggregates were removed by centrifugation at 120,000 ***g*** at 4° for 1 h.

Binding of FITC-protein conjugates by cells

The monocyte suspension (2×10^6 cells/ml) was filtered through a nylon mesh (50 μm) and incubated with FITC-IgG1 or -F(ab')$_2$ at 25° for 1 hr. The cells were analysed without further washing for fluorescence and light scattering distributions at 25° in a flow cytometer within half an hour.

Flow cytometric measurements

The flow cytometric measurements were carried out in a FACS III fluorescence-activated cell sorter (Becton-Dickinson). The 488 nm line of the argon ion laser was set for excitation. A neutral density filter (Absorbance: 1·0) was used in the scatter channel and a 520 nm 'cut-on' filter with a 520 series D coloured glass filter in the fluorescence channel (Ditric Optics). The scattered light and the fluorescence were detected with a photodiode and a photomultiplier tube, respectively. The intensities (both scattered light and fluorescence) were

stored in a multichannel analyser and displayed as frequency distribution histograms. Chicken red blood cells (CRBC) fixed with glutaraldehyde (Herzenberg & Herzenberg, 1978) were used as a standard for daily set up of the instrument. This way, each experiment was standardized and could be related to earlier experiments.

Analysis of binding experiments

The IgG1 molecules bound to a simple cell (r) and the free IgG1 in the surrounding medium (c) were calculated for each experimental point. A Scatchard plot of r/c versus r was constructed, with the relation $r/c = nK - rK$ (Arend & Mannik, 1973). The n value (the x intercept) represents the number of receptor sites per cell, assuming that only one IgG1 molecule occupies one receptor site. The slope of the line, K, stands for the effective association constant expressed in M^{-1}. All curve fittings were done by the least squares method with a computer.

RESULTS

The human peripheral monocytes suspensions were analysed with FACS III. The scattered light intensity distribution histograms of the cells are shown in Fig. 1. The light scattering was proportional to the crossectional area of the cells (Salzman, Mullaney & Price, 1979). Three peaks were observed on the frequency distribution histograms. The cells were separated according to their light scattering. The characteristics of the cell fractions were further defined by (a) non-specific esterase staining (α-napthylacetate esterase), (b) microscopic visualization of the phagocytozed latex and (c) the reaction of Fc receptors with sSRBC. The first peak belonged to the red blood cells and debris; the second one to lymphocytes and small monocytes; and the third one to large monocytes.

Table 1 shows the staining and functional characteristics of the non-fractionated monocytes and cell fractions obtained from twelve cell separations of the monocyte suspension of twelve different individuals. In non-separated monocytes and in the third fractions latex phagocytosis closely approximated the percentage of monocytes subsequently demonstrated by esterase staining. In the second fractions the percentage of the esterase-stained monocytes is lower than that of their latex phagocytosis.

Among the non-fractionated cells containing 86·1% esterase positive cells, 62% of the cells were Fc receptor positive. In the second fractions the percentage of the Fc positive cells was smaller than in the third fractions.

The results of these experiments showed that the esterase staining and the EA uptake of the small monocytes were lower than that of the large ones.

The monocytes suspension was stained with FDA and the cell distributions were examined according to their size and fluorescein content. The data analysis was triggered with fluorescence intensity and only two peaks could be observed on the scattered light distribution histograms and two on the fluorescence intensity distribution histograms (Fig. 2). The first peak belonging to the red blood cells and debris (Fig. 1) disappeared because these cells and debris did not accumulate the fluorescein (Rotman & Papermaster, 1966). In order to measure the size distribution of monocytes with high fluorescence intensity separately,

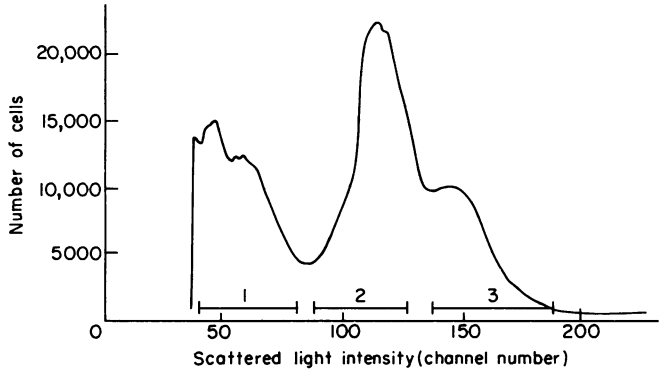

Figure 1. Scattered light intensity distribution of purified monocytes. Fraction 1, red blood cells and cell debris; fraction 2, lymphocytes and small monocytes: fraction 3, large monocytes.

Table 1. Staining and functions of monocytes

Cell Fraction	% Cells positive (mean ± SD) Esterase	Latex	EA*
Unseparated monocyte	86·12 ± 9·2	89·87 ± 7·2	61·92 ± 8·2
Small monocyte	50·5 ± 5·3	68·5 ± 8·2	16·3 ± 2·5
Large monocyte	83·2 ± 7·6	82·2 ± 9·4	50·3 ± 7·4

Means were derived from duplicate determinations for twelve different separations.
* EA, sensitized SRBC.

data collection was triggered with a discriminated fluorescence signal as indicated in Fig. 2. It became evident that cells with low scattered light intensities (small size ones) had low, whereas the large ones had high fluorescence intensities.

During FDA hydrolysis the fluorescence intensity distribution of cells changes with time. The average fluorescence intensity of small monocytes and that of the large ones, determined from the distribution histograms, increased at the beginning of the reaction and reached different plateaus (Fig. 3). The exact fluorescein content of cells could be determined by comparing the average fluorescence intensities of cells to that of CRBC used as a standard. In separate experiments, carried out with steady state fluorimeter as well as with FACS III, we concluded that the average fluorescence intensity of CRBC corresponds to the fluorescence intensity of 260,000 fluorescein molecules. Small monocytes contained four times less fluorescein than large ones. This finding indicates two and a half times higher intracellular fluorescein concentration in large monocytes compared to that in small ones, taking into account the differences in size. (The ratio of the cross-sectional areas of large to small monocytes was 1·3, i.e. the ratio of cell volumes was 1·5.)

The FITC-IgG1-stained monocyte suspension was analysed with FACS III both for light scattering and fluorescence (Fig. 4). By setting the threshold of light scattering, red blood cells and debris were excluded from the analysis. Both the light scattering and the fluorescence distributions of cells show two peaks. Similar to the results of FDA hydrolysis, the triggered data collection revealed that the cells with low light scattering had low, while the large monocytes had high fluorescence intensity.

Monocytes were incubated with increasing amounts of FITC-IgG1 (or with medium alone) and analysed for fluorescence (Fig. 5). Cells lacking FITC-IgG1 had a sharp distribution with relatively low fluorescence intensity, and, with increasing amounts of FITC-IgG1, the peak broadened and moved to higher channel numbers. The average fluorescence intensities were calculated from the two peaks of the fluorescence intensity distribution and were compared with the average fluorescence intensity of CRBC in order to determine the amount of bound IgG1 molecules per cell. (The slight spectral differences between FITC-conjugates and fluorescein were taken into account.)

Figure 6 shows the specific binding data displayed as Scatchard plots. Small and large monocytes had a

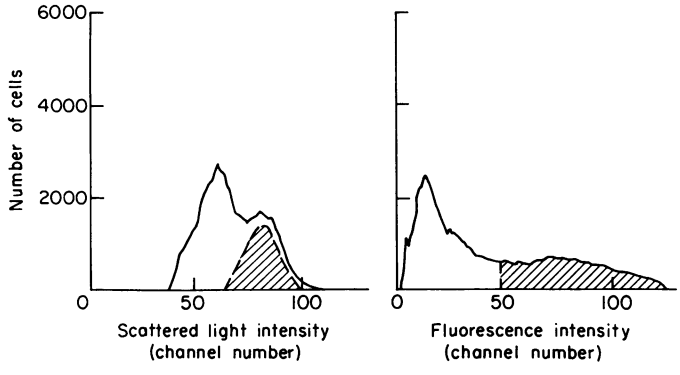

Figure 2. Distribution histograms of scattered light and fluorescence intensity of cells stained with FDA. (——) Data collection was triggered by light scattering; (■) data collection was triggered by the hatched region of fluorescence intensity.

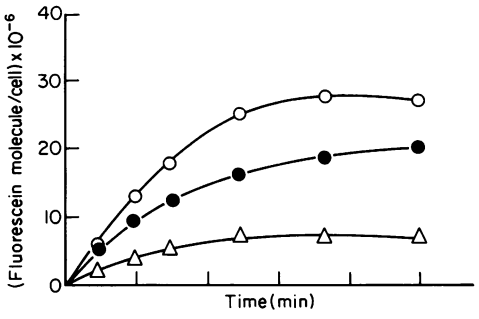

Figure 3. Average fluorescense intensities of monocytes during FDA hydrolysis. (–○–) Large monocytes; (–●–) total monocyte; (–△–) small monocytes.

different number of binding sites ($3{\cdot}3 \times 10^5$ and 1×10^6, respectively), but practically identical association constants ($1{\cdot}28 \times 10^5$ M^{-1} and $1{\cdot}17 \times 10^5$ M^{-1}). The cell surface density of the Fc receptors of large monocytes is 2·3 times higher than that of the small ones, even after normalizing the differences in size.

Figure 7 demonstrates the Scatchard plot of FITC-F (ab′)$_2$ binding to monocytes with low association constant ($1{\cdot}6 \times 10^4$ M^{-1}) and with high numbers of binding sites ($3{\cdot}3 \times 10^6$ M^{-1}). These values were calculated from the average intensity of the whole fluorescence intensity distribution curve. Similarly to the above results, a very low association constant and very high numbers of binding sites were obtained in the FITC-BSA binding experiments (data not shown).

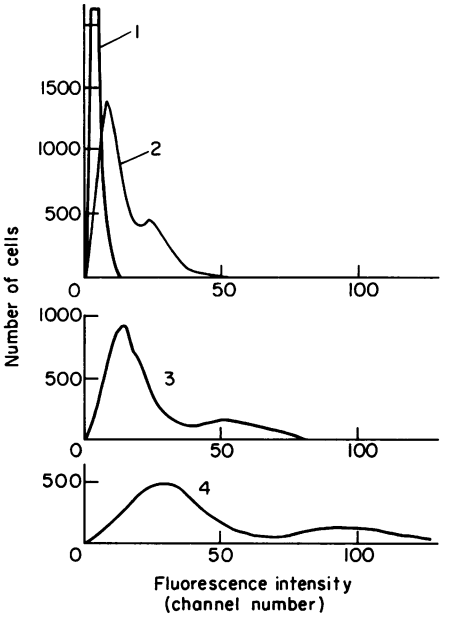

Figure 5. Distribution histograms of fluorescence intensity of cells incubated with different amount of FITC-IgG1. 1, Without IgG1; 2, 9×10^{-7} M, 3, $1{\cdot}8 \times 10^{-6}$ M; 4, $3{\cdot}6 \times 10^{-6}$ M IgG1.

Summarizing the data of eight separate experiments the average association constant of the Fc receptors fell in the range of $1\text{–}2 \times 10^5$ M^{-1} and the numbers of Fc receptors of small and large monocytes were within the range of $2\text{–}5 \times 10^5$ and $8\text{–}13 \times 10^5$, respectively.

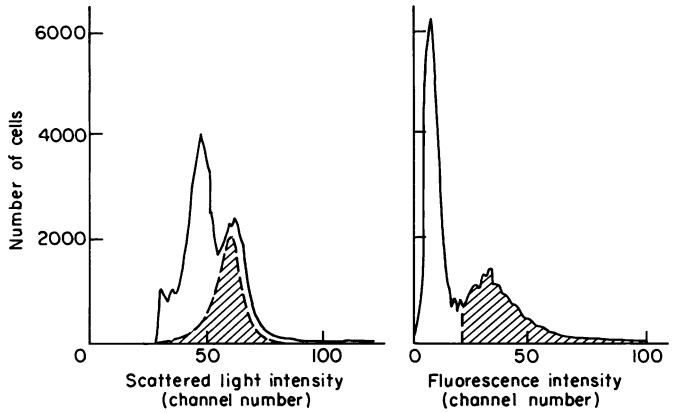

Figure 4. Distribution histograms of light scattering and fluorescence intensity of cells reacted with FITC-IgG1. (——) Data collection was triggered by light scattering; (■) data collection was triggered by the hatched region of fluorescence intensity.

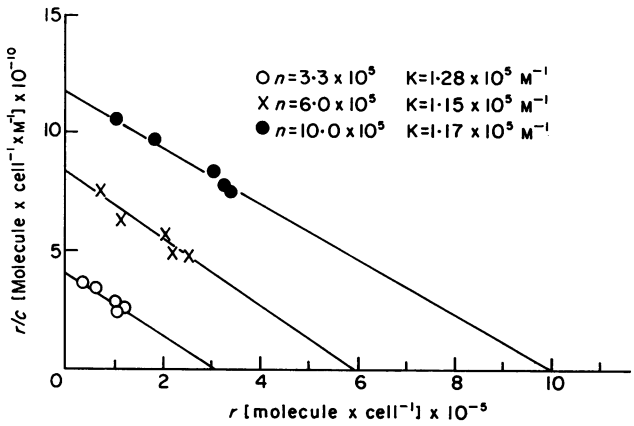

Figure 6. Scatchard plots demonstrating the variation in numbers of receptor sites for IgG1 (*n*) without significant difference in average association constants (K). (●) Large monocyte; (O) small monocyte; (X) total monocyte.

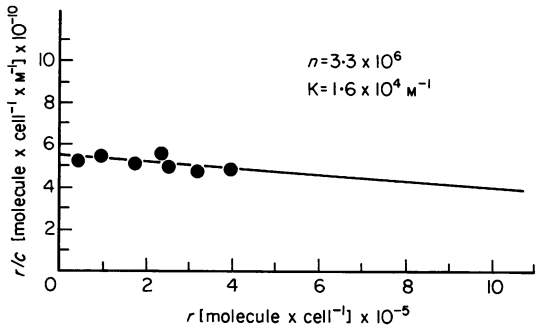

Figure 7. Scatchard plot demonstrating the number of receptor sites for F(ab')2 (*n*) and the average assotiation constant (K).

DISCUSSION

The heterogeneity of peripheral blood monocytes was established by identifying two distinct cell populations with different size and functional characteristics. Although both small and large monocytes were phagocytic, the majority of the large ones stained well for non-specific esterase as well as having surface receptors for IgG, whereas these two functions of the small monocytes could only be detected at a lower degree.

There is a close correlation between the non-specific esterase activity and the fluorescein content of the cells. The intracellular fluorescein concentration of cells is determined by the rates of FDA hydrolysis and the efflux of fluorescein (Bresler, Bresler, Konbekov, Nikiforov & Vasileva, 1979; Szöllösi, Kertai, Somogyi & Damjanovich, 1981). Assuming that the rate of fluorescein efflux has similar value for small and large monocytes we can state that the esterase activity of large monocytes is significantly higher than that of the small ones.

Arenson *et al.* (1980), who analysed the human monocyte with similar technique as we did, described three monocyte subpopulations with different functional characteristics. The monocytes in our experiments were isolated by Ficoll-Hypaque and the commonly used adherence technique before their analysis in FACS III. Therefore one must consider that, on one hand, certain monocyte subpopulations may not readily adhere to the selecting surface, and on the other hand, the adherence-purified monocyte population may have its native function altered by the adherence procedure (Stevenson, Katz, Wright, Contreras, Jemionek, Hartwig, Flor & Fauci, 1981). Though adherence can stimulate procoagulant activity and protein synthesis in monocytes (van Ginkel, van Aken, van Oh & Vreeken, 1977), this activation process can hardly make 'homogeneous' monocyte populations become heterogeneous. Our results are consistent with the work of Norris *et al.* (1979), who demonstrated two monocyte subpopulations isolated by Ficoll-Hypaque without adherence before elutriation centrifugation. Several authors observed only a normal cell distribution with smaller and larger monocytes in elutriation centrifugation. With this technique Weiner & Shah (1980) found two cell populations to be separable according to size.

Norris *et al.* (1979) identified large monocytes to be predominantly Fc receptor positive and the small ones to be largely Fc receptor negative. The present work gives further illustration of the number of the Fc receptors on the cell surface and the association constants of their reaction with IgG1. The data by Alexander, Andrews, Leslie & Wood (1978) differ from ours. Their Scatchard analysis of IgG1 binding to peripheral blood monocytes indicated Ka $10{\cdot}7 \times 10^7 \pm 3{\cdot}9$ litre/mol, with $3{\cdot}1 \times 10^4 \pm 1{\cdot}6$ sites/cell. A possible explanation for this difference is that our measurements were carried out at 25° instead of 0°. Furthermore, when the unwashed cells were analysed with FACS III, the equilibrium between bound and free ligands was not perturbed. The triggered data analysis made it possible not to take red blood cells and the cell debris into account. At the same time the number and association constant of the Fc receptors of living monocytes could be investigated at population level.

The observation that the number of receptors per cell was lower among small monocytes than among large ones, and, at the same time, the association constants were not significantly different, might be of great interest. The number of the Fc receptors of large monocytes was found to be about three times higher than that of the small ones. Taking the differences in size into account, this means, that the density of the Fc receptors is about 2·3 times higher for large monocytes than for small ones. The ratio of the cross-sectional areas of the large and small monocytes and the ratio of cell volume can be directly calculated from the data obtained in FACS III; therefore we expressed the esterase activity and the receptor number on a cellular basis instead of the protein basis of the cells.

The studies suggest that the activity of the cell surface receptors in terms of the relative adherence of IgG1 was related to the number of the receptor sites and not to their affinity, regarding each subpopulation of monocytes. The investigation on the number of the Fc receptors is an addition to morphological and biochemical characteristics of monocytes.

The different Fc receptor numbers on monocyte subpopulations being associated with the different maturation of the mononuclear phagocytic cell line (van Furth, Raeburn & van Zwet, 1979; Kwan, Epstein & Norman, 1976) or with different states of activation of monocytes may probably serve as an explanation for these findings.

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