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Isolation of Functionally Different Human Monocytes by Counterflow Centrifugation Elutriation

By Carl G. Figdor, Willy S. Bont, Ivo Touw, Johan de Roos, Eddy E. Roosnek, and Jan E. de Vries

Human peripheral blood monocytes were isolated by counterflow centrifugation elutriation (CCE). This technique was modified in such a way that various monocyte fractions (viability >99%) could be elutriated by increasing the density of the CCE-medium in steps of 0.0027 g/ml. All monocytes showed the same size distributions as determined by electronic sizing, which indicated that they differed in their density only. Both cytoplasmic esterase and peroxidase activity increased with the density of the cells. Furthermore, the monocytes with the highest density were 2.3-4 times more active in an antibody-dependent cellular cytotoxicity (ADCC) assay than those with the lowest density. In contrast, the monocytes with the highest density were less capable to induce the proliferation of lymphocytes in mixed leukocyte cultures (MLC) than those with the lowest density. This observation could not be attributed to differences in the expression of HLA-DR determinants, since a monoclonal antibody directed against HLA-DR antigens reacted equally well with the monocytes in different fractions. These results provide evidence for the existence of functionally different subsets of monocytes or different stages of differentiation or maturation.

Monocytes/macrophages have been shown to exert various important immunoregulatory functions; for instance, they are required for optimal T-cell responsiveness and T-cell differentiation induced by mitogens and (allo)antigens and they play an intermediary role in the passage of suppressor signals between T-cell subsets. Until now it has not been clear whether the various functions are mediated by the same monocytes/macrophages or whether distinct monocyte/macrophage subsets are involved. Human peripheral blood monocytes have been found to be heterogenous, both in functional and biochemical properties. Furthermore, subpopulations of HLA-DR-bearing monocytes that failed to support antigen-induced T-cell proliferation and that were ineffective in lymphokine production have been described. Monocyte heterogeneity has further been demonstrated with monoclonal antibodies that reacted with a 120,000-dalton determinant present on a proportion of the peripheral blood monocytes. Only those monocytes that displayed both the 120,000-dalton determinant and HLA-DR but not those that had HLA-DR only, are effective in antigen-induced T-cell proliferation.

The aim of the present study was to investigate whether functionally different monocytes can be isolated from human blood. We have previously shown that counterflow centrifugation elutriation (CCE) can be used successfully for the isolation of human monocytes and lymphocytes. Here we describe a modified CCE technique that enabled us to isolate various monocyte fractions according to their density. These monocytes are shown to be different in their enzyme activity, antibody-dependent cellular cytotoxicity (ADCC), and their capacity to induce lymphocyte proliferation in mixed leukocyte cultures.

Materials and Methods

Media and Reagents

Phosphate-buffered saline (PBS) with 0.14% bovine serum albumin (BSA) (fr. V, Sigma, St. Louis, Mo.), penicillin (100 IU/ml), streptomycin (50 µg/ml) was used as standard CCE medium. Elutriation with medium of a higher density was achieved by mixing the standard CCE medium with CCE medium containing 12.5% Percoll (Pharmacia, Uppsala, Sweden). This medium was designated CCE-P-medium. ADCC assays and mixed leukocyte cultures (MLC) were carried out in Dulbecco's modification of Eagle's Minimal Essential Medium (DMEM) supplemented with 5% fetal calf serum (FCS), glutamin, and antibiotics, all obtained from Gibco-Biocult, Glasgow, Scotland. Anti-D antibody was obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands. Tritiated thymidine (³H-TdR, 6.7 Ci/mole) was purchased from New England Nuclear (Boston, Mass.) and FITC-conjugated goat anti-mouse immunoglobulin (GAM) from Nordic Diagnostics, Tilburg, The Netherlands. Maxidens was obtained from Nyegaard and Co, Oslo, Norway.

Cell Preparations

Buffy coat (BC-1) cells prepared from 500 ml blood containing acidic citrate dextrose (ACD) were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam. A second buffy coat (BC-2) was prepared in a newly developed cell separator. This sandglass-resembling apparatus (manufactured by De Koningh B.V., Arnhem, The Netherlands) is shown in Fig. 1 and was made of glass. The cell separator (without fractionation device) was filled with BC-1. The volume of the lower compartment was adjusted by means of Maxidens (a nontoxic autoclavable fluid, which is immiscible with blood and has a density...
of 1.9 g/ml) in such a way that during centrifugation BC-2 was formed just below the narrow tube. The volume of Maxidens required could be calculated after determination of the hematocrit of BC-1. The apparatus was placed in a centrifuge (MSE-Mayor, MSE, Sussex, England), and after centrifugation for 30 min 500 g at room temperature, BC-2 was isolated as shown in Fig. 1.

Counterflow Centrifugation Elutriation

In order to be able to vary the density of the CCE medium, the elutriation system as previously described had to be adapted as shown in Fig. 2. The reservoirs R1 and R2, containing CCE medium and CCE-P-medium, respectively, are large flat plastic bags placed at a height of 180 cm and filled with 2 liters of medium. The thickness of the bags is approximately 4 cm. Since elutriation is carried out at a constant flow rate of 18 ml/min and the pressure is 180 cm, the flow rate will decrease at about 1%/hr. These modifications made it possible to exclude the pump and the overflow as used previously. In this way the system was largely simplified.

Elutriation was carried out under sterile conditions. Autoclavation of the shaft and extensive rinsing of the other parts and tubing with 70% ethanol was sufficient to permit long-term cultures of the fractionated lymphoid cells. During elutriation, the centrifuge was cooled to 0°C, and the medium was cooled by passage through a spiral placed in alcohol of 0°C. This resulted in a temperature of the medium of 4°C just before entering the rotor, and 10°C just after leaving the rotor.

Characterization of Fractionated Cells

The fractionated cells were differentiated according to the following criteria: (1) May-Grünwald-Giemsa (MGG) staining of cytocentrifuge preparations. (2) Staining for nonspecific cytoplasmic esterase. (3) Electronic sizing on a Coulter counter ZF with a pulse-height analyzer (Chanalyzer model C-1000). (4) Pulse-cytophotometry for esterase and peroxidase staining with a Hemalog-D apparatus (Technicon, New York). (5) Immunofluorescent staining with monoclonal anti-HLA-DR antibodies.

ADCC of Fractionated Cells

The various cell fractions were tested for their ADCC capacity in a 51Cr release assay in which antibody-coated human erythrocytes were used as target cells. A quantity of 106 fresh autologous human erythrocytes (HE) were resuspended in 0.2 ml medium and labeled with 100 μCi 51Cr (sodium chromate, New England Nuclear, Boston, Mass.) for 60 min at 37°C. During the last 30 min of the labeling period 0.2 ml of undiluted anti-D antibody was added to the HE. The 51Cr-labeled antibody-coated HE were washed 3 times with PBS, and 2.5 x 10^6 erythrocytes (in 100 μl DMEM) were seeded per well of an U-bottomed microtiter test plate (Linbro, no. 76-213-05, Hamden, Conn.). Various concentrations of effector cells were added in 100 μl medium. The plates were centrifuged for 2 min at 150 g and incubated for 24 hr at 37°C in 100% humidity and 5% CO2. At the end of the incubation, 100 μl of supernatant fluid was collected and counted in a gamma counter. The ADCC was expressed as the percentage of 51Cr release, which was calculated as follows: (AB/(AT-BT)) x 100, where A is mean cpm of test sample; B, mean cpm of spontaneous 51Cr release (i.e., 51Cr release by the labeled target cells in medium only); 7, mean cpm of the maximal 51Cr release that was obtained after addition of 1% Triton X to the target cells. The average spontaneous release ±SD of the HE was 8% ± 2%.

The Capacity of Monocytes to Induce Lymphocyte Proliferation in Mixed Leukocyte Cultures

The monocytes in the various fractions were tested for their capacity to induce lymphocyte proliferation in mixed leukocyte cultures. Mononuclear cells (2 x 10^6) were incubated with 10^3, 5 x 10^3, 2.5 x 10^3, and 1.25 x 10^3 irradiated (3000 rad) monocytes in a final volume of 200 μl DMEM in flat-bottomed microtiter plates (3040 Falcon Plastics, Oxnard, Calif.). After 6 days of incubation at 37°C and 5% CO2, the cultures were pulsed for 3 hr with 0.4 μCi 3H-thymidine (3H-TdR) per well and harvested onto glass fiber filter strips with an automatic sample harvester (MASH II, Microbiological Associates, Walkerville, Md.). 3H-TdR incorporation was counted in a liquid scintillation counter (Packard Instrumental Company, Downers Grove, Ill.). Each test was carried out in triplicate.

Immunofluorescent Staining of Monocytes With Monoclonal Anti-HLA-DR Antibodies

The presence of HLA-DR antigens on the monocytes in the various fractions was determined by direct membrane immunofluorescence using a monoclonal anti-HLA-DR antiserum (anti p29,34, kindly provided by Dr. Cox Terhorst, Sidney Farber Cancer Insti-
The monocytes were resuspended in DMEM supplemented with 5% inactivated (45 min, 56°C) human AB serum and incubated with anti-p29,34 for 30 min at 0°C. After this incubation period, the monocytes were extensively washed in PBS containing 0.01 M NaN₃ and incubated again with 1:10 diluted FITC-conjugated GAM for 30 min at 0°C. The excess of the conjugate was removed by extensive washing with PBS containing 0.01 M azide. After this washing procedure, the monocytes were collected, mounted, and counted under a fluorescence microscope as described previously.² Monocytes incubated with FITC-conjugated GAM only were used as controls.

RESULTS

Isolation of Subsets of Human Peripheral Blood Monocytes

Cells of a second buffy coat (BC-2), as defined in Materials and Methods, were used as starting material. This cell suspension was highly enriched for leukocytes. The leukocyte-erythrocyte ratio was approximately 1:10 (compared with 1:1000 in peripheral blood). Eighty-five percent of these leukocytes are mononuclear cells (Table 1) (representing 90% of the mononuclear cells present in BC-1), whereas the other cells were granulocytes. If, however, BC-2 cells were used as starting material, the granulocytes present in this cell suspension were found to contaminate the monocyte fractions (data not shown), indicating that the sedimentation velocities of both cell types are almost equal.

By manipulating the density of the CCE medium, we were able to isolate various monocyte fractions, which in addition were almost completely depleted of granulocytes (Table 1). The BC-2 cells were fractionated at a constant flow rate of 18 ml/min in the following way: The cell sample was introduced at 3200

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<th>Table 1. Characterization of the Isolated Fractions</th>
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<td>Rotor Speed (rpm)</td>
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<td>Starting material</td>
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<td>Mean ± standard deviation of 6 experiments is shown. Starting with fraction 4, the density of the elutriation medium was raised as shown in Fig. 1. For further details of the isolation procedure, see Materials and Methods and Results.</td>
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rpm to remove the thrombocytes and most of the erythrocytes. The first cell fraction was collected at 3050 rpm and contained the rest of the erythrocytes and approximately 40% of the lymphocytes. The fractions 2 and 3 were elutriated at 2900 and 2750 rpm, respectively, and contained lymphocytes. At this point of the separation procedure, the density of the CCE medium was increased, whereas the rotor speed was kept constant. A stepwise increase in density of the elutriation medium was achieved by mixing the CCE medium and CCE-P-medium at different ratios, as described in the legend of Fig. 2. To isolate monocyte fractions, the following ratios of CCE and CCE-P-medium (ml/min) were used: 15:3, 12:6, 9:9, 6:12, 3:15, and 0:18. This implies that the density of the medium increased with steps of 0.0027 g/ml (Table 1). Fraction 4 is an intermediate fraction (Table 1) and contained few cells. In fraction 5, consisting of 85% monocytes, 19% ± 9% of the monocytes were recovered. Fractions 6 and 7 contained 88%–96% monocytes and 24% ± 6% and 30% ± 10% of the monocytes were recovered in these fractions, respectively. Fraction 8 was contaminated with granulocytes and contained 11% ± 4% of the monocytes. Fraction 9 was mainly composed of granulocytes. The monocytes present in this fraction represented only 4% ± 3% of the total monocyte number. The monocytes recovered in fractions 5–8 represent about 75% of all monocytes present in BC-1.

The viability of the cells in all fractions was >99%. Electronic sizing of the fractions demonstrated the same size distribution of all monocyte fractions (4–8) (not shown).

These results indicate that monocyte fractions that differ in density can be isolated by means of a stepwise increase of the density of the medium.

**Enzyme Activity of the Monocyte Fractions**

Both cytoplasmic peroxidase and nonspecific esterase activity were measured in a Hemalog-D pulse-cytophotometer. The peroxidase activity of fractions 5, 6, 7, and 8 was shown to increase in parallel with the increase of the density of the monocytes (Fig. 3). This phenomenon could not be attributed to an increase in cell size, because all monocyte fractions had the same size distribution profile as measured electronically. The fractions 5, 6, and 7 had the same size distribution as measured by light scatter in the Hemalog-D (Fig. 3). The increase in size of the cells in fraction 8 (illustrated by the upward shift of the scatter along the Y axis) has to be attributed to a 42% contamination

**Fig. 3.** Peroxidase activity in the monocyte fractions. The peroxidase activity of the cells present in fractions 5, 6, 7, and 8 were measured with a Hemalog-D pulse-cytophotometer. The Y axis represents the scatter (cell size) and the X axis represents the enzyme content per cell. The granulocytes were displayed as larger cells than the monocytes, due to scattering of internal structures of these cells.
with granulocytes, which also contain peroxidase activity (see legend to Fig. 3).

The content of cytoplasmic nonspecific esterase of the various monocyte fractions was also shown to increase in parallel with the increase in density of these cells (Fig. 4). The small scatter spots in the lower left corners of fractions 5 and 8 were due to minimal lymphocyte contaminations (see also Table 1). In spite of the fact that the same chemicals were used, the percentage of esterase-positive cells detected by the Hemalog-D (Table 2) was always lower than the percentage esterase-positive cells obtained with the esterase staining procedure described by Lawrence and Grossman. Since the latter procedure was carried out on cytocentrifuge preparations that were counted under the microscope (Table 1), the discrepancy could be explained by the lower sensitivity of the Hemalog-D apparatus (due to threshold setting) compared to the human eye, which is illustrated by the relatively large number of "large unstained cells" scored by the Hemalog-D apparatus (Table 2). Furthermore, it is shown that the number of "large unstained cells" decreased with increasing cell density, indicating again that enzyme activity increased with the cell density.

**ADCC of the Fractionated Cells**

The monocyte fractions obtained by elutriation were tested on their capacity to lyse anti-D-coated autologous erythrocytes. The effector/target cell ratios were 1, 0.5, 0.3, 0.2, and 0.1. Afterwards, the E/T ratios

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<th>Table 2. Percent Monocytes and Large Unstained Cells Determined by the Hemalog-D Pulse-Cytophotometer</th>
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<td><strong>Percent Monocytes</strong></td>
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The sum of the percent of monocytes and large unstained cells equals the amount of monocytes as determined by the manual esterase staining (see Table 1).
were converted to 100% monocytes in each fraction (as judged by esterase staining), so that the number of lytic units/10^7 monocytes could be calculated. In this way, the various fractions were directly comparable (Table 3). Maximal ADCC was observed in the monocyte fractions with the highest density. These cells were 2.3–4 times more active than the cells with the lowest density. Purified granulocytes and lymphocytes, which contaminate these fractions, are ineffective in this ADCC. From these data it can be concluded that there is a positive correlation between the density of the monocytes and their ADCC activity. Activation of the monocytes by Percoll, which possibly might influence the ADCC, could be excluded, since incubation of monocytes for 3 hr with 5%, 10%, or 15% Percoll had no effect on their activity in ADCC. \( \text{Percoll} \) Furthermore, identical results were obtained if the separation procedure was carried out with Isopaque instead of Percoll.

The lack of ADCC activity of highly purified lymphocytes could not be attributed to lymphocyte defects introduced by the separation procedure, since stimulation with phytohemagglutinin (PHA) in the presence of monocytes resulted in \(^3\text{H}\)-thymidine uptake, which was comparable to that of the corresponding unfractionated lymphocytes (De Vries et al., unpublished results). Finally, the low ADCC of fraction 4 cells could not be attributed to suppressive effects of the lymphocytes present in this fraction, since no decrease in ADCC of the monocyte fractions 5, 6, and 7 was observed after addition of fraction 4 cells.

The Capacity of the Monocytes in the Various Fractions to Induce Lymphocyte Proliferation in MLC

Various concentrations of the monocytes in fractions 5, 6, and 7 were used as stimulator cells in MLC in 5

\[
\begin{array}{ccccccc}
\text{Fraction Number} & \text{exp 1} & \text{exp 2} & \text{exp 3} & \text{exp 4} & \text{exp 5} & \text{exp 6} \\
\hline
\text{Starting material} & 221 & 99 & 146 & 223 & NT & 240 \\
4 & 111 & NT & NT & 129 & 137 & 148 \\
5 & 161 & 55 & 100 & 200 & NT & 210 \\
6 & 266 & 78 & 153 & 210 & 210 & 267 \\
7 & 162 & 106 & 190 & 250 & NT & 307 \\
8 & 287 & 235 & 368 & 333 & 320 & 400 \\
\end{array}
\]

\[^{51}\text{Cr} \text{release of autologous anti-D-coated erythrocytes was measured by adding cells from fractions 4 to 8. Effector/target cell ratios were 1, 0.5, 0.3, 0.2, and 0.1. To compare the results from the different fractions, the E/T ratios were converted afterwards to 100% monocytes in each fraction (as judged by esterase staining), so that the number of lytic units/10^7 monocytes could be calculated. One lytic unit equals the number of monocytes necessary to give 25% lysis of the target cells.\]

DISCUSSION

CCE has been shown to be a very efficient procedure for the isolation of large numbers of human lymphocytes and monocytes from peripheral blood.\(^{11,12,16-18}\) We previously described that lymphocytes and monocytes can be separated from mononuclear leukocyte samples that were obtained by centrifugation of peripheral blood over Ficoll/Hypaque mixtures.\(^{12}\) Here we demonstrate that this laborious Ficoll/Hypaque step can be omitted and that the BC-2 cells can be introduced directly into the elutriator rotor if they are...
prepared with the cell separator as described in Materials and Methods. By means of this technique, BC-2 cells can be collected without exposure to body foreign substances that might affect their physical and functional properties.

Increase in density of the CCE medium during elutriation not only prevents contamination of the monocytes with granulocytes but also enables us to separate monocyte subpopulations that differ in density.

Introduction of a density gradient in the system requires a second reservoir with CCE-P-medium. However, introduction of a second reservoir in the system described previously would make it too complex to handle. Therefore, in the present system, the pump and the overflow were replaced by large, flat, soft plastic containers. The constancy of the flow rate was not significantly affected by these alterations in the system, since the decrease of the flow rate was only 1%/hr. This is acceptable, especially if the simplification of the system is taken into account.

To prevent lymphocyte contamination of the monocyte fractions, the increase in density of the CCE medium can only be started after the lymphocytes have been elutriated. A stepwise increase in the density of the medium of only 0.0027 g/ml proved to be sufficient to isolate various monocyte fractions. The monocytes in these fractions had the same size distribution as judged by electronic sizing, indicating that they only differ in their density. The results showed that a physical property of the monocytes, like their density, is correlated with their functions; a positive correlation between density enzyme content and ADCC activity was observed. Preliminary data furthermore indicated that hydrogen peroxide production during phagocytosis of serum-treated zymosan (STZ) increased in parallel with the increase in density of the monocytes (Roosnek et al., manuscript in preparation).

Less dense monocytes (fraction 5) induced significantly stronger T-cell proliferation in MLC than did the more dense monocytes (fractions 6 and 7). We previously reported that the T-cell-induced proliferation by monocytes in MLC was mediated by HLA-DR antigens present on the monocytes. However, the differences in T-cell-inducing capacity of the monocyte fractions 5, 6, and 7 could not readily be attributed to differences in the expression of HLA-DR antigens, as judged by fluorescence microscopy, and are probably due to other metabolic differences between these cells.

Norris et al., and recently also Yasaka et al., reported the isolation by CCE of "small" and "large" monocytes that differed in function. The results obtained by Norris et al. may be explained by the fact that the cell population designated by these authors as small monocytes actually represent the contaminating lymphocyte population, which implies that the bimodal size distribution profiles observed here to be attributed to lymphocytes and monocytes and not to "small" and "large" monocytes. In addition, the size distribution profile of the intermediate fraction (containing lymphocytes and monocytes) of Norris et al. indicates that the monocytes in this fraction are primarily large monocytes instead of small ones, as was concluded by these authors. Our results are in line with those of Contreras et al., who were unable to demonstrate bimodal size distribution of small and large monocytes.

Furthermore, it has to be stressed that it is not allowed to determine the volume of monocytes directly from the size distributions of mixtures of lymphocytes and monocytes. Since the peaks of these two populations overlap, a careful analysis of the profiles is required. The apparent differences in size of the various monocyte subpopulations, as reported by Yasaka et al., can therefore be attributed to lymphocyte contamination. This can be illustrated by the size distributions of their unfractionated mononuclear cell suspensions; the volume of the monocytes in these cell suspensions (65% lymphocytes, 35% monocytes) equals the volume of the "small" monocytes, although this type, according to the authors, represents only 25% of the total monocyte populations. Our results indicate that the physical differences between the various subsets of monocytes are primarily related to density and not to size.

The present study strongly suggests that monocytes that differ in morphological and functional criteria exist. It was not clear until now whether these monocytes represent various subsets or different maturation/differentiation stages of these cells. Because macrophage differentiation was shown to be accompanied by a loss of peroxidase positive cytoplasmic granules, it may be concluded that the low density monocytes represent the most and the high density monocytes the least differentiated monocytes.

However, since tissue macrophages, which are considered to be the differentiation endpoint of monocytes, still have been shown to contain considerable quantities of both esterase and peroxidase, it is not unlikely that the maturation or differentiation of the monocytes is following an opposite direction than was suggested by in vitro studies. This could imply that the most dense monocytes containing the highest peroxidase and esterase activity are the most mature cells. Accurate estimation of the esterase and peroxidase activities...
of isolated tissue macrophages may help us obtain a better insight in the maturation pathway of monocytes.

Finally, preliminary analysis of the distribution of monocytes according to their differentiation/maturation stages in the hemalog-D showed abnormal distribution profiles. Depending on the individual donors, shifts to more mature or more immature monocytes have been observed (unpublished observations). Shifts in these populations may explain the defects in monocyte functions described in cancer patients and deserve further investigation.

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