A Method for the Recognition and Separation of Human Blood Monocytes on Density Gradients

By Hans Loos, Bep Blok-Schut, Rineke van Doorn, Regina Hoksbergen, Aarf Brutel de la Rivière, and Louis Meerhof

The density distribution of human mononuclear blood leukocytes was studied in order to define the optimal conditions for the separation of monocytes and lymphocytes by isopycnic centrifugation. Under standardized conditions, two populations of cells with partially overlapping, normally distributed densities were consistently found. The cells with the lowest density were recognized as monocytes, using phagocytosis and size distribution analysis as criteria. Since the density of monocytes continuously increased during the centrifugation, optimal separation of monocytes and lymphocytes could only be achieved by limiting the time of centrifugation to 10 min at 2200 g and 4°C. The separation on discontinuous density gradients decreased when the load exceeded $8 \times 10^6$ mononuclear cells per sq cm.

Analysis of the composition of the two cell populations obtained after separation on a three-layer discontinuous gradient revealed that the contamination of the monocytes with lymphocytes was due to the partial overlapping density distributions of both cell types. A small and a large scale method for isolation of monocytes from blood on discontinuous density gradients are presented. Under the described conditions, a preparation of functionally intact monocytes can be obtained which is comparable, both in yield and purity, to those obtained by methods based on surface adherence without the drawbacks of the latter methods.

The usual methods for isolation of lymphoid cells are based on differences in size or density. However, little attention has been paid to the fact that cell size and cell density are not constant properties, since these parameters may change independently for each cell type during an isolation procedure. Even in careful studies on the density distribution of mononuclear blood cells, the possibility that the optimal conditions for isopycnic equilibrium centrifugation of lymphocytes need not be identical with those required for monocytes has been neglected.

We have studied the sedimentation behavior of lymphocytes and monocytes in continuous and discontinuous density gradients. Monocytes were localized in the density distribution profiles on the basis of phagocytosis and by electronic sizing. Under proper conditions, monocytes and lymphocytes could be distinguished as distinct cell populations on the basis of density.

The results of this study allowed us to devise a method for the preparative isolation of monocytes exclusively on the basis of density. The yield and purity of monocytes thus obtained was comparable with those obtained after surface adherence.
MATERIALS AND METHODS

Eagle's minimum essential medium for spinner cultures (MEM-S) and trypsin 1:250 were purchased from Gibco, Grand Island, N.Y. Fetal calf serum (FCS) was obtained from Flow Laboratories, Ayshire, Scotland. Ficoll (polysucrose, MW 400,000) was purchased from Pharmacia, Uppsala, Sweden and Isopaque (1.156 M metrizoate solution) from Nyegaard A/S, Oslo, Norway. 6-3H-Thymidine was obtained from the Radiochemical Centre, Amersham, Great Britain, and carbonyl iron powder (code 1-1-63.763) was obtained from General Aniline and Film Corp., Linden, N.J. Human albumin solution was obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam.

Preparation of Density Gradients

Linear density gradients, isotonic with human serum and ranging from 1.055 g/ml (at 4°C) at the top to 1.095 g/ml at the bottom were prepared, as described previously,6 from two solutions of Ficoll-Isopaque containing 1% human serum albumin (see Table 1). In comparison to the original method, the gradient volume was increased from 8 to 12.8 ml. This modification increased the resolution of the cell separation. Discontinuous density gradients were obtained by successive layering of Ficoll-Isopaque mixtures with decreasing density on top of each other in 13-ml siliconized glass tubes. In order to prepare solutions of any desired density within the range 1.055-1.095 g/ml (at 4°C), the mixing ratio \(a\) of the stock solutions was calculated with the equation

\[
\text{Required density} = a(1.095) + (1 - a)1.055.
\]

The final density was verified by measuring the refractive index at 25°C (see Table 1).

Determination of Density Profiles

Analysis of the density profile was performed as described previously.6 In brief, 20–25 x 10^6 cells were layered on top of a gradient of 12.8 ml and centrifuged for 10 min at 4°C. The separated cells were harvested by pumping out the gradient. To this end, a long steel needle was lowered toward the bottom of the gradient tube and 40 fractions of 0.32 ml were collected. The cell concentration of a 100-fold dilution of each fraction was counted with a Coulter Counter (ZF); a sample volume of 0.5 ml was used. In order to make the density distributions mutually comparable, the countings were expressed as a percentage of the total number of cells recovered after the analysis. The recovery was 80%-90%, thus 1% in the density distribution curves represented 2000–3000 cells counted electronically in the actual dilution of the fractions. The

Table 1. Recipe for Ficoll-Isopaque Mixtures Iso-osmotic With Human Serum

<table>
<thead>
<tr>
<th>I.</th>
<th>Density 1.055 g/ml (4°C), osmolality 294 mOsm, pH 7.4 (4°C)</th>
<th>Fill to 1100 ml with H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ficoll (MW 400,000)</td>
<td>28.2 g</td>
<td></td>
</tr>
<tr>
<td>Isopaque (1.156-M metrizoate)</td>
<td>110.3 ml</td>
<td></td>
</tr>
<tr>
<td>Tris-HCl (0.175 M, pH = 7.4 at 4°C)</td>
<td>74.3 ml</td>
<td></td>
</tr>
<tr>
<td>Krebs-Ringer-Tris solution*</td>
<td>202.4 ml</td>
<td></td>
</tr>
<tr>
<td>After Millipore filtration (0.22-µm pore size), 950 ml of this solution is mixed under sterile conditions with 50 ml human serum albumin (20% w/v).</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>II.</th>
<th>Density 1.095 g/ml (4°C), osmolality 294 mOsm, pH 7.4 (4°C)</th>
<th>Fill to 1100 ml with H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ficoll (MW 400,000)</td>
<td>195.8 g</td>
<td></td>
</tr>
<tr>
<td>Isopaque (1.156-M metrizoate)</td>
<td>88.0 ml</td>
<td></td>
</tr>
<tr>
<td>Tris-HCl (0.175 M, pH = 7.4 at 4°C)</td>
<td>82.3 ml</td>
<td></td>
</tr>
<tr>
<td>After Millipore filtration (0.22-µm pore size), 950 ml of this solution is mixed under sterile conditions with 50 ml human serum albumin (20% w/v).</td>
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The relation between the density and the refractive index of these mixtures is given by \(Y = (0.2304) X + 31.3\), where \(Y = \text{density at } 4°C - 1.0\) x 10^3, \(X = \text{refractive index at } 25°C - 1.34\) x 10^4

*Krebs-Ringer-Tris solution: 125 mM NaCl, 5 mM KCl, 1.2 mM MgSO_4_, 33 mM Tris, 1 mM Na phosphate (pH 7.4 at 4°C)
density of the fractions was derived from the refractive index measured with an Abbé refractometer.

**Preparation of Escherichia coli Labeled with $^3$H-Thymidine**

K 12, a thymine auxotroph strain of *Escherichia coli*, kindly provided by Dr. P. H. Pouwels, RVO/TNO, Rijswijk, The Netherlands, was cultured in the presence of 4 mM $^3$H-labeled thymidine (specific activity 26 μCi/m mole) on a synthetic minimal medium without unlabeled thymidine for 24 hr. The bacteria were killed by boiling for 20 min, spun down, and washed with saline solution until no further radioactivity could be eluted. The pellet was resuspended to a final concentration of $3 \times 10^8$ bacteria/ml and a radioactivity of 25 μCi/ml. The bacterial suspension was used after dilution with unlabeled *E. coli* and MEM-S supplemented with 10% (v/v) fetal calf serum at a final concentration of $2 \times 10^8$ bacteria/ml and a radioactivity of 0.1 μCi/ml.

**The Isolation of Mononuclear Leukocytes From Defibrinated Blood**

The isolation procedure, as described previously, was slightly modified, i.e., the incubation with iron carbonyl particles was omitted, no calcium-containing media were used, and the mononuclear cells were finally resuspended in either Krebs-Ringer-Tris solution (see Table 1) with 5.5 mM glucose and 10% (v/v) human AB serum (pH = 7.4 at 37°C), or Eagle's MEM-S + 10% (v/v) human AB serum. This method gave yields of $1.5 \pm 0.12 \times 10^6$ mononuclear leukocytes obtained from 1 ml of defibrinated blood (mean ± SEM, n = 30). The monocyte content of these preparations was 21% ± 1% (mean ± SEM, n = 49), contamination with platelets, erythrocytes, and granulocytes was less than 5%. Viability was 97%-99% (trypan blue exclusion). Cells were enumerated electronically (Coulter Counter model ZF). Differentiation of the various types of leukocytes was performed microscopically after staining with Türk's Blue solution.

**The Isolation of Mononuclear Leukocytes From ACD Blood**

Blood was collected in ACD (2.7% [w/v] anhydrous disodium citrate and 2.3% [w/v] anhydrous glucose) in a ratio of 6 to 1. After centrifugation, (5 min, 1000 g at 20°C) plasma and cells were separated; 50 ml of the upper layer of the packed blood cells was collected (buffy coat). Theuffy coat preparation was diluted with 500 ml phosphate-buffered saline. The elimination of granulocytes and erythrocytes was performed in the same way as described, with one exception. To avoid clotting of the thrombocytes, human albumin solution was used instead of human AB serum throughout the whole procedure. In the final preparation, the thrombocytes were eliminated by aggregation induced with ADP as described previously. After washing, the mononuclear leukocytes were resuspended in Krebs-Ringer-Tris solution with 5.5 mM glucose and 10% (v/v) human AB serum (pH = 7.4 at 37°C). The yield obtained by this method was $1.0 \pm 0.08 \times 10^6$ mononuclear leukocytes (mean ± SEM, n = 12) from 500 ml ACD blood. Monocyte content, contamination with other blood cells, and viability were comparable to the values obtained with defibrinated blood.

**Isolation of Monocytes**

In order to reverse any possible change in density introduced by the isolation procedure, mononuclear leukocyte suspensions, as described above, were incubated for 30 min in a 37°C water-bath, under continuous shaking, at a cell concentration of $6 \times 10^8$/ml. Discontinuous density gradients were prepared by successive layering of solutions with decreasing density on top of each other as specified in Table 2. In order to prevent disturbance of the interfaces, the layers in the 50-ml tube had to be pumped with the aid of a proportional pump, connecting tubing, and a needle. The needle was inserted into the tube to the bottom, and the mixtures were pumped in successively, starting with the lightest. Care was taken that no air bubbles were trapped in the tubing system during the change from one mixture to the next.

The centrifugation procedure, as described previously, was modified as specified in Table 2. The speed was monitored by viewing with a stroboscope through the transparent lid of the centrifuge.

The monocytes sedimented in the upper ring and the monocyte-poor fraction at the second interface. Both ring fractions were harvested separately, diluted with twice the volume of MEM-S
**RESULTS**

**The Density Distribution Profile of Mononuclear Leukocytes**

Mononuclear blood cells were isolated from defibrinated blood as described under Methods. The cells were resuspended in Eagle’s MEM-S with 10% (v/v) fetal calf serum to a final concentration of 6 x 10⁶ cells/ml and incubated in a shaking waterbath for 30 min at 37°C. Thereafter, density analysis was performed as described under Materials and Methods. Figure 1 shows that a density distribution with two peaks was obtained. The mean density of the major population varied from 1.068 to 1.070 g/ml in 11 similar analyses, and the mean density of the minor fraction from 1.062 to 1.064 g/ml. By normalizing the shape of the curves to equal-sided triangles, the surface area covered by the light cell fraction was estimated to be 23% ± 3% (mean SE, n = 11).

![Fig. 1. Phagocytic cells in mononuclear human blood leukocyte suspensions. Abscissa: density in g/ml; ordinate: number of mononuclear cells/fraction (per cent of total recovered cells). Density distributions of mononuclear leukocytes, untreated (○—○), and preincubated (■—■) with iron carbonyl particles.](image)
Fig. 2. Phagocytosis of labeled E. coli bacteria by human mononuclear leukocytes. Abscissa: density in g/ml; ordinate: recovery of cells or radioactivity (per cent of total). Density distribution of cells incubated without (o-o) or with (e-e) 3H-labeled E. coli bacteria, and the radioactivity in each fraction (a-a-a).

When the mononuclear leukocyte suspension was incubated with 4-mg iron carbonyl particles/ml for 30 min at 37°C in a shaking waterbath and subsequently submitted to density analysis, the light cell fraction was almost eliminated (Fig. 1). It is known that phagocytic cells are effectively eliminated by the combination of iron particle treatment and isopycnic centrifugation. Therefore, this experiment suggested that monocytes could be recognized as a distinct light cell population in the density distribution profile of mononuclear blood leukocytes.

Localization of the Phagocytic Mononuclear Leukocytes

Phagocytosis of labeled E. coli bacteria. In order to analyze further the two peaks in the density distribution profile, the mononuclear leukocytes were isolated as described before, the cells were suspended in Eagle's MEM-S with 10% (v/v) FCS to a final concentration of 6 x 10⁶ cells/ml and subsequently incubated without and with labeled E. coli bacteria (10⁶ bacteria/ml; 0.1 μCi/ml) in a shaking waterbath at 37°C for 30 min. After density analysis, the radioactivity of each fraction was estimated as described elsewhere. The result is depicted in Fig. 2. The major part of the radioactivity was recovered within the light leukocyte fraction. The mean density of the bacteria was found to be 1.068 g/ml (not shown here). This suggested that only leukocytes of the light cell fraction were able to phagocytize, since the radioactivity, not associated with the light cells, reflected the noningested bacteria. This suggestion was confirmed by repeating the experiment with a light and heavy mononuclear leukocyte fraction separated on a discontinuous density gradient prior to incubation with labeled E. coli (not shown here). After density analysis of both fractions, the distribution of radioactivity was found to coincide with the density distribution of the light cell fraction only.
The Percentage of Phagocytes in Mononuclear Leukocyte Populations of Different Densities

As described previously, nuclei of chicken erythrocytes, coated with human gamma globulin and stained with ethidium bromide, can be used to estimate the number of phagocytic cells in mononuclear cell preparations. Therefore density analysis was performed on mononuclear leukocyte suspensions which had been incubated with or without fluorescent nuclei. The incubation mixture consisted of 5 x 10^6 mononuclear cells/ml Eagle's MEM-S + 10% (v/v) AB serum with or without 6 x 10^6 nuclei/ml. The cell suspension, incubated with the nuclei for 30 min at 37°C, was diluted with 0.5% (w/v) trypsin in MEM-S (ratio 1:5.5), and the incubation was continued for 10 min in order to detach the noningested nuclei adhering to cell surfaces. Both cell suspensions were then submitted to density analysis, as described above. Although treatment with trypsin slightly changed the density distribution of the mononuclear leukocytes, more than 90% of the total cells was recovered. The peak fractions of the light and of the heavy cells were pooled separately. After washing, these fractions were submitted to fluorescence microscopy. Ninety-one per cent of the light cells had ingested nuclei, whereas in the heavy cell fraction only 9% of the cells were phagocytic (500 cells counted).

Recognition of Monocytes in the Density Distribution Profile by Electronic Sizing

We have shown that monocytes can be distinguished from lymphocytes on the basis of electronic sizing. In order to localize the monocyte peak in the density distribution profile of mononuclear leukocytes, we analyzed the size of...
MONOCYTES: RECOGNITION AND SEPARATION

Fig. 4. Differentiation by size distribution of the cells present in the original mononuclear cell suspension and in the light and heavy fractions obtained by separation on a three-layer discontinuous density gradient. Abscissa: channel number indicating the pulse height of the light scatter, which is proportional to the cell size; ordinate: number of pulses per channel (per cent of total). Original (——o), light (——l), and heavy (——c) mononuclear leukocyte fraction, obtained by separation on a discontinuous density gradient, as described in Table 2. Sizing was performed with a Cytograph 6300A, as described elsewhere. The data specified separately were obtained by morphological differentiation after staining with Türk's Blue solution in a sample of the same cell suspensions. The percentages of large cells (between channels 25 and 50) were found to be identical with the percentages of monocytes determined morphologically.5

The cells after separation into different fractions on a continuous density gradient. Size distribution analysis was performed with a Coulter Counter, as described before, and density analysis was performed as described in Materials and Methods. The mean size of the lymphocytes in the different fractions is shown in Fig. 3A, the density distribution of the mononuclear leukocytes in Fig. 3B, and the percentage of monocytes in each fraction in Fig. 3C. From this figure two conclusions may be drawn. First, the size of the main part of the lymphocytes was inversely proportional to their density (Figs. 3A and 3B). Second, the appearance of the peak of the monocytes in the size distribution profile coincided with the appearance of the shoulder of light cells in the density distribution profile (compare Figs. 3B and 3C). The small number of cells with a size larger than that of lymphocytes, which was found in the heaviest lymphocyte fraction, may have been due to the granulocyte contamination of the original mononuclear cell preparation.5

We predicted from the density distribution profile and our foregoing experiments that almost pure monocytes could be separated from mononuclear leukocytes on a Ficoll/Isopaque mixture with a density of 1.062 g/ml at 4°C. This thesis was proven by separating mononuclear human blood leukocytes into a heavy and light cell fraction on a discontinuous density gradient, as described above. The composition of the original, the light, and the heavy cell fraction was analyzed by electronic sizing and nuclear morphology, as shown in Fig. 4. It was found that the light cell fraction consisted of almost pure monocytes and that the heavy cell fraction was slightly depleted of monocytes. Both phenomena were in complete accordance with our prediction, since we con-
Fig. 5. Effect of increasing centrifugation time on the density distribution profile of mononuclear blood leukocytes. Abscissa: density in g/ml; ordinate: recovery of mononuclear leukocytes per fraction (per cent of total).

Sisently found a partial overlap between monocytes and lymphocytes in the density distribution profiles. We concluded that the shoulder of light cells in the density distribution of mononuclear cells was caused by monocytes.

Factors Influencing the Separation of Lymphocytes and Monocytes

Changes induced by centrifugal stress. The sedimentation behavior of monocytes and lymphocytes was studied by measuring the density distribution profile after various centrifugation times (5–25 min). The result is given in Fig. 5. It was clear that the apparent density of the monocytes increased continuously. The lymphocytes remained in isopycnic equilibrium during 20 min, as reported previously. Thus, isopycnic centrifugation influenced the density of monocytes. However, the reproducible density distribution profile, described above, was not surprising, since our analytical procedure was carefully standardized.

Changes induced by discontinuous density gradients. Discontinuous density gradients may easily influence the density distribution by trapping cells at the interfaces. This trapping is the result of overloading of the gradients, since the interfaces probably become less penetrable as soon as they are covered completely with cells. We studied this phenomenon by separation of different num-
MONOCYTES: RECOGNITION AND SEPARATION

Fig. 6. Density distribution of the light mononuclear leukocyte fraction after loading discontinuous density gradients with 12 × 10⁶ cells (○--○), 18 × 10⁶ cells (△--△), or 30 × 10⁶ cells (△--△). Separation area 1.5 sq cm. Abscissa: density in g/ml; ordinate: recovery of mononuclear leukocytes per fraction (percent of total).

bers of mononuclear cells on discontinuous gradients and analysis of the density distribution profile of the monocyte-rich fraction. The intermediate layer of the three-layer gradient had a density of 1.064 g/ml at 4°C. Figure 6 shows that the contamination with lymphocytes increased with the amount of mononuclear cells applied to the gradients. Incomplete separation of monocytes and lymphocytes was obtained when the cell load exceeded 8 × 10⁶ cells/sq cm. It may be noted that the mean density of the light cell fraction exceeded the original density of 1.064 g/ml. A similar result was obtained previously during a rebanding experiment with pure lymphocytes.

Preparation of Monocytes on the Basis of Density

Monocytes were isolated on discontinuous density gradients from blood collected in two different ways, as described in Materials and Methods. Sixty-four different preparations were made, and the yield and purity were determined. The results are summarized in Table 3. In the fractions depleted and enriched in monocytes, together, the recovery of the cells was 59% ± 5% (mean ±

<table>
<thead>
<tr>
<th>Starting Material</th>
<th>Purity* (% Monocytes)</th>
<th>Yield†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defibrinated blood</td>
<td>75 ± 2 (44–96)</td>
<td>34 ± 3 (10–75)</td>
</tr>
<tr>
<td>10 = 49</td>
<td>n = 29</td>
<td></td>
</tr>
<tr>
<td>ACD blood</td>
<td>82 ± 2 (59–95)</td>
<td>28 ± 4 (12–55)</td>
</tr>
<tr>
<td>10 = 15</td>
<td>n = 13</td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± SEM; ranges are shown in parentheses; n is the number of experiments.
†Harvested monocytes expressed as a percentage of the number present in the mononuclear leukocyte preparations.
Figure 7 also shows that there was an individual variation in the location of the size distribution peak of lymphocytes and monocytes in the original mononuclear leukocyte suspension (the range was 20% for both cell types). Therefore, a similar variation is to be expected in the degree of overlap of the density distribution profiles of both cell types. This variation may be the main cause of the variability in yield and purity of the monocytes obtained from different donors. So far, no significant difference has been found between the yield and purity of the monocytes obtained from defibrinated blood or ACD blood; about 20% of the monocytes present in whole blood can be isolated by both...
methods. Thus, from 50 ml of blood about $5 \times 10^6$ monocytes, and from one unit of blood $50 \times 10^6$ monocytes can be obtained. The entire procedure from venipuncture to enumeration of the cells took about 6 hr for the bulk procedure and 4 hr for the small scale procedure.

Phagocytosis is a sensitive criterion for the functional activity of monocytes. We found that in nine different preparations, 91%-100% (97% ± 3.5% = mean ± SE) of the monocytes, recognized and quantitated by electronic sizing, were able to phagocytose nuclei of chicken erythrocytes coated with gamma globulin.5

The lymphocytes contaminating the monocyte preparations were consistently found to be larger than those present in the original mononuclear leukocyte suspensions. We found that this type of large lymphocyte had a spontaneous $^{3}$H-thymidine incorporation and response to mitogens and antigens 1.5-6 times higher than the original lymphocytes (unpublished observations).

DISCUSSION

The density distribution profile of human mononuclear blood leukocytes consistently revealed two cell populations with partially overlapping, normally distributed, densities. Using three different criteria, it was shown that the phagocytic mononuclear leukocytes were located in the light cell fraction. In addition, the combined analysis of size and density confirmed that the light mononuclear blood cell fraction was identical to the monocytes. In fact, it appeared that the size and density distribution profiles could be described as a mirror image of each other, provided the proper centrifugation conditions with respect to speed and time were used. These findings were in striking contrast to the data obtained by Williams et al.4 These investigators reported that lymphocytes and monocytes were heterogeneous cell populations with substantial overlapping densities. Since we demonstrated that the apparent density of monocytes changed continuously during centrifugation, this discrepancy became understandable; Williams et al. used a higher centrifugal force and a longer centrifugation time. Moreover, our Ficoll-Isopaque gradients were nonagglutinating at a pH of 7.4, while their albumin gradients had to be kept at pH 5.1 to minimize cell aggregation,10 which may also have contributed to this discrepancy.

The purity and yield of the monocytes obtained by the preparative methods described here closely approached the values described in the literature2 and obtained by the combination of density separation and surface adherence. The improvement in the preparative separation on the basis of density only, as described in this report, could be achieved merely by our study on the sedimentation behavior of monocytes and lymphocytes in continuous and discontinuous density gradients. A sensitive and accurate method for the detection of monocytes5 enabled us to monitor the effect of every single step of the preparation procedure. No significant changes in the size distribution profile of the mononuclear cells was noted during the different steps prior to the final separation. The sizing before and after the final separation revealed that the contamination of monocytes with lymphocytes resulted from the overlapping density of monocytes and lymphocytes.
Moreover, this analysis stressed the fact that any attempt to separate cells with a partially overlapping density distribution would lead to a selection and cross contamination on the basis of density. Our finding, that the monocytes were contaminated with a subpopulation of lymphocytes, which was highly reactive towards antigens and mitogens in vitro, has great importance for studies on the actual role of monocytes in immune reactions. We feel that many of the immune reactive properties commonly attributed to monocytes may be due to this minor contamination of highly reactive lymphocytes.

In conclusion, we have presented a method for the separation of monocytes from lymphocytes on the basis of density only. The yield and purity were comparable to the values that can be achieved after surface adherence. The monocytes in these preparations were functionally active as judged from the fact that all cells recognized as monocytes were able to phagocytize. This method gave comparable results whether or not small or large quantities of blood were used.

Since the monocytes were harvested entirely in suspension, they could not have been changed by surface adherence. Therefore, this kind of monocyte preparation can be used to study the metabolic changes introduced by surface adherence.

ACKNOWLEDGMENT

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