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Comparison of human monocytes isolated by elutriation and adherence suggests that heterogeneity may reflect a continuum of maturation/activation states

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SUMMARY

Monocytes are heterogeneous both in terms of physical properties and in their functional capacity. Isolation of monocytes from peripheral blood may perturb the observed heterogeneity for purified cell preparations. To explore this possibility we examined monocytes prepared by two techniques, counter-flow centrifugation elutriation (CCE) and fibronectin adherence, in terms of cell-surface molecule expression and several physical properties. Although such cells would be expected to represent dissimilar cross-sections of the total monocyte population, they were found to have similar cell-surface antigenic profiles. Observed differences in levels of expression of several molecules (CR1, CR3 and the antigen recognized by LP9 antibody) were found to be a temperature-related phenomenon. These results indicate that monocytes are not divisible into 'subpopulations' on the basis of cell-surface molecule expression and suggest that heterogeneity of monocytes may reflect the presence in the circulation of a continuum of maturational/activation states.

INTRODUCTION

Monocytes and macrophages play a fundamental role in the expression of an immune response, displaying antibody-dependent cellular cytotoxicity (Poplack *et al.*, 1976), direct tumouricidal activity (Adams & Nathan, 1983) and involvement in the phagocytic destruction of cellular debris and invading microorganisms. In addition, they interact with other cells of the immune system, presenting antigen to T-helper cells (Unanue, 1984) and modulate cellular responses by production and release of a variety of cytokines (Davies & Allison, 1976). Monocytes are continually released from the bone marrow into the circulation, with an average half-life of 71 hr in the peripheral blood before migrating into tissues and differentiating to become macrophages (Van Furth, Raeburn & Van Zwet, 1979).

The diverse range of functions displayed by these cells makes dissection of heterogeneity difficult, although subsets of monocytes that differ in size (Yasaka *et al.*, 1981) or density (Picker *et al.*, 1980) have been isolated that show differential hydrogen peroxide production (Turpin, Hersh & Lopez-Berestein, 1986), phagocytosis (Chiu *et al.*, 1984), cytotoxic ability (Normann & Weiner, 1983), cytokine release (Khansari, Chou & Fudenberg, 1985) and markers for activation or differentiation (Akiyama *et al.*, 1983). *In vitro*-cultured monocytes become less dense than those freshly isolated (Elias *et al.*, 1985), and it has been suggested that density-defined populations of monocytes may reflect their maturation states (Figdor *et al.*, 1986). Generally, low-density monocytes exhibit reduced functional activity when compared with high-density cells, for example in peroxidase activity, OKM1 expression, accessory cell function (Akiyama *et al.*, 1985) and IL-1 release (Elias *et al.*, 1985). In addition, low-density monocytes are not activated by LPS or IFN-γ (Figdor *et al.*, 1986). However, 5′ nucleotidase activity, FcRII expression and phagocytic ability were found to be similar for both cell fractions. Figdor *et al.* (1986) suggested that cell maturity is a factor in monocyte responses to activation and that, in contrast to *in vitro* maturation, the least dense monocytes actually represent the least mature cells.

In order to examine monocyte functional heterogeneity many different procedures have been used to separate monocytes from other peripheral blood cells, introducing several factors that may affect observed heterogeneity. Monocyte yield is less than 100% and any given isolation procedure will select for cells with particular characteristics, e.g. adherent properties are often exploited in monocyte isolation (Treves *et al.*, 1980).

Abbreviations: BSA, bovine serum albumin; CCE, counterflow centrifugal elutriation; CR1, complement receptor type 1; CR3, complement receptor type 3; EDTA, ethylenediamine tetraacetic acid; FcR, receptor for the Fc region of immunoglobulin G; FITC, fluorescein isothiocyanate; FN, fibronectin; HIFCS heat-inactivated fetal calf serum; IFN-γ, interferon-gamma; LPS, lipopolysaccharide.

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Isolation of monocytes has been shown to have effects upon the levels of expression of certain cell-surface molecules (Fearon & Collins, 1983) and it is possible that some perturbation of function may result. In addition, differential contamination of preparations of monocytes with other cell types may account, in part, for functional capabilities ascribed to the purified population, as has been found for lysis of NK-sensitive targets by preparations of adherent cells (Koren & Herberman, 1985). Furthermore it is not yet clear whether or not isolated subsets represent distinct subpopulations of monocytes or differences in the maturation or activation states caused in part by isolation (Bodel, Nichols & Bainton, 1977). For any continuum it is of course always possible to choose an arbitrary cut-off point, either side of which pooled populations exhibiting different characteristics may be generated. However, such populations do not qualify for the designation of 'subpopulations' or 'subsets' as conventionally applied to cells, compare for example lymphocyte subsets.

To investigate further the problem of separation-induced heterogeneity, we undertook a comparative study of two commonly used isolation techniques, adherence to fibronectin-coated surfaces as described by Ackerman & Douglas (1978) (FN adherent) and counterflow centrifugal elutriation (CCE) (Stevenson, 1984). These techniques enrich for monocytes in very different ways, selecting on the basis of adherent properties and cell size, respectively, and therefore it is extremely unlikely that they will select for the same populations of cells. Cell-surface antigenic profiles of monocytes prepared by FN adherence and CCE from the same donor on the same day were examined with a comprehensive panel of anti-monocyte monoclonal antibodies.

Although examination of cells prepared by the different methods for a number of markers indicated heterogeneity, this was seen as a continuum rather than distinct subpopulations. For certain cell-surface molecules, consistent differences in the levels of expression were found for the two preparations, but were shown to be essentially a temperature-related artefact, and in terms of surface-molecule expression the two preparations were remarkably similar. The results of this study support the suggestion that heterogeneity of monocytes is not due to the presence of defined functionally distinct populations of cells, but that phenotypic and functional differences are a reflection of a continuum of maturational/activation states.

MATERIALS AND METHODS

Subfractionation of peripheral blood mononuclear cells

Buffy-coat leucocyte concentrates, obtained from the Trent Regional Blood Transfusion Service, Sheffield, were centrifuged over Ficoll–Hypaque to isolate mononuclear cells. Adherence preparation of monocytes was performed as described by Ackerman & Douglas (1978). Mononuclear cells in RPMI (Gibco Europe, Paisley, Renfrewshire) supplemented with 10% heat-inactivated fetal calf serum (HIFCS) were allowed to adhere for 60 min at 37°, 5%CO_2. After removal of non-adherent cells and three washes in warm RPMI, removal of adherent cells was effected using a Ca^{2+}/Mg^{2+}-free balanced salt solution (BSS), pH 7·4, containing 5 mM EDTA, for 45 min at 4°.

For separation by CCE, mononuclear cells were resuspended in BSS containing 5 mM EDTA and were loaded onto a Beckman JE6B elutriator rotor equipped with a 'Sanderson' separation chamber at a rotor speed of 2500 ± 20 r.p.m. at 10°, with an initial flow rate of 8 ml/min. Elutriation of cells was achieved essentially as described by Stevenson (1984), by stepwise increments in the flow rate whilst the rotor speed was maintained at a constant rate. Fractions exiting the chamber were continuously monitored by cell-size analysis using a model ZM Coulter Counter interfaced to a BBC microcomputer with a pulse-height analyser program. Calibration was achieved using 5·5 μm and 8·7 μm diameter polystyrene microspheres. 'Monocyte' fractions were collected when cells with a volume greater than 300 μm^3 were the predominant cell type exiting the chamber (CCE monocytes). After collection of cells at a flow rate of 19 ml/min, the centrifuge was switched off and cells collected termed the 'rotor off' fraction. Pooled CCE monocytes were resuspended in RPMI/10% HIFCS and prior to testing were either kept on ice, incubated for 60 min in sterile siliconized vessels at 37°, or allowed to adhere to FN-coated surfaces as described above.

Histochemical staining

Cytocentrifuge preparations of cells were stained for the presence of non-specific esterase, as described by Horowitz *et al.* (1977), and counter-stained with 1% methyl green, or were stained with Geimsa.

Monoclonal antibodies

Antibodies used in this study are described in Table 1. Where appropriate, CD groupings are shown. 8EB1, a CD19 antibody, was obtained from Dr N. R. Ling, Dept. of Immunology, University of Birmingham, Birmingham. LP9 (Partridge *et al.*, 1987) reacts with an antigen found on the cell surface of monocytes, macrophages and several cell lines, K562, EB4 and HUT78. The antigen is also found in the cytoplasm of monocytes, macrophages, granulocytes and the cell lines HL60 and U937. In tissue sections, LP9 stains macrophages, follicular dendritic cells and interdigitating reticular cells in B- and T-cell areas of lymphoid tissue, respectively. Langerhans' cells in skin are also weakly stained. LP9 also stains some endothelial cells, proximal and distal tubules in kidney and cells lining bile duct in the liver.

FACS analysis

Cells to be tested for the presence of cell-surface antigen ($0{\cdot}5 \times 10^6$ per test) were washed in BSS containing 0·2% BSA and 0·1% sodium azide (BBN) and resuspended in test antibody or suitable control, and incubated for 30 min on ice with vortexing every 10 min. Cells were then washed twice in BBN and resuspended in FITC-conjugated $F(ab')_2$ goat anti-mouse immunoglobulin antibody (Cappel Laboratories, Downington, PA) and incubated a further 30 min on ice. After two washes in BBN, cells were analysed for fluorescence using a FACS 420 (Becton-Dickinson) fitted with a linear fluorescence amplifier. Controls included omission of primary antibody and incubation of cells with parent myeloma culture supernatant instead of primary antibody. 4U is an IgG2a control antibody for UCHM1. Results were recorded as the percentage of cells with a fluorescence greater than 95% of background staining (percentage positive) and mean fluorescent intensity of stain. The relative level of antigen present on each cell preparation was

Table 1. Monoclonal antibodies used in this study

Antibody	CD group	Molecule recognized	Reference
UCHT1	CD3	p20, 26	Beverley & Callard (1981)
Mol‡	CD11b	CR3	Todd & Schlossman (1982)
44	CD11b	CR3	Malhotra, Hogg & Sim (1986)
3.9	CD11c	p150, 95	Malhotra *et al.* (1986)
WM-15 (213)*	CD13	p150	Hogg & Horton (1987)
UCHM1†	CD14	p55	Hogg *et al.* (1984a)
anti-LeuM3‡	CD14	p55	Dimitriu-Bona *et al.* (1983)
29*	CD15	anti-X	Hogg *et al.* (1984a)
IV3‡	CDw32	FcRII	Anderson *et al.* (1986)
E11	CD35	CR1	Hogg *et al.* (1984b)
aCR1	—	CR1	Becton-Dickinson (Cowley, Oxon)
10.1	—	FcRI	Dougherty *et al.* (1987)
32.2	—	FcRI	Anderson *et al.* (1986)
52§	—	HLA-D/DR, DQ	Allen & Hogg (1987)
LP9*	—	—	Partridge *et al.* (1987)
JW6*	—	—	Partridge *et al.* (1987)
JW7	—	—	Partridge *et al.* (1987)
24	—	p175	Hogg & Selvendran (1985)
4U†	—	IgG2a control	N. Hogg unpublished data

Molecules recognized and cluster designations are shown for antibodies for which these have been determined. All antibodies are of IgG1 isotype except those marked* (IgM),† (IgG2a)‡ (IgG2b) and § (IgG3).

expressed as a ratio of the mean fluorescent intensities observed for FN-adherent and CCE monocytes.

RESULTS

Distribution of cell types separated by CCE

Peripheral blood mononuclear cells were separated in the elutriator rotor by sequential increase in the flow rate whilst centrifuge speed was maintained constant. The number of cells eluting at a particular flow rate was measured using a Coulter Counter, adjustment of the flow rate being made when the majority of the cells eluting at that flow rate had been collected. The proportions of monocytes, lymphocytes, and polymorphonuclear cells present in collected fractions were determined by examination of morphology and esterase activity of cytocentrifuge preparations. Some variation in the elutriation characteristics of mononuclear cells was observed for different donors. However, the overall pattern of cell distribution in eluted fractions was consistent from one separation to another.

Results of cell type analysis for a typical elutriation run are shown in Fig. 1. Platelets and contaminating red blood cells were eluted at the initial 8 ml/min flow rate virtually free of mononuclear cells. Fractions collected at 12–14 ml/min were 95–100% lymphocyte, as determined by the absence of esterase-positive cells and morphology. The modal volume of eluted lymphocytes varied from 140 μm^3 in early fractions to 175 μm^3 in later fractions, with an average modal volume for pooled lymphocytes of 158 ± 11 μm^3. Subsequent increases in the flow rate resulted in the elution of increasing proportions of monocytes. Fractions containing monocytes were pooled (CCE monocytes) when the larger cells (> 300 μm^3, as determined by cell-size analysis) represented the majority of cells exiting the chamber, as shown in Fig. 1. The cells in the 'rotor off' fraction frequently contained large numbers of polymorphonuclear cells and were not included in the pooled monocyte fraction. Cell-size analysis was routinely used to estimate the cell composition of the different eluted fractions.

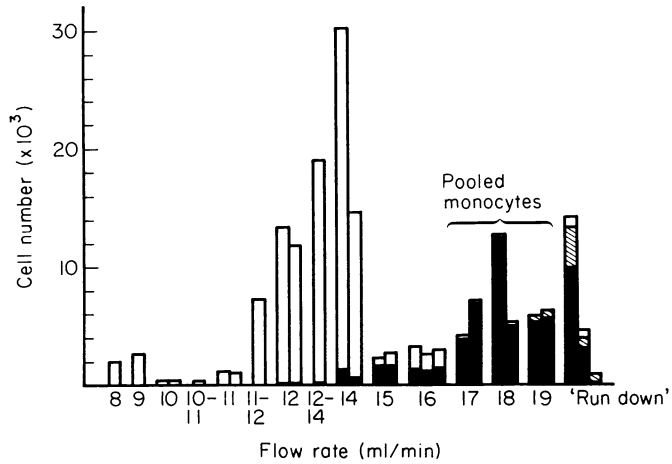

Figure 1. Histogram to show the numbers and types of cells eluting at the various flow rates for a typical elutriation run. Cell-type distribution was determined by examination of morphology and esterase staining, as described in the Materials and Methods. □ Lymphocytes, ▨ polymorphonuclear cells, ■ monocytes.

Table 2. Cell type distribution in purified cell preparations

Cell preparation	% monocyte	% lymphocyte	% polymorpho-nuclear	Modal volume (μm^3)	% monocyte yield	*n*
FN adherent	92±2	7±1	1±0·5	369±14	60±14	6
Non-adherent	4±2	93±3	3±1	154±12	18±5	6
Pooled CCE monocytes	95±2	2±1	3±1	344±29	67±13	8

Estimation of the numbers and types of cells present were made by examination of cytocentrifuge preparations that had been stained for non-specific esterase activity or with Geimsa.

Comparison of cells prepared by FN adherence and CCE

Monocytes adherent to FN-coated surfaces, non-adherent cells and pooled CCE monocytes were also examined for the presence of cytoplasmic esterase and for morphology. Results of the cell-type analysis for the different preparations are shown in Table 2. These data show that the purity of monocytes prepared by FN adherence and CCE, as assessed by non-specific esterase staining, are similar. However, there were differences in the types of cells that contaminated the preparations. Esterase-negative cells in FN-adherent preparations were mostly lymphocytes, whereas approximately equal numbers of polymorphonuclear cells and lymphocytes were present in the CCE preparations. Both techniques were found to yield between 50% and 80% of the monocytes initially present in the Ficoll–Hypaque mononuclear cells. In the FN preparations, monocytes were present in the non-adherent population and in subsequent washes prior to removal with EDTA. Monocytes lost during CCE separation included those cells with elutriation characteristics similar to lymphocytes and cells in the 'rotor off' fraction; such cells were separable only by further isolation procedures and were not included in further analyses.

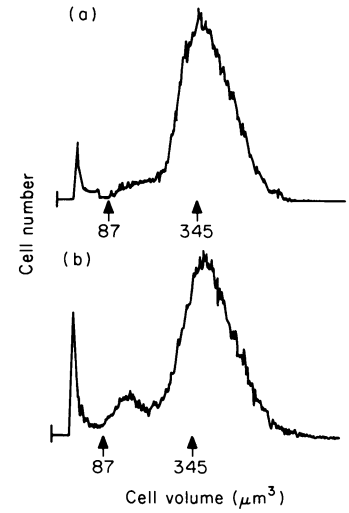

Figure 2. Cell size profiles of purified cell preparations (a) CCE monocytes, (b) FN-adherent monocytes. Modal volumes for 87 μm^3 and 345 μm^3 volume calibration beads are also shown.

Cell-size analysis

Although the volume of cells prepared from different donors varied slightly (Table 2), the modal volumes of cells prepared from the same donor by CCE and FN adherence were similar. Figure 2 shows typical cell-size profiles obtained for pooled CCE monocytes and FN-adherent monocytes, with modal volumes of 374 μm^3 and 380 μm^3, respectively. Average modal volumes for monocytes prepared by the two techniques were not significantly different (Table 1). However, FN-adherent monocytes often contained a small peak modal volume of 160 μm^3, corresponding well with the size of lymphocytes separated by CCE and the cell-type analysis data showing lymphocytes as the major contaminant. Cell-size analysis can be compared with analysis of light-scattering properties of cells, known to be related to the size and density of the light-scattering particle (Parks, Lanier & Herzenberg, 1986). Typical light-scatter profiles for lymphocytes, FN-adherent and CCE monocytes (Fig. 3) show very similar light-scattering properties of monocytes prepared by the two techniques. These data also suggest the greater lymphocyte contamination of the FN-adherent cells, as judged by the presence of cells with scattering properties similar to purified lymphocytes. Light-scatter profiles obtained for FN-adherent cells stained with CD3 T-cell antibody (UCHT1) and a CD19 B-cell antibody (8EB1) by FACS analysis confirmed that these cells were mainly T lymphocytes (data not shown).

Antibody reactivity with FN-adherent and CCE monocytes

Cells were tested with a panel of monoclonal antibodies representative of many of the anti-monocyte antibodies submitted to the Third International Leukocyte Differentiation Antigen Workshop in 1986. Most antibody staining profiles showed a single peak of fluorescence, suggesting a uniform staining pattern, although several antibodies displayed a broader range of fluorescence than others, e.g. CR1, CR3, 52 and WM-15, suggesting heterogeneity of expression. Fluorescent-staining profiles for CCE and FN-adherent monocytes with antibodies

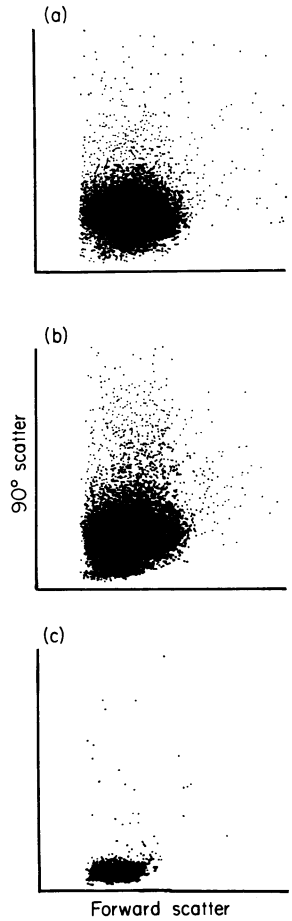

Figure 3. Light-scattering profiles obtained from FACS analysis of purified cell preparations. (a) CCE monocytes, (b) FN-adherent monocytes, (c) lymphocytes prepared by CCE.

representative of the types of profiles observed are shown in Fig. 4. Comparisons of the percentage of cell populations staining positive shows between 80% and 90% staining for most antibodies (Table 3). However, the relative expression of the molecules recognized by the antibodies, as indicated by fluorescence intensity, showed a consistent pattern of results, as shown in Table 3. Many molecules were expressed at equivalent levels on the two preparations, e.g. 32.2/10.1 and IV3 recognizing FcRI and FcRII, respectively, or slightly increased levels on adherent cells with a relative expression of around 1·2, e.g. antibody UCHM1 [the staining of the cell preparations with contol IgG2a antibody, accounting for a part of that observed with UCHM1, is presumably due to the interaction of the antibody via the Fc receptor of the monocyte, a possibility noted by Woof *et al.* (1986)]. Consistently increased expression of CR1 and CR3 was observed for adherent cells, whilst LP9 showed reduced expression.

One major difference between the two preparative techniques is the temperature to which the cells are exposed during isolation. CCE is performed at 10° whereas adherence involves incubation at 37° for 60 min. Cells exposed to *in vitro* temperature changes are known to alter levels of expression of certain cell-surface molecules. Fearon & Collins (1983) showed up-regulation of complement receptors on polymorphonuclear

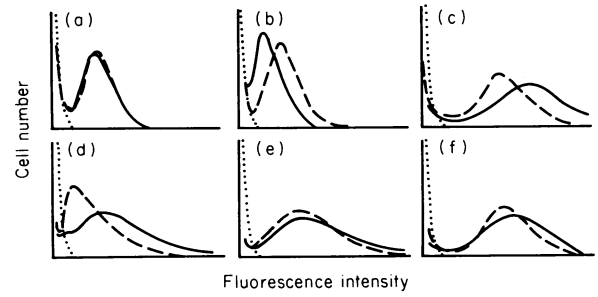

Figure 4. FACS profiles of monoclonal antibody staining with FN-adherent (—), and CCE monocytes (- - -) in indirect immunofluorescence. (a) 32.2, (b) LP9, (c) 44, (d) E11, (e) WM-15 (213), (f) UCHM1. (. . .) Shows staining with control antibody.

cells and monocytes after exposure of unfractionated cells to 37°. Similarly, Figdor *et al.* (1986) showed induction of HLA-D/DR and HLA-D/DQ on the monocyte cell surface after adherence. To investigate the effects of incubation of CCE monocytes at 37° on cell-surface molecule expression, cells were incubated in RPMI/HIFCS at 4° (control), at 37° in siliconized vessels to prevent adherence, or allowed to adhere to FN-coated tissue culture dishes and removed using EDTA treatment. Cells were then tested for relative expression of cell-surface molecules as described above.

Results from these experiments using antibodies to CR1, CR3, LP9 and several other antibodies are shown in Table 4. It can be seen that exposure of CCE monocytes to 37°, with or without adherence, causes alterations in the levels of expression of CR1, CR3, and LP9 comparable to differences observed for CCE and FN-adherent monocytes. The levels of expression of other molecules is not affected by incubation at 37°, e.g. 10.1 and JW6. These results indicate that differences observed between FN-adherent and CCE monocytes in terms of cell-surface molecule expression are due primarily to the temperature that cells are exposed to during preparation and cannot be ascribed to the presence of cell populations that express different levels of the complement receptors types 1 and 3 and LP9 antigen.

DISCUSSION

Isolated monocytes are known to be functionally heterogeneous, although the origin of this heterogeneity is still uncertain. A potential problem in understanding the significance of this heterogeneity is that the isolation procedure may select for certain subpopulations of cells, making extrapolation to the *in vivo* situation uncertain. We have explored this problem using two isolation techniques, FN adherence and CCE, which are highly unlikely to enrich for the same populations of cells. For example, monocytes that co-elute with lymphocytes during separation by CCE ['intermediate' monocytes as described by Akiyama *et al.*, (1985)], although not significantly different in terms of cell size, are low-density cells that account for roughly 20% of the initial monocyte population. They can be enriched for after CCE by plastic adherence (McCarley, Shah & Weiner, 1983) and should therefore be present in the FN-adherent population. Indeed adherence-prepared cells can be subfractionated into density-defined populations with similar characteristics to 'intermediate' monocytes (Schreiber *et al.*, 1983). 'Intermediate' monocytes therefore should form a signifi-

Table 3. Monoclonal antibody staining in indirect immunofluorescence with monocytes prepared by FM adherence and CCE

Antibody	Protein recognized	% of cells staining positive		Relative intensity of staining adherent/elutriator	*n*
		Adherent	Elutriator		
Mol	CR3	88	92	1·5±0·16	8
44		83	89		
E11	CR1	84	88	1·5±0·17	7
a CR1		89	90		
UCHM1	p55	82	83	1·17±0·05	4
LeuM3	p55	63	78	1·08±0·07	6
JW6	—	99	99	1·13±0·1	6
JW7	—	90	93	1·1 ±0·02	4
52	MHC class II	85	94	1·26±0·02	4
3.9	p150,95	87	91	1·2 ±0·13	4
32.2	FcR_I	77	84	1·05±0·1	3
10.1	FcR_I	80	81	0·98±0·02	4
IV3	FcR_{II}	93	94	0·92±0·1	3
WM15 (213)	p150	90	91	1·03±0·2	4
LP9	—	78	85	0·54±0·1	9
24	p175	18	7	—	3
29	anti-X	1	3	—	6
4U	IgG2a control	17	9		3

Mean percentage fluorescence and relative intensity of stain expressed as the ratio of fluorescence intensity observed for adherence-prepared monocytes relative to that observed for elutriator.

Table 4. Effects of temperature and adherence on cell-surface molecule expression of CCE-prepared monocytes

Anti-body	Relative intensity of staining to elutriator monocytes (4°)			
	N-adherent	Adherent-elutriator	37° elutriator	*n*
Mol	1·5	1·4	1·4	3
E11	1·5	1·6	1·4	3
LP9	0·54	0·52	0·64	3
10.1	0·95	0·96	1·04	3
JW6	1·02	1·1	1·04	3
3.9	1·3	—	1·23	3

Relative intensity of staining in indirect immunofluorescence using monoclonal antibodies expressed as the ratio of fluorescence intensity observed for adherent, CCE-37° monocytes and CCE-adherent monocytes compared with that of CCE monocytes held at 4°.

cant part (>20%) of the FN-adherent preparation but be absent from the CCE preparation. Similarly, one might expect monocytes that are not adherent or are lost during washing, representing 20–40% of the initial population, to be present significantly in CCE-prepared cells. Monocytes prepared by the two techniques therefore would be expected to contain different populations of cells, representing dissimilar cross-sections of the total monocyte population.

Somewhat surprisingly, our results show that the cell-surface antigenic profiles of the two preparations, as detected by monoclonal antibodies, are very similar, arguing against the existence of subsets. Previously, subsets have been proposed based on the expression of HLA-D/DQ (Gonwa & Strobo, 1984) and complement receptors (Whisler, Newhouse & Lachman, 1982). However, expression of these molecules at the cell surface has been shown to be subject to *in vitro* alteration (Figdor *et al.*, 1986; Fearon & Collins, 1983). The existence of FcR-negative monocytes is still not resolved, although our study would suggest that any such cells would represent a very minor proportion of the total population. Anti-FcRII shows all monocytes positive and anti-FcRI antibodies show approximately 80% positivity and low-intensity fluorescence that does not exclude the possibility of low levels of expression on the negative cells. Another criterion for defining monocyte subsets has been cell size. Indeed it is clear that monocytes present in the circulation are of widely varying volume but that there is little evidence of bimodality of cell size. From the results of this study, division of monocytes into 'small' and 'large' subsets (Norris *et al.*, 1979) is not justifiable, lymphocyte contamination accounting for the presence of the population of smaller cells. Subsets have been defined in terms of function but this has always related to differential ability rather than the presence or absence of function.

In conclusion, the division of a heterogeneous population into subsets defined by continuously variable physical and functional parameters is artificial and such cells do not represent true subpopulations. We suggest that this is the case for monocytes, heterogeneity existing as a continuous spectrum of

physical and functional properties that are related to cell maturation/activation.

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