Characterization of a Human Blood Monocyte Subset with Low Peroxidase Activity

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ABSTRACT Two human monocyte subsets from the peripheral blood of healthy donors have been isolated in >90% purity by countercurrent centrifugal elutriation and human serum albumin gradients and their functional capabilities have been assessed.

We have demonstrated that one subset ("regular" monocytes, RM) showed intense cytoplasmic peroxidase staining and contained substantial peroxidase activity. In contrast, another subset ("intermediate" monocytes, IM) stained poorly for peroxidase and had low peroxidase activity. By electron microscopic analysis combined with peroxidase localization, it was found that IM had fewer peroxidase-positive granules per cell than did RM. IM coelutriated with some lymphocytes and by cell sizing analysis were shown to be slightly smaller than RM.

Functional and cytochemical analysis of these subsets indicated that IM had less activity than RM in assays such as accessory cell function for mitogen-induced T lymphocyte proliferation and antibody-dependent cellular cytotoxicity, and that fewer IM expressed OKM1 antigen and pokeweed mitogen (PWM) receptors on their membranes than did RM. The subset of IM not bearing either the PWM receptor or the OKM1 antigen had very low peroxidase activity. IM also were found to have a greater sensitivity to polyriboinosinic and polyribocytidilic acid (100 $\mu\text{g}/\text{ml}$)-induced secretion of interferon. There was no significant difference in the phagocytic capability, the percentage

of Fc receptor-positive cells, 5'-nucleotidase activity, DR antigen expression, or the responsiveness to migration inhibitory factor of IM as compared with RM. Furthermore, it was found that the ratio of IM to RM increased after prolonged cytapheresis, which suggests that IM are more mobilizable than RM from the extravascular reservoirs of human monocytes.

INTRODUCTION

Leukocytes of the mononuclear phagocyte series (monocytes, monocytes, and macrophages) are known to participate in critical components of the immune response. These macrophage or monocyte-mediated functions include antigen presentation (1), production of a wide range of biological response modifiers (2, 3), direct cytotoxic function against tumor cells (4, 5), and antibody-dependent cellular cytotoxicity (ADCC)¹ (6). It is unclear, however, whether each monocyte can perform all of these functions or whether there exist functional subsets within the total monocyte pool. Although there has been no direct evidence for antigen-specific clones in the mononuclear phagocyte series (as exists for the B and T lymphocyte series), it is well

¹ *Abbreviations used in this paper:* ADCC, antibody-dependent cellular cytotoxicity; CCE, countercurrent centrifugal elutriation; Con A, concanavalin A; FACS, fluorescence-activated cell sorter; FcR, receptors for Fc portion of IgG; FCS, fetal calf serum; HSA, human serum albumin; IFN, interferon; IM, "intermediate" monocyte; IMF, "intermediate" monocyte fraction; LCL-LIF, lymphoblastoid cell lines-leukocyte inhibitory factor(s); MIF, monocyte migration inhibitory factor(s); MMI, monocyte migration inhibition; 5'-N, 5'-nucleotidase; PHA, phytohemagglutinin; poly I:C, polyriboinosinic and polyribocytidilic acid; PWM, pokeweed mitogen; RM, "regular" monocytes; RMF, "regular" monocyte fraction; 7S-EA, IgG-coated ox erythrocytes.

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known that cells from the mononuclear phagocyte series can differ from one another by virtue of their stage of differentiation and/or activation (7-9).

Several laboratories have defined monocyte subsets in the circulating peripheral blood of normal humans on the basis of histochemical and surface marker procedures, functional data, or both (10-12). Various criteria have been used to define human monocyte subsets, including the presence of complement receptors (13), certain histocompatibility gene products (10), size (11, 12, 14, 15), and density (16, 17).

Most reported techniques for isolating human monocyte subsets do not allow for sufficient purification of the monocyte subset cells from other contaminating leukocytes (usually lymphocytes), and those techniques that attempt to purify human monocyte subsets frequently employ an adherence step which could conceivably alter the native function of these cells. In addition, once monocyte subsets are purified by plastic or glass adherence, they are very difficult to enumerate, use in add-back experiments, and process through the fluorescence-activated cell sorter (FACS). The present study extends previous indications that monocytes of different sizes and densities can be differentially separated by a technique known as counter-current centrifugal elutriation (CCE) (11, 12, 17, 18). Monocyte subsets separated from one another by CCE have been reported to have different functions (11, 12, 17), but it has been difficult to prove this point because the monocyte subsets obtained were not sufficiently purified from other contaminating mononuclear leukocytes.

In the present study, methods were developed for obtaining large numbers of two monocyte subsets by using CCE and human serum albumin (HSA) gradients in >90% purity in suspension, which allowed extensive cytochemical and functional characterization of the cells. In addition, an analysis was made of the numbers of these subsets that entered the circulation from the extravascular pool after prolonged cytappheresis.

METHODS

Cell identification procedures. The purity of the monocyte preparations was determined by histological analysis of Wright's-stained cytocentrifuge preparations, nonspecific esterase staining (19), peroxidase staining (Histozyne Kit 390-A, Sigma Chemical Co., St. Louis, MO), and ability to phagocytose IgG-coated ox erythrocytes (7S-EA) and latex particles. Fc receptors (FcR) for IgG were enumerated by the ability of these cells to form rosettes with 7S-EA after 30-min incubation at 37°C, as previously described (20). To demonstrate phagocytosis, cells that had bound 7S-EA during the determination of FcR, as described above, were allowed to incubate at 37°C for 1 h, and the percentage of cells that had ingested two or more erythrocytes was determined. Latex phagocytosis was determined as the percentage of cells able to ingest three or more latex particles (0.6 μ m,

Sigma Chemical Co.) within 1 h at 37°C. Cell viability was determined by trypan blue dye exclusion.

Donor selection. Leukocytes were removed from normal adult volunteers by cytappheresis after informed consent as previously reported (21). These individuals were assessed to be normal on the basis of a complete history and physical examination, chest x-ray, electrocardiogram, complete blood count with platelet count and differential leukocyte count, urine analysis, 18-item serum chemistry profile, clotting parameters, immunoglobulin levels, and serum complement profile. All donors were known to be free of recent intercurrent infections and not to have ingested drugs of any type within 24 h of the procedure.

Cytapheresis technique. Cytappheresis was performed on adult volunteers by using a double needle continuous flow technique and a Celltrifuge II Leukapheresis machine (Baxter Travenol Laboratories, Deerfield, IL) as previously described (18). All donors were cytapheresed for a 2-h period.

Monocyte isolation. Monocytes were isolated from the cytappheresis specimen by first diluting the leukocytes to 5×10^6 cells/ml in sterile phosphate-buffered saline (PBS). These cells were then placed on standard Ficoll-Hypaque gradient to obtain unfractionated mononuclear leukocytes. Unfractionated mononuclear leukocytes were then washed in RPMI 1640 medium (Hem Research, Inc., Rockville, MD), resuspended at 5×10^6 cells/ml in elutriation medium which consisted of endotoxin-free sterile PBS with 2% clinical grade HSA (pH 7.4, 300 mosmol; Cutter Laboratories, Inc., Berkeley, CA). These cells were entered into a Beckman standard elutriation chamber (Beckman Instruments, Inc., Palo Alto, CA) which was spun at 2,020 rpm at 18°C. The initial entering flow rate into the elutriation chamber was 5.0 ml/min. As elutriation proceeded, the medium flow rate was increased by 0.5 ml increments by use of a Sarns cardiovascular pump (Sarns, Inc., St. Paul, MN). The size of the cells exiting the chamber was constantly monitored using an Elzone cell-sizing system (Particle Data, Inc., Elmhurst, IL). Lymphocytes were collected by this procedure up to the elutriation fraction which first contained detectable numbers of monocytes as determined by the cell-sizing system. After this phase of elutriation, a variable number of elutriation aliquots followed which contained mixtures of lymphocytes and monocytes; we termed these aliquots the "intermediate" monocyte fraction (IMF). The next series of cells to exit the elutriation chamber were purified monocytes; we termed these aliquots the "regular" monocyte fraction (RMF). All cells were washed in RPMI 1640 medium.

Monocyte subset purification. Discontinuous HSA gradients were used to purify monocytes in IMF from contaminating lymphocytes while still maintaining the monocytes in suspension. The gradients were made by using clinical grade HSA (25% solutions), five concentrations of albumin (ranging from 2 ml of 17% albumin at the top to 2 ml of 25% albumin at the bottom of the gradient), and sterile PBS as the diluent. Elutriated mononuclear cells (2.5×10^7 in 2 ml of 15% albumin) were carefully layered on the top of the gradient and centrifuged at 420 *g* for 30 min at 4°C. The cells were recovered from each interface of the gradient and were maintained at 4°C. The RMF also was purified further by HSA gradients.

Peroxidase assay. The peroxidase activity of cell suspension was assayed by the method of Baggiolini et al. (22). 2×10^5 cells were lysed with 0.1% Triton X-100 and the production of oxidized *o*-toluidine was measured. *o*-Toluidine and H_2O_2 were used as the substrates and horseradish peroxidase (Sigma Chemical Co.) was used as the standard. Enzyme activity was expressed as nanograms per milliliter (of horse-

radish-peroxidase equivalents) found in a suspension of 10^6 monocytes.

5'-Nucleotidase (5'-N) assay. The assay used to measure 5'-N activity was the technique of Shenoy and Clifford (23) as modified by Reaman et al. (24). Briefly, 0.5×10^6 cells were incubated for 30 min at 37°C with 0.1 ml of a 0.1-mM solution of [U- ^{14}C]AMP (New England Nuclear, Boston, MA). The resulting reaction mixture was precipitated with 0.5 M lanthanum chloride at 4°C overnight and then centrifuged at 2,000 g for 10 min. The radioactivity of the resulting supernatants was counted in a liquid scintillation counter and the results are expressed as nanomoles of AMP degraded per 10^6 monocytes per minute.

Cell sizing. Purified monocytes were sized on an Elzone cell-sizing system. The cells were suspended in medium RPMI 1640 at a concentration of 10^6 cells/ml of medium, and the mean cell volume was determined.

Electron microscopy. Cells processed for peroxidase localization were fixed in 1.5% distilled glutaraldehyde in 0.1 M sodium cacodylate-HCl buffer (pH 7.4) containing 1% sucrose at 4°C for 10 min. After fixation, cells were washed three times in sodium cacodylate-HCl buffer (pH 7.4) with 7% sucrose. They were then incubated at 22°C in Graham and Karnovsky's medium (pH 7.4) for peroxidase for 1 h (25). Enzyme preparations were postfixed for 1 h at 4°C in 1% OsO_4 in acetate-Veronal buffer (pH 7.6; Winthrop Laboratories, Evanston, IL) with or without staining en bloc in uranyl acetate for 60 min and then were processed and examined as previously described (26).

Immunofluorescence analysis. The distribution of cells bearing membrane antigens that reacted with monoclonal antibodies and fluoresceinated pokeweed mitogen (PWM) prepared as previously described (27) was investigated using a FACS IV fluorescence-activated cell sorter (Becton-Dickinson, FACS System, Sunnyvale, CA) as previously described (28). The monoclonal antibodies used were anti-HLA-DR (Becton-Dickinson, Monoclonal Antibodies, Inc., Sunnyvale, CA), OKM1, OKT3 (Ortho Pharmaceutical Corp., Raritan, NJ). Fluorescence was only analyzed in those cells of a volume $>200 \mu\text{m}^3$ (to "gate out" debris). An additional type of FACS experiment was performed to determine the degree of overlap of the various markers among the monocyte subsets. Because our FACS was only capable of sorting one fluorescent marker, fluorescein isothiocyanate (FITC)-labeled PWM and OKM1 were used. "Intermediate" monocytes (IM) and "regular" monocytes (RM) were first stained with FITC-PWM and sorted into PWM-positive and PWM-negative cells. Cell sizing and peroxidase activity were performed on both populations. In addition, IM and RM were stained both with FITC-PWM and FITC-OKM1 and the positive and negative cells were analyzed for cell size and peroxidase activity.

Assay of mitogen-induced T lymphocyte proliferation. T cells were obtained by rosetting lymphocytes obtained by CCE with sheep erythrocytes as previously described (18). All cultures were carried out in a volume of 0.2 ml of complete culture medium of RPMI 1640 supplemented with 10% heat-inactivated human AB serum (Medical Specialties Laboratory, Inc., Boston, MA), gentamycin (10 $\mu\text{g}/\text{ml}$), and L-glutamine (2 mM). 1×10^5 T cells were cultured with or without 1×10^4 monocytes in triplicate in flat-bottomed well microtitre plates (Costar, Cambridge, MA) with phytohemagglutinin (PHA; HA-16, Wellcome Research Laboratories, Beckenham, England), concanavalin A (Con A; Calbiochem-Behring Corp., La Jolla, CA), or PWM (Grand Island Biological Co., Grand Island, NY) dissolved in 20 μl of medium or an equal volume of medium as control. The cultures

were incubated for 3 d in humidified atmosphere of 95% air and 5% CO_2 at 37°C . 1.0 μCi of [^3H]thymidine (New England Nuclear, Boston, MA) was added to each well for the final 4 h of culture. The cells were harvested with a cell harvester (Brandel, Gaithersburg, MD); [^3H]thymidine incorporation was determined in a liquid scintillation counter.

Interferon (IFN) induction and assay. 1,000,000 monocytes per culture were incubated in triplicate cultures in 24 well plates (Costar) in a volume of 1.0 ml complete culture medium (RPMI 1640 supplemented with 10% human AB serum) with or without 100 or 200 $\mu\text{g}/\text{ml}$ of polyriboinosinic and polyribocytidilic acid (poly I:C; Sigma Chemical Co.). After 4 h, the cells were washed thoroughly with RPMI 1640 and incubated for additional 32 h (optimal time period) in a volume of 1.0 ml of complete culture medium. IFN activity of culture supernatants was assayed (Biofluids, Inc., Rockville, MD) and expressed in antiviral units by reference to the human leukocyte IFN standard G-023-901-527 (as supplied by the National Institute of Allergy and Infectious Diseases, Antiviral Substances Program, Bethesda, MD).

Monocyte migration inhibition (MMI) assay. Evaluation of the responsiveness of monocytes to monocyte migration inhibitory factor(s) (MIF) was performed according to a modification of the agarose droplet technique of Harrington and Stastny (29) as described below. Since it was determined that lymphocytes had no effect on the migration pattern of monocytes, cells from IMF and RMF were used without further purification.

The elutriated IMF and RMF were washed once by centrifugation at 200 g in RPMI 1640, resuspended, counted, and adjusted to 10^6 viable cells per milliliter and transferred to 1.5 ml Eppendorf microfuge tubes (Brinkmann Instruments, Inc., Westbury, NY). They were then centrifuged at 200 g for 10 min at 4°C . After total removal of the supernatant, the cell pellet was quickly warmed to 37°C in a water bath and 0.02 ml of Sea Plaque agarose was added per 10^7 cells. The Sea Plaque agarose (FMC Corp., Marine Colloids Division, Rockland, ME) was prepared at 0.7% in a boiling water bath and then held at 37°C . An equal volume of RPMI 1640 with 2% fetal calf serum (FCS; HyClone, Sterile Systems Inc., Logan, UT) at 37°C was added to the agarose. The cells were resuspended gently in the agarose at 37°C using a Hamilton repeating dispenser with a 0.05-ml gastight syringe (Hamilton Co., Reno, NV), and 1- μl droplets were centrally placed in the wells of flat-bottomed microtitre plates (Falcon Labware, Oxnard, CA). The droplets were allowed to solidify at 4°C for 5 min and 0.05 ml of RPMI 1640 was added to each well. The MIF preparation that was used was obtained from the human cell line RPMI 1788 (Preparation LCL-LIF, Organon, OSS, the Netherlands) and 0.05 ml of lymphoblastoid cell lines-leukocyte inhibitory factor(s) (LCL-LIF) was added to the wells; 0.05 ml of medium alone was added in the control wells. Samples were tested in triplicate or quadruplicate. The microtitre plates were incubated for 24 h in a humid, 5% CO_2 in air atmosphere on a perfectly level shelf of an incubator. The areas of migration were computed on a TI-59 programmable calculator from the dimensions measured by projecting the image of the droplet and cells on a grid, using a Bausch and Lomb Trisimplex Micro projector (Fisher Scientific Co., Pittsburgh, PA). The shape of the migration was assumed to be elliptical; the major and minor axes of the agarose droplet and the cell migration pattern minus the area of the agarose droplet were determined. The percent monocyte migration inhibition (MMI) was calculated as: $\% \text{ MMI} = 100 - [\text{Area (test material)}/\text{Area (medium)}] \times 100$. Inhibition

FIGURE 1 Representative cell sizing analysis of unfractionated mononuclear leukocyte preparation obtained from cytopheresis separated on Ficoll-Hypaque gradient. P, platelets; L, lymphocytes; M, monocytes. Number beneath letter indicates average size (cubic micrometers) of cells in peak.

of migration was defined as a significantly smaller ($P < 0.05$ by t test) area of test migration than that with medium alone.

ADCC assay. In this assay, the complement-independent antibody-dependent lysis of ^{51}Cr (chromium)-labeled target cells was measured after an 18-h incubation with effector monocytes as previously described (6). Effector cells were diluted in RPMI 1640 with 10% heat-inactivated FCS (KC Biological Inc., Lenexa, KS) to a final concentration ranging from $2.5 \times 10^5/\text{ml}$ to $2 \times 10^6/\text{ml}$. Targets were fresh human

type-B erythrocytes obtained from heparinized human blood and washed three times in PBS. 200 μl of target cell pellets were labeled with 200 μCi of ^{51}Cr (New England Nuclear) during a 30-min incubation at 37°C . The cells were then washed three times in PBS to remove unbound chromium and resuspended in RPMI 1640 with 10% FCS. Antibody for the human erythrocyte targets was obtained from the Dade Hospital Corporation (Division of American Hospitals, Miami, FL) and was diluted 1:10 in RPMI 1640 with 10% FCS. 2.0×10^5 target cells in 100 μl medium and effector cells in 100 μl medium were added per well to round-bottomed microtiter plates (Falcon Labware) with or without 50 μl of antibody solution. Cultures were incubated at 37°C in 5% CO_2 in air for 18 h. Plates were then centrifuged, the supernatant was removed by Titertek Supernatant Collection System (Flow Laboratories, Inc., McLean, VA). The percent cytotoxicity was determined by the formula: cytotoxicity (%) = $[(\text{supernatant cpm} - \text{spontaneous release cpm}) / (\text{total counts cpm} - \text{spontaneous release cpm})] \times 100$. Total counts were obtained after addition of 10% sodium dodecyl sulfate to target cells. In all cases, spontaneous release was $<10\%$.

Analysis of the entry into the circulation of human monocyte subsets from mobilizable monocyte pools. Experiments were performed to analyze the entry of IMF monocytes into the circulation from the extravascular reservoirs of normals during cytopheresis. Different normal donors were cytopheresed identically for 1.5 h. The cytopheresis specimen from the first 1.5 h was elutriated as described above to obtain IMF and RMF. All donors were then

FIGURE 2 Representative cell sizing analysis of elutriated lymphocyte fractions (1-6) using unfractionated mononuclear leukocytes obtained from cytopheresis as starting material. P, platelets; L, lymphocytes; M, monocytes. Number beneath letter indicates average size (cubic micrometers) of cells in peak.

FIGURE 3 Representative cell sizing analysis of elutriated IMF fractions (7-10) using unfractionated mononuclear leukocytes obtained from cytophoresis as starting material. L, lymphocytes; M, monocytes. Number beneath letter indicates average size (cubic micrometers) of cells in peak.

cytopheresed for an additional 0.5 h and these cells were elutriated separately to obtain a second set of IMF and RMF.

Statistical evaluation. The *t* test was used to evaluate the data sets.

RESULTS

Isolation of monocyte subsets by CCE. After Hypaque-Ficoll gradient purification, mononuclear leu-

kocytes from each donor were entered into the CCE apparatus. Figs. 1-4 illustrate the representative Elzone cell sizing graphs that were obtained from each of the fractions isolated sequentially from the elutriator. The first six collection tubes contained purified lymphocytes (<1% monocyte contamination). Fractions 7-10 contained mixed populations of large and small sized cells (~50% monocytes) and these were

FIGURE 4 Representative cell sizing analysis of elutriated RMF fractions (11-13) using unfractionated mononuclear leukocytes obtained from cytophoresis as starting material. M, monocytes. Number beneath letter indicates average size (cubic micrometers) of cells in peak.

TABLE I
Characteristics of the Two Monocyte Fractions
Obtained by CCE

Cell fraction	Percent of monocytes		
	Wright's stain	Esterase	Peroxidase
IMF	58.6±7.9*	60.4±8.1	34.6±9.9
RMF	90.5±2.7	92.1±3.7	90.4±4.7

* Values are means±SD of fractions obtained from eight separate CCE.

combined to form the IMF. Tubes 11–13 contained highly purified monocytes (RMF). To document the degree of purity of these fractions obtained from the elutriator, Wright's stains, esterase stains, and peroxidase stains were performed as summarized in Table I. These data indicate that the RMF contained an average of ~91% monocytes. In contrast, the IMF contained only 59% monocytes, with the vast majority of the contaminating cells being lymphocytes. It was interesting that the IMF contained fewer peroxidase-positive cells when compared with the RMF. Usually $500 \pm 161 \times 10^6$ (mean±SD, $n = 25$) IMF and $415 \pm 94 \times 10^6$ ($n = 25$) RMF cells were obtained by CCE isolation from each donor.

Purification of monocyte subsets. To perform side-by-side functional comparisons of the monocyte subsets obtained by CCE, a negative selection method to further purify the monocytes was developed. By centrifugation at low speeds on HSA gradients, we were able to obtain highly purified monocyte preparations

TABLE II
Purification of Two Monocytes Fractions from CCE
on HSA Discontinuous Gradients

Fraction	HSA	Gradient distribution (percent of recovered cells)		Percent of monocytes by morphology	
		IMF	RMF	IMF	RMF
	%				
A*	15	3.3†	7.2	93.1‡	95.5
B	17	42.2	65.5	90.8	94.6
C	19	35.1	18.4	43.8	80.2
D	21	7.6	5.0	38.2	69.7
E	23	7.3	2.0	29.0	64.1
F	25	4.5	1.9	28.6	61.2

* Letter denotes leukocyte band harvested immediately below the corresponding percent HSA in the gradients.

† Values are percentage means of four separate experiments. Percent recovery of cells applied to HSA gradient was 87%.

‡ Differential analysis was performed on Wright's stained aliquots of cell fractions.

(Table II). Greater than 90% pure monocytes in suspension were obtained only from the first two gradient fractions (A and B). We termed these purified monocyte preparations from the IMF "intermediate" monocytes (IM). Purified cells from the RMF were termed "regular" monocytes (RM). These monocytes were used for further characterization. The remaining fractions of the gradients (C, D, E, and F) contained monocytes that were less pure, and thus these were not used for further study.

Characterization of purified monocyte subsets. The results in Table III indicate that IM and RM were very similar in regard to the degree of phagocytosis of latex beads and 7S-EA and the percentage of FcR-positive cells. However, the percentage of peroxidase-positive cells in IM was lower than that of RM ($P < 0.005$). Furthermore, IM lacked the intensity of peroxidase staining demonstrated by RM. To document such differences, peroxidase activity per 10^6 monocytes was measured, as shown in Table IV. It was found that IM had only ~60% of the activity of RM. These results show that IM differed from RM in peroxidase activity as well as in the percentage of peroxidase-positive cells.

5'-N activity which correlates with macrophage activation and differentiation (9, 30, 31) was also assayed. No significant difference between IM and RM was evident with respect to this enzyme activity (Table IV).

Cell size evaluation indicated that IM are only an average of $10 \mu\text{m}^3$ smaller than RM. There was a considerable overlap of the sizing curves of these two subsets (IM = $406 \mu\text{m}^3$, RM = $416 \mu\text{m}^3$, $n = 5$), which makes size differences insignificant. Bimodal size distribution of small and large monocytes was not seen when using monocyte fractions obtained from fractions C–F from HSA gradients.

Electron microscopy combined with peroxidase localization. Analysis of monocyte peroxidase localization by electron microscopy, using the criteria of Bainton and Golde (26), revealed that peroxidase is localized only in cytoplasmic granules and not in other organelles, e.g., cisternae of the rough endoplasmic reticulum and Golgi complex, in IM and RM (Fig. 5A and B). This indicated that IM and RM are not promonocytes or macrophages, but are typical blood monocytes. As shown in Fig. 6, the average number of peroxidase granules per cell was lower in IM than RM (IM = 22, RM = 36). This result correlates with the differences in peroxidase activity and peroxidase staining noted between IM and RM.

Comparison of surface membrane antigen expression on monocyte subsets. We analyzed the distribution of several surface membrane antigens on IM and RM by using the monoclonal antibodies, OKM1, OKT3, anti-HLA-DR, and fluoresceinated PWM. As shown in Table V, the percentage of OKT3-positive

TABLE III
Characteristics of IM and RM Obtained by CCE and HSA Gradient

Cell fraction	Percent of monocytes			Phagocytosis		
	Wright's stain	Esterase	Peroxidase	Latex*	7S-EA†	FcR‡
				% positive		% positive
IM	91.5±2.4 (12)	92.7±3.8 (12)	66.9±17.3 (12)	89.8±3.7 (5)	81.9±6.0 (5)	89.2±3.4 (5)
RM	95.8±1.5 (12)	95.6±2.4 (12)	94.8±1.9 (12)	94.4±2.8 (5)	87.5±4.7 (5)	91.9±2.8 (5)

Values are percentage means±SD for the number of experiments indicated in parentheses. Cell viability assayed by trypan blue exclusion was >98%.

* Ingestion of three or more latex particles.

† Ingestion of two or more IgG-sensitized ox erythrocytes.

‡ FcR(+) determined by rosetting with IgG-sensitized ox erythrocytes.

cells was very low in both IM and RM and most of the cells in both subsets were HLA-DR positive. However, a significant difference between IM and RM was observed with regard to the binding of OKM1 and PWM. Fewer IM expressed the OKM1 antigen and receptor(s) for PWM than RM.

Next, by double labeling of monocytes with PWM and OKM1 followed by FACS separation, we analyzed the possible association of the presence or absence of these markers on the monocyte subsets for cell size and peroxidase activity. RM demonstrated 130.6 ng/ml peroxidase activity. When reacted with PWM, the PWM-positive IM (65.5% of IM) expressed 94.7 ng/ml peroxidase activity; in contrast, the PWM-negative IM (35.5% of IM) had only 27.5 ng/ml peroxidase activity. Among the PWM-negative IM cells, 42% were also negative for the OKM1 antigen; these IM cells negative for both PWM and OKM1 demonstrated a peroxidase activity of only 7.9 ng/ml, whereas IM bearing both markers demonstrated 78.0 ng/ml peroxidase activity. The cell size (in cubic micrometers) of the PWM-negative, OKM1-negative IM was 15.3% smaller than that of the total IM. We were not able to complete a similar

analysis of RM since too low a proportion of these cells were negative for both antigens.

Accessory function of monocyte subsets. Several monocyte functions such as accessory function, IFN production, MIF responsiveness, and ADCC activity of monocyte subsets were measured. The accessory function of these cells in PHA, Con A, and PWM-induced autologous T cell proliferation was tested by adding 10% monocytes to purified T cells (Table VI). With T cells alone, all three mitogens induced only a very low level of [³H]thymidine incorporation, whereas the addition of 10% monocytes led to high level of mitogen-induced T cell proliferation. IM had less accessory function than RM in all three mitogen systems. The lower accessory function of IM was also evident when the percentage of monocytes added was changed to 5 and 25% and when a low, suboptimal concentration of the mitogens were used (data not shown).

IFN production by monocyte subsets. The results in Table VII indicate that the monocytes did not produce IFN without stimulation, but they did produce IFN when stimulated by poly I:C. IM were found to produce 113% more IFN in response to 100 µg/ml of poly I:C than RM ($P < 0.025$). However, when 200 µg/ml of poly I:C was used, higher levels of IFN were produced and no difference between IM and RM was evident. As a control, purified lymphocytes were found to produce ~2% of the amount of IFN produced by RM after poly I:C stimulation (data not shown).

Responsiveness of monocyte subsets to MIF. RMF cells have excellent spontaneous random migration and are very sensitive to MIF, such as that contained in our standard preparation, LCL-LIF. The percent inhibition in response to LCL-LIF of monocytes in the IMF was essentially identical to the percent inhibition seen with RMF cells (Fig. 7). These results indicate that IM and RM appear to express a similar pattern of MIF responsiveness.

Function of monocyte subsets in ADCC. Mono-

TABLE IV
Peroxidase and 5'-N Activity of IM and RM

Cell fraction	Peroxidase activity per 10 ⁶ monocytes	5'-N activity per 10 ⁶ monocytes
	ng/ml of horseradish- peroxidase equivalents	nmol × 10 ³ of AMP degraded/min
IM	65.6±11.1* (8)	4±2 (5)
RM	108.6±12.7 (8)	3±2 (5)
	$P < 0.005$	NS†

* Values are means±SD for the number of experiments indicated in parentheses.

† P values not statistically significant (>0.05).

FIGURE 6 Comparison of numbers of peroxidase-positive granules per cell in IM (□) and RM (▨). About 50 cells from each subset were compared for the number of peroxidase-positive granules per cell. IM have approximately a 39% reduction in the number of granules seen in RM.

cytes are effective cytotoxic effector cells for antibody-coated human erythrocytes. The cytotoxic capabilities of IM in ADCC was 41% less ($P < 0.05$) than RM at the 1:4 effector to target cell ratio and 52% less ($P < 0.05$) at the 1:8 effector to target cell ratio (Fig. 8). Addition of 10% lymphocytes to RM did not affect the ADCC activity of RM.

The entry into the circulation of monocyte subsets during cytapapheresis. It is of interest to determine whether the entry into the circulation is different for the two monocyte subsets. Therefore, we analyzed the monocyte cell number recovered during cytapapheresis and the peroxidase activity of IM and RM by using cytapapheresis specimens that were collected early and late during a 2-h cytapapheresis procedure. In these 2-h experiments, cytapapheresis removed $62.8 \pm 9.4\%$ (mean $\pm$ SD, $n = 5$) of the monocytes known to be circulating in the peripheral blood of normal humans. This number was calculated by correcting the monocyte recovery by CCE by the efficiency factor of 1.26 as previously reported (18), divided by 1.7×10^9 (total number of monocytes in peripheral blood according to prior published studies) (32). Table VIII demonstrates that there was a higher ratio of IM to RM in late cytapapheresis specimens, which indicates that an appreciably higher proportion of IM entered the circulation from the mobilizable reservoirs during the cytapapheresis.

DISCUSSION

The results of our experiments indicate that human blood monocytes are composed of at least two subsets, a peroxidase-rich subset (RM) and a slightly smaller,

low peroxidase subset (IM). We have developed methods for purifying large quantities of IM and RM by negative selection techniques (CCE plus HSA gradients) and have found that IM and RM possess quantitatively different functional capacities. IM and RM also differed with respect to the expression of certain membrane determinants; OKM1 antigen and PWM receptor positive cells were more frequent in RM than IM. Analysis of the entry into the circulation of these subsets from the extravascular monocyte reservoirs of normal human donors suggested that IM are more mobilizable than RM after prolonged cytapapheresis.

The existence of functional subsets of human monocytes seems well established from the report in the literature (11–17). Although only two or three distinguishable subsets have been reported by most authors, there is considerable disagreement as to the identifying characteristics of the subsets obtained. Our IM and RM subset classification most closely corresponds to that of Yasaka et al. (11), who used CCE and adherence methods to purify monocyte subsets, since “small” monocytes differ from “large” monocytes principally on the basis of having lower peroxidase activity. As has been noted by Figdor et al. (17), however, the reported striking size differences between “large” and “small” monocytes (11, 12, 15) almost certainly represents the size differences between monocytes and lymphocytes in the preparation used. This hypothesis was testable since we could purify the monocytes in the IMF from the contaminating lymphocytes by HSA gradients. Purified IM had less peroxidase activity than purified RM. This observation was confirmed at the single cell level with direct peroxidase stains and peroxidase localization analysis by using electron microscopy. We were able to demonstrate that the purified IM are only slightly smaller than RM, in contrast to previous reports (11, 12, 15). Also, there was no difference in FcR expression between these subsets, in contrast to previous reports (12, 15). The ADCC activity of IM was only slightly but significantly reduced when compared with RM, in contrast to the markedly reduced ADCC previously observed when monocytes and lymphocyte mixtures were studied (12).

IM share other histological and functional properties with RM. They both have the same degree of esterase staining and are similar in their phagocytic capabilities for latex beads and 7S-EA. Since phagocytosis and esterase staining are the most reliable, current functional standards for monocyte identification, it is significant that the lymphocytes separated from the IMF dem-

FIGURE 5 Electron micrograph of a typical cell of RM (A) and IM (B) stained for peroxidase. Peroxidase is present only in cytoplasmic granules in both IM and RM. Bars, 1 μ m. A, $\times 9,600$; B, $\times 14,000$.

TABLE V
Percentage of IM and RM Reactive with Fluorescent Probes
for Surface Membrane Antigens

Cell fraction	Percent of positive cells			
	OKM1	anti-HLA-DR	OKT3	PWM
IM	69.8±8.5* (5)	86.4±4.3 (5)	0.8±1.6 (5)	53.5±17.8 (4)
RM	88.2±3.4 (5)	91.0±3.2 (5)	0.4±0.8 (5)	85.3±7.0 (4)
	<i>P</i> < 0.005	NS†	NS	<i>P</i> < 0.05

* IM and RM were assayed for their reactivity with monoclonal antibodies, OKM1, anti-HLA-DR, OKT3, and fluorescinated PWM by using FACS. The results represent percentage means±SD for the number of experiments indicated in parentheses.

† *P* values not statistically significant (>0.05).

onstrated neither of these properties. IM and RM also demonstrated similar responsiveness to MIF in our human MMI assay.

The purified IM proved ideal for FACS analysis since they were isolated in suspension. Using antibodies to HLA-DR, we found no significant differences between IM and RM in the expression of this histocompatibility antigen. In contrast, the number of OKM1 antigen and PWM receptor positive cells were significantly decreased in the IM subset. Two functional correlates of the membrane composition of IM and RM were measured in our experiments. It is of note that monocyte-dependent tetanus toxoid T lymphocyte proliferation was similar for IM and RM containing cell cultures (data not shown). This function is known to be dependent (at least in part) on monocyte HLA-DR antigen expression (1). In contrast, the ac-

cessory cell function of IM to support lymphocyte proliferation to PWM was lower than that of RM. Since this function is known to be not only monocyte-dependent but also reflective of the ability of monocytes to bind PWM on their membranes (27), the observed lower accessory function of IM to support PWM-induced T lymphocyte proliferation may be partly due to the lower percentage of PWM receptor positive cells in the IM population. When IM cells that were negative for the PWM receptor and the OKM1 antigen were obtained by sorting, it was evident that this small subpopulation of monocytes was particularly deficient in peroxidase activity. The relationship of these cells to the marker-positive cells in IM and RM will require further in-depth investigation.

Cytapheresis is not only a valuable technique for isolating large numbers of purified monocyte subsets, it has also proven useful in analyzing the subset distribution of monocyte mobilizable reservoirs in man. We calculate that we removed approximately half of the monocytes that circulate in the peripheral blood

TABLE VI
Reconstitution of Mitogen-induced T Cell Proliferation
by IM and RM*

Donor	Cell population	[³ H]Thymidine incorporation (Δcpm)		
		PHA (1 μg/ml)	Con A (10 μg/ml)	PWM (1:200)
1	T	1,764	20	101
	T and IM	27,234	7,058	4,323
	T and RM	36,167	9,749	7,115
2	T	1,903	149	26
	T and IM	99,821	26,269	16,828
	T and RM	138,449	43,509	22,252
3	T	1,392	81	376
	T and IM	16,878	5,541	6,797
	T and RM	25,522	10,135	9,575

* 1×10^5 T cells were incubated with or without 1×10^4 monocytes in the presence and absence of mitogen. Values show the mean of triplicate cultures above unstimulated levels.

TABLE VII
IFN Production by IM and RM*

Cell fraction	poly I:C (μg/ml)		
	0	100	200
IM	Negative	281±88†	430±153
RM	Negative	132±41	468±278
		<i>P</i> < 0.025	NS§

* 1×10^6 monocytes were cultured with or without poly I:C. After 4 h, the cells were washed thoroughly and the incubation was continued for an additional 37 h. Supernatants were harvested and measured for IFN titre.

† Each value represents IFN titre (U/ml) and the mean±SD derived from five separate experiments are shown.

§ *P* values not statistically significant (>0.05).

FIGURE 7 Responsiveness of monocytes in IMF and RMF to MIF. The elutriated IMF and RMF were cultured with or without LCL-LIF (micrograms per milliliter) (partially purified MIF obtained from supernatants of a human lymphoblastoid cell line) as the MIF preparation. Data are means of triplicates in a representative experiment. In all cases, SD in triplicates was <17%. ■, monocytes in IMF; ●, monocytes in RMF. The percent inhibition in response to LCL-LIF of monocytes in the IMF was essentially identical to the percentage inhibition seen with RMF cells.

during the first 90 min of cytophoresis, although the circulating monocyte counts did not fall. The ratio of IM to RM rose appreciably after this monocyte-depletive procedure. Since there is no evidence for vascular margination of monocytes in man (32), this suggests that IM may be more prevalent in the mobilizable monocyte reservoirs of man than in the normal circulating peripheral blood and/or that they may more readily enter the circulation. Similarly, Alteri et al. (33) recently reported that the subset of human monocytes that is deficient in fMethionyl-Leucyl-Phenylalanine chemotactic responses is enriched during cytophoresis procedures similar to those reported in this

FIGURE 8 The cytolytic capabilities of IM (□) and RM (○) in a monocyte-mediated antibody-dependent cytotoxicity (ADCC) system against IgG-coated human erythrocyte targets. Data represent the mean±SD of four separate experiments. At effector/target ratios of 1:4 and 1:8, ADCC activity of IM was significantly less than RM ($P < 0.05$).

study. Since no other details are available on this chemotaxis-deficient monocyte subset, the relationship to IM is unclear.

The exact location of the reservoir of IM and RM and the differentiation status of IM relative to RM are not known at this time. The main reservoir for human monocytes and promonocytes is the bone marrow (33, 34), but from our electron microscopic analysis of IM, it is clear that they were not promonocytes as defined by previously established criteria (26). The observed diminished peroxidase activity in IM suggests an alternative possibility; these cells may have begun initial maturation steps towards becoming macrophages because this differentiation process is known to be accompanied by a loss of monocyte peroxidase activity (31, 35). However, maturation from monocytes to macrophages is also known to be accompanied by an increase in 5'-N activity (31, 35) and this was not found in our purified IM populations. A more precise understanding of the differentiation status and tissue reservoir sites of IM would be greatly enhanced by detection of a stable, unique differentiation antigen with monoclonal antibodies; attempts to develop such reagents are currently underway.

Perhaps the human monocyte function most relevant to current cancer therapy trials is their ability to release a number of potent biological response modifiers (monokines). Among these, IFN has probably received the most current clinical attention (36-38). It is interesting to note that IM produced 113% more IFN than did RM in response to intermediate concentration of poly I:C (100 μg/ml), although the responses to 200 μg/ml of poly I:C were equivalent. This difference was shown not to be attributable to contaminating lymphocytes. The implications of this finding are that monocyte subsets may vary in their ability to release IFN and possibly other biological response modifiers in reaction to immunostimulating agents.

With mounting evidence for the critical role of monocytes in the initiation, modulation, and final effector phase of many components of the human immune response (1, 2, 8, 9), more emphasis has been placed on understanding the physiologic intricacies of this multifunctional cell. Although no evidence for "clonality" of human monocytes is yet available, we have demonstrated a distinct subset of human monocyte (IM) that is low in peroxidase activity, low in accessory cell function and ADCC, and has a lower proportion of OKM1 and PWM receptor positive cells. IM had a greater sensitivity to the induction of IFN release by poly I:C and were more mobilizable than regular monocytes (RM) into the peripheral blood after cytophoresis. The distinctive properties of monocyte subsets reported here and by others may represent different activation and/or differentiation states of this

TABLE VIII
Entry into the Circulation of Human Monocyte Subsets during Cytophoresis*

Donor		First cytophoresis		Second cytophoresis	
		IM	RM	IM	RM
A	Peroxidase activity†	57.3	100	64.8	102.7
	Total No. of cells ($\times 10^{-6}$)	119	284 (42)§	64	80 (80)
B	Peroxidase activity	61.1	100	65.7	100.8
	Total No. of cells ($\times 10^{-6}$)	176	314 (56)	121	156 (78)
C	Peroxidase activity	60.7	100	59.6	101.9
	Total No. of cells ($\times 10^{-6}$)	296	248 (119)	117	40 (293)
D	Peroxidase activity	64.7	100	58.7	99.5
	Total No. of cells ($\times 10^{-6}$)	77	229 (34)	99	114 (87)
E	Peroxidase activity	41.8	100	61.4	92.7
	Total No. of cells ($\times 10^{-6}$)	239	227 (105)	131	41 (320)

* Five normal donors were cytopheresed for 1.5 h. All donors were then cytopheresed for an additional 0.5 h. Both cytophoresis specimens were elutriated as described in Methods to obtain IMF and RMF. Aliquots of these fractions were purified by HSA gradient centrifugation and measured for peroxidase activity.

† Peroxidase activity of RM obtained from first cytophoresis was set at 100% in each experiment. The percent peroxidase activity is shown.

§ Cell number ratio, IM/RM $\times 100$.

cell. Since we have shown that IM have several quantitatively different functional properties as compared with RM, it is important to explore further the physiologic basis for this functional discrepancy. In addition, the deficiency of monocyte function reported in cancer patients and other disease status patients (39–41) may now be carefully analyzed for alterations of the normal balance of monocyte subsets. The technology developed for this study will allow us to make comparisons between the functional activities of monocyte subsets present in cancer patients with the above results obtained in normal donors.

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