

THE SEPARATION, LONG-TERM CULTIVATION, AND MATURATION OF THE HUMAN MONOCYTE*

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Monocytes and macrophages, collectively termed the mononuclear phagocyte system, are intimately involved in a variety of physiological and pathological events (1-5). They participate in the inflammatory process, secrete numerous biologically active products, and are recognized as major effectors of immunity against neoplastic disease and infectious agents. Our present understanding of the physiology and biochemistry of this cell system is based in large part on studies of rodent cells. Although the circulating human monocyte is readily available and is recognized as the precursor of tissue macrophages, its study has been impeded by inefficient isolation and cultivation methods. The yield of monocytes from blood has ranged from 30 to 75% with different isolation procedures (6-11), with the subsequent loss of up to 90% of monocytes during the first 3 days of in vitro cultivation (8).

In the present report, we have evaluated the yield of monocytes obtained with Ficoll-Hypaque and albumin gradients and defined optimal conditions for the cultivation of monocytes in vitro. The physiologic changes which occur during the in vitro differentiation of monocytes into macrophages were also examined with respect to the secretory enzyme lysozyme, the lysosomal enzyme myeloperoxidase and plasma membrane markers, such as the Fc and complement receptors and 5'-nucleotidase. A marked increase in 5'-nucleotidase activity was observed during cultivation which was further studied in terms of the effects of phagocytosis, serum deprivation, and inhibition of protein synthesis.

Materials and Methods

Reagents. Fetal calf serum (FCS), Earle's balanced salt solution (EBSS), serumless medium (Neuman-Tytell [NT]), and RPMI-1640 (RPMI) were obtained from Grand Island Biological Co., Grand Island, N. Y. Horseradish peroxidase (HRP), diaminobenzidine, *o*-dianisidine, Triton X-100, cycloheximide, AMP, Tris (hydroxymethyl) aminomethane, and Tris buffer solution were obtained from the Sigma Chemical Corp., St. Louis, Mo. Hen egg white lysozyme, twice crystallized, and *Micrococcus lysodeikticus*, spray dried, were obtained from the Worthington

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Abbreviations used in this paper: AS, fresh autologous serum; BSA, bovine serum albumin; E, sheep erythrocytes; EBSS, Earle's balanced salt solution; FCS, fetal calf serum; HRP, horseradish peroxidase; NT, serumless medium (Neuman-Tytell).

Biochemical Corp., Freehold, N. J. Bovine serum albumin, fraction V (BSA) was obtained from Miles Laboratories, Inc., Miles Research Products, Kankakee, Ill. Polystyrene latex beads were obtained from Amersham Searle Corp., Arlington Heights, Ill. Dextran 500 was obtained from Pharmacia, Uppsala, Sweden. Sheep erythrocytes (E), rabbit anti-sheep IgG and IgM, and fresh C5-deficient mouse serum were generously provided by Dr. Celso Bianco, Rockefeller University, New York. All other reagents or chemicals were reagent grade and purchased from commercial suppliers.

Flotation of Leukocytes in a Continuous Albumin Gradient. The optimal density of an albumin gradient for the separation of human monocytes was determined in the following manner. Venous blood (20–40 ml) was collected from healthy adults in syringes containing one part acid citrate dextrose to five parts whole blood, mixed with an equal vol of 3% dextran 500, and allowed to stand at room temperature for 20 min. The leukocyte-rich plasma was centrifuged (160 g, 15 min) and the cell pellet resuspended in 5 ml of BSA (1.10 g/ml) and placed on ice. One reservoir of a gradient maker was filled with 8 ml of BSA (1.10 g/ml) and the other received 8 ml of BSA (1.05 g/ml) which resulted in an effluent BSA of continually decreasing density. This BSA was layered on top of the 5 ml of BSA containing the leukocytes. After centrifugation (10,000 g, 30 min, 4°C) in a Sorval RC2-B, successive 1-ml aliquots were removed with a fraction collector. The refractive index of a drop of each of the 21 aliquots was determined with a refractometer and the density calculated from a standard curve. Platelet and total leukocyte counts were performed on each of the gradient fractions. Differential cell counts were performed on Giemsa-stained centrifuge preparations.

Monocyte Isolation. Mononuclear cells were separated from blood with either albumin or Ficoll-Hypaque gradients and monocytes isolated by adherence to glass or plastic substrates.

ALBUMIN GRADIENTS. Leukocytes from 20 to 40 ml of citrated, dextran sedimented blood were suspended in 5–7 ml of BSA (1.10 g/ml) as described in the previous section. The addition of the cells and associated fluid decreased the albumin density to 1.090–1.093 g/ml. An even distribution of the cells in the BSA was achieved by repeated aspiration with a pipette. The BSA-leukocyte mixture was then carefully overlaid with 2 ml of diluted BSA, prepared by mixing equal volumes of BSA (1.10 g/ml) with Mg⁺⁺- and Ca⁺⁺-free phosphate-buffered saline (PBS). After centrifugation in a Sorval RC2-B (10,000g, 30 min, 4°C) a discrete band of cells was visible at the interface of the BSA solutions. The cell band, in 2–3 ml of BSA, was carefully removed with a Pasteur pipette, placed in 10 ml of EBSS with heparin (7.5 U/ml) and centrifuged for 13 min at 160 g. The cell pellet was resuspended in RPMI containing 10% heat-inactivated autologous serum ([HIAS] 56°C, 1 h), adjusted to a density of $3-5 \times 10^6$ cells/ml and 1.4 ml plated in 35-mm plastic culture dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.), or 0.5 ml was plated in 16-mm wells of a plastic Multi-Dish Dispo-Tray (Linbro Chemical Co., New Haven, Conn.). The cells were incubated for 30 min (37°C, 5% CO₂/95% air), washed three times with EBSS to remove nonadherent cells, and then either studied or cultured. Culture media consisted of 1.0–2.5 ml of NT medium supplemented with 10% fresh or frozen autologous serum (AS) which had not been heat-inactivated. Culture media was replaced every 3–4 days, usually without washing the cells.

FICOLL-HYPAQUE GRADIENT. Heparinized blood (7.5 U/ml) was layered on a mixture of Ficoll-Hypaque (24 vol 9% Ficoll:10 vol 34% Hypaque; 5 vol blood: 4 vol Ficoll-Hypaque) and centrifuged (300 g) for 45 min at room temperature (12). The mononuclear cell band was resuspended in PBS containing 7.5 U heparin/ml and centrifuged at 500 g for 10 min. The pelleted cells were washed and centrifuged three times in PBS containing 0.3 mM EDTA (100 g, 10 min), and resuspended in media for plating as already described.

Cell Counting. Monocyte cultures were examined daily with an inverted phase contrast microscope ($\times 100-200$) (Wild Heerbrugg Instruments, Inc., Switzerland). The number of adherent cells was determined by direct cell counts of at least eight representative microscope fields and from daily Polaroid photographs of etched fields of cell cultures. These cell counts were confirmed by counting nuclei in cell lysates as described by Unkeless and Eisen (13).

Enzyme Assays

LYSOZYME. Lysozyme secretion was assayed in culture supernates by measuring the initial rate of lysis of a suspension of *M. lysodeikticus* with a Gilford 240 spectrophotometer (Gilford Instruments Laboratories, Inc., Oberlin, Ohio), according to the method described by Parry et al. (14) and modified by Gordon et al. (15). Egg white lysozyme in media containing 5% FCS was

used as the standard. Controls included media containing human serum which was incubated without monocytes. Activity is expressed in terms of human lysozyme which has a 3.3-fold higher activity than the egg lysozyme standard.

5'-NUCLEOTIDASE. 5'-nucleotidase was assayed by the method of Avruch and Wallach (16), by using [³H]adenosine monophosphate as substrate in a 54 mM Tris (hydroxymethyl) aminomethane buffer pH 9.0, containing 12 mM MgCl₂. Cells to be assayed were washed in EBSS and lysed in 0.3–1.0 ml of freshly prepared 0.05% Triton X-100. In general, 0.1 ml of cell lysate was assayed for activity in 0.5 ml substrate. After incubation at 37°C for 30 min the unhydrolyzed substrate was precipitated with zinc sulfate and barium hydroxide, and separated from the supernate by centrifugation. A 0.5 ml sample of the supernate was mixed with 10 ml of Bray's solution (New England Nuclear, Boston, Mass.), and counted in a Nuclear-Chicago scintillation counter. Enzyme blanks were included in each assay. Over 97% of the substrate radioactivity was precipitated with barium sulfate.

PEROXIDASE. Myeloperoxidase was assayed by the method of Steinman and Cohn (17), with *o*-dianisidine and H₂O₂ as substrates, HRP as the standard, and measuring the rate of production of oxidized *o*-dianisidine with a Gilford 240 spectrophotometer. Peroxidase activity was always measured within 3 h of cell lysis with Triton X-100. Activity in cell lysates was optimal at pH 5.0, H₂O₂ dependent, and directly correlated with the number of cells lysed. Enzyme activity was not detected in supernates of cell cultures.

Protein Determination. Protein was assayed by the method of Lowry et al. (18), using egg lysozyme as the standard.

Cytochemistry. Monocytes were stained for myeloperoxidase by the method of Graham and Karnovsky (19) after fixation in 1.25% glutaraldehyde buffer with 0.1 M Na cacodylate (pH 7.4) at room temperature for 5 min. Cells were washed with 0.05 M Tris buffer solution, exposed to 50 mg/100 ml diaminobenzidine in 0.05 M Tris buffer (pH 7.6) and 0.01% H₂O₂ for 10 min and examined by light microscopy.

Phagocytosis. E were coated with rabbit anti-sheep IgG [E(IgG)] or with rabbit anti-sheep IgM [E(IgM)] as described by Bianco et al. (20). E(IgM) exposed to fresh C5-deficient mouse serum became complement-coated and were designated as E(IgM)C. Monocyte cultures were incubated with suspensions of E, E(IgG), E(IgM), or E(IgM)C for 1 h at 37°C, washed three times with EBSS, fixed with glutaraldehyde, and examined by phase contrast microscopy for attachment and ingestion of E. Duplicate monocyte cultures were incubated with 0.83% ammonium chloride immediately before fixation to lyse extracellular erythrocytes and facilitate the counting of intracellular erythrocytes.

Polystyrene latex beads of 1.1 μm diameter (1.3 × 10⁹ particles/1,000 μg) were washed twice in EBSS and resuspended in NT-5% AS. Beads (1 ml) in concentrations of 250, 500, and 1,000 μg/ml were incubated with monocyte cultures for 1 h at 37°C. Monocytes were washed and either lysed or further incubated in fresh media.

Results

Monocyte Isolation with Albumin or Ficoll-Hypaque Gradients. The distribution of leukocytes within a continuous albumin gradient is shown in Table I. The percent of leukocytes recovered in fractions 1–7 was 2.3 and 19.4% in fractions 8–13, and 78.3% in fractions 14–21. Fractions 8–13 contained almost all of the mononuclear cells isolated, with 1/3 identified as monocytes, and the remainder identified as either lymphocytes or unclassified mononuclear cells. The unclassified cells were small, darkly stained, lacked visible cytoplasm, and probably represented lymphocytes. The granulocytes were recovered in fractions 13–21 and 87% of the platelets (not shown) were recovered in fractions 1–7. Therefore, subsequent studies employed discontinuous albumin gradients composed of a dense BSA preparation (1.092 g/ml) which was overlaid with diluted BSA (<1.060 g/ml). Platelets and granulocytes would be concentrated at the top or bottom of this gradient, respectively, and mononuclear cells would be concentrated at the interface of the two albumin preparations.

TABLE I
Human Leukocyte Separation in a Continuous Albumin Gradient*

Fraction	Density	Leukocytes ‡ recovered	Differential			
			Monocytes	Lympho- cytes	Unclassi- fied	Granulo- cytes
	<i>g/ml</i>	%	%	%	%	%
1-7	1.049-1.064	2.3	—	—	—	—
8	1.069	0.7	53	32	15	0
9	1.074	3.3	61	33	6	0
10	1.077	6.8	38	49	13	0
11	1.082	4.5	34	46	19	1
12	1.088	3.5	7	81	12	0
13	1.092	0.6	5	67	9	19
14-21	1.094-1.099	78.3	0	0	1	99

* A mean of 83% of the leukocyte contained in 20-40 ml of whole blood were recovered from the gradient in three experiments.

TABLE II
Monocyte Isolation with Albumin and Ficoll-Hypaque Gradients

Steps in separation procedure	Mean number of total leuko- cytes per milliliter whole blood ($\times 10^{-6}$)		Mean number of monocytes per milliliter whole blood ($\times 10^{-6}$)	
	Albumin	Ficoll-Hy- paque	Albumin	Ficoll-Hy- paque
Starting whole blood	6.8 \pm 1.5*	6.6 \pm 1.8	0.34 \pm 0.22	0.33 \pm 0.21
Leukocyte-rich plasma	6.3 \pm 2.0	—	—	—
Leukocytes obtained from gra- dient and resuspended in buffer	1.6 \pm 0.4	2.7 \pm 1.1	—	—
Final leukocyte suspension in culture media‡	1.4 \pm 0.4	1.3 \pm 0.5	0.28 \pm 0.08	0.23 \pm 0.09
Leukocytes adherent to cul- ture vessel	0.27 \pm 0.10	0.23 \pm 0.08	0.25 \pm 0.02	0.21 \pm 0.08

* 1 SD.

‡ Leukocytes from Ficoll-Hypaque gradients were washed three times before suspension in culture medium and leukocytes from albumin gradients were washed once. The number of platelets remaining in both preparations was comparable after these washes.

The mean number of leukocytes and monocytes recovered from 1 ml of whole blood by albumin and Ficoll-Hypaque gradient separation is shown in Table II. The monocyte yield was 74% (albumin) and 64% (Ficoll-Hypaque) of the number present in the starting whole blood. Both procedures yielded cell monolayers which contained 91-93% monocytes by phase contrast microscopy and by cytochemical staining for myeloperoxidase activity. One-half of the myeloperoxidase-negative adherent cells had the morphologic appearance of monocytes, i.e. cytoplasmic veils and indented nuclei. 90% of cells also ingested 3 or more E(IgG) and 95% of cells had 3 or more E(IgM)C attached without ingestion. Polymorphonuclear leukocytes were rarely seen and constituted less than 0.5% of the cells.

Ficoll-Hypaque separated mononuclear cell preparations contained three-to five-fold more platelets than the albumin gradient preparations. Platelets or associated factors decreased monocyte adherence and necessitated extra washes of the Ficoll-Hypaque cells.

Cultivation of Human Monocytes. Multiple variables were examined to determine optimal conditions for the cultivation of monocytes in vitro. Equal numbers of monocytes initially adhered to plastic and glass surfaces but cells detached from glass more rapidly during cultivation. Cell survival was not improved by prior washing, boiling (6), or acid treatment (21) of the glass coverslips. Maximum numbers of monocytes of uniform appearance were obtained when the environment was 5% CO₂/95% air during the early cultivation period. There was little increase in the number of adherent monocytes if the plating period was extended from 30 min to 2 h. Monocytes could be cultivated in serumless medium (NT) for 2-3 days before assuming a spindly appearance and detaching from the culture vessel. Monocytes cultured in RPMI or MEM without serum detached within 1-2 days. The presence of heated autologous serum (56°C, 1/2-3 h) in the cultivation media promoted clumping of monocytes which was evident after 6-12 h in culture. These cell clumps subsequently detached from the culture vessel. This clumping phenomenon persisted even when serum was dialyzed, depleted of gamma globulin by heat-aggregation and ultracentrifugation, or absorbed with autologous erythrocytes or mononuclear cells. The clumping of monocytes was markedly decreased when fresh serum was employed. Fetal or newborn calf, dog, horse, and guinea pig sera did not support monocyte cultures for more than 2-4 days. The optimal cell density of monocytes designated for long-term cultivation was $4 \times 10^5/16$ mm plastic well. The aforementioned observations were true for monocytes obtained from both albumin and Ficoll-Hypaque gradients in a series of three or more experiments.

Morphology and Spreading of Human Monocytes. After 30 min of adherence to plastic or glass 90-95% of monocytes were well spread with extensive ruffled plasma membrane and mean cell diameters of 30-40 μ m. Cell diameters and the percentage of spread cells were maximal when the medium (NT) was supplemented with fresh AS. During the first 24 h cells contracted to 1/2-2/3 of their initial diameter and only 10-30% of cells had visible ruffled membrane. After 2-3 days in culture ruffled membranes, often unipolar, were again apparent and cell diameters returned to their original value. A heterogeneous population of cytoplasmic organelles including lipid droplets and peroxidase-negative phase dense granules appeared and occasional multinucleate giant cells (150-200 μ m) were now present. Mean cell diameters doubled to 60-70 μ m between days 3 and 7 in culture. Large amounts of lipid frequently obscured intracellular structures at this time. We have cultured cells for up to 3 mo, changing media twice weekly. In these long-term cultures 50-70% of nuclei were contained in giant cells, with individual cells possessing up to 20 nuclei. It is not rare in these cultures to see multinucleate cells in direct contact with uninucleate cells, possibly undergoing fusion.

Effect of Cultivation on Cell Number and Protein. There was close correlation between the number of adherent monocytes counted under phase contrast

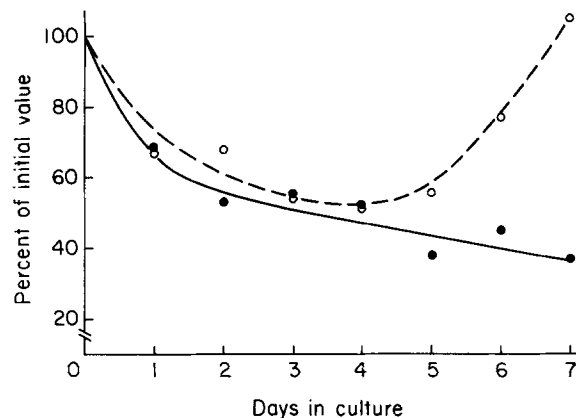


FIG. 1. Effect of monocyte cultivation on cell number (●—●) and protein (○—○). 2.5×10^6 mononuclear cells obtained from albumin gradients were plated in 16-mm plastic wells and cultured in NT-10% AS with replacement of media every 3-4 days. Direct cell counts were performed daily and the protein content of parallel cultures was determined. Cell cultures on day 0 (1 h after cell plating) contained an average of 5×10^5 monocytes/well and the mean cell protein was $46 \mu\text{g}/\text{well}$. Results are the mean of 3-23 observations for each time point.

microscopy and the cell protein during the first 4 days of in vitro cultivation (Fig. 1). Cell number and protein indicate that over 50% of the initially adherent cells were present on the 4th day of cultivation. Thereafter, the two parameters diverge with a marked increase in cell protein and a relatively stable cell count. Cell survival, as determined by direct counting, was underestimated in the older cultures because of the presence of multinucleate giant cells. The survival of monocytes from Ficoll-Hypaque gradients was comparable to that of albumin separated cells.

Lysozyme Secretion. Lysozyme activity in the media of monocyte cultures increased at a constant rate. However, since the number of cells remaining in culture after 1 wk was 40-50% of the original number, secretion of lysozyme per cell had doubled during this period. The cumulative total amount of human lysozyme was $9.6 \pm 2.1 \mu\text{g}/1 \times 10^6$ monocytes after 1 wk (Table III).

Myeloperoxidase Assay and Cytochemistry. Myeloperoxidase activity in cell lysates progressively decreased as cells were continued in culture (Table IV). The initial activity of $8.1 \pm 2.2 \text{ ng HRP}/100 \mu\text{g}$ cell protein fell to $1.4 \pm 0.8 \text{ ng}$ after 3 days. This decreasing activity paralleled the disappearance of peroxidase-positive granules in cell cultures.

5'-Nucleotidase Activity. The 5'-nucleotidase activity of monocytes adherent to culture vessels for 30 min was $3.9 \pm 3.1 \text{ nmol AMP hydrolyzed}/\text{min}/\text{mg}$ of cell protein. Cultivation of monocytes in NT-10% AS for 2 days resulted in an 11-fold increase in enzyme activity (Table V). There was no further increase in activity with longer cultivation.

5'-nucleotidase activity of nonadherent mononuclear cells (lymphocytes) ranged from 0.4 to 1.6 nmol/min/mg protein and the activity of polymorphonuclear leukocytes ranged from 0.2 to 0.5 nmol AMP/min/mg protein. The 5'-nucleotidase activity of monocyte lysates was not decreased by tartrate, an

TABLE III
Lysozyme Secretion by Cultured Human Monocytes

Days of cell cultivation	Cumulative total μg human lysozyme per 1×10^6 monocytes*
0-2	$2.5 \pm 1.1\ddagger$
2-5	7.9 ± 1.5
5-7	9.6 ± 2.1

* Results are expressed per number of monocytes initially present in culture and are not corrected for subsequent cell loss.

‡ Lysozyme secretion was $35 \pm 13 \mu\text{g}/\text{mg}$ cell protein remaining after 2 days.

TABLE IV
Peroxidase Activity in Cultured Human Monocytes

Number of days in culture	Nanogram horseradish peroxidase activity per 100 μg cell protein
0	$8.1 \pm 2.2^*$
1	4.6 ± 4.7
2	2.3 ± 1.5
3	1.4 ± 0.8

* 1 SD.

TABLE V
Effect of Cultivation on Monocyte 5'-Nucleotidase Activity

Number of days in culture	5'-nucleotidase activity (<i>nmol AMP/min per mg protein</i>)
0	3.9 ± 3.1
1	17.1 ± 6.8
2	42.7 ± 12.6
3	39.3 ± 11.0
4	46.3 ± 14.2

* 1 SD.

inhibitor of acid phosphatase, or by β -glycerophosphate, an inhibitor of alkaline phosphatase (Table VI). Fluoride, a general phosphatase inhibitor, and Zn^{++} , an inhibitor of 5'-nucleotidase, reduced enzyme activity to 43 and 14% of control levels, respectively.

The increase in 5'-nucleotidase activity of monocytes was not because of the loss of an enzyme inhibitor or the appearance of an activator. In mixing experiments employing cell lysates of freshly explanted and 2- or 4-day monocyte cultures the absolute activity equalled the sum of the activities present in the individual cell lysates.

EFFECT OF SERUM ON MONOCYTE 5'-NUCLEOTIDASE ACTIVITY. Monocytes were cultured for 48 h in serum-free media (NT) or in media supplemented with 0.1-50% AS (Table VII). Enzyme activity did not increase in cells cultured without serum and was greatest in cells cultured in 5-50% serum. Repeated changes of media with fresh serum did not further increase enzyme activity. The enzyme

TABLE VI
Effect of Inhibitors on 5'-Nucleotidase Activity

Inhibitor	5'-nucleotidase activity <i>percent of control*</i>
None	100
Tartrate, 10 mM	118
Fluoride, 10 mM	43
Zn ⁺⁺ , 0.1 mM	14
β -glycerophosphate, 5 mM	104

* 5'-nucleotidase activity was assayed at pH 8.5. Inhibitors were added to the final concentrations listed above and enzyme activity is expressed as percentage of control without inhibitor. Results are the mean of three experiments.

TABLE VII
5'-Nucleotidase Activity of Monocytes Cultivated in Various Concentrations of Fresh Autologous Serum

Serum concentration %	5'-nucleotidase activity <i>nmol AMP/min per mg protein*</i>
50	40
20	72
10	60 \pm 19
5	41 \pm 7
1	13 \pm 2
0.1	11
0	8 \pm 6

* 1 SD is indicated when the mean is derived from more than three separate experiments.

activity of fresh human serum alone ranged from 0.03 to 0.1 nmol AMP/min per mg protein.

5'-NUCLEOTIDASE ACTIVITY OF MONOCYTES EXPOSED TO CYCLOHEXIMIDE. Monocytes cultured for 48 h increased their 5'-nucleotidase activity from 2.8 to 47.6 nmol AMP/min per mg protein (Table VIII). The cells were then washed and cultured for an additional 24 h in NT-5% AS (1.5 ml) containing 0.5–2.5 μ g of cycloheximide/ml. Enzyme activity decreased to 27–59% of control levels. When cycloheximide was removed and the cells were cultured in drug-free media, enzyme activity returned to control levels within 24 h except in cells exposed to the highest cycloheximide concentration (2.5 μ g/ml). These latter cultures had few viable well-spread cells.

EFFECT OF PHAGOCYTOSIS ON 5'-NUCLEOTIDASE ACTIVITY. Monocytes were cultured for 3 days and then pulsed for 1 h with latex beads in NT-5% AS. Cells were washed to remove noningested latex and continued in culture with fresh media. 5'-nucleotidase activity decreased in proportion to the size of the latex challenge (Table IX). The enzyme activity in cultures 6 h after exposure to 250, 500, or 1,000 μ g of latex was 75, 63, and 43%, respectively, of the activity in the control cultures. Enzyme activity returned toward control levels within 24 h.

TABLE VIII
5'-Nucleotidase Activity in Monocytes Exposed to Cycloheximide

Concentration of cycloheximide $\mu\text{g/ml}$	5'-nucleotidase activity	
	Cycloheximide exposure from h 48 to 72 in culture	Additional cultivation in drug-free medium from h 72 to 96
None	100	100
0.5	59	98
1.5	47	107
2.5	27	38

* Cells were cultured in drug-free medium (NT-5% AS) for 48 h before exposure to various concentrations of cycloheximide. During this initial period 5'-nucleotidase activity increased from 2.8 to 47.6 nmol AMP hydrolyzed/min per mg cell protein. Results are the mean of three experiments.

TABLE IX
*Effect of Latex Ingestion on Monocyte 5'-Nucleotidase Activity**

Amount of polystyrene latex $\mu\text{g/ml medium}$	5'-nucleotidase activity % of control \ddagger
None	100
250	75
500	63
1,000	43

* Assayed in 3-day monocyte cultures 6 h after latex ingestion.

\ddagger Mean of three experiments.

Discussion

This report defines conditions for monocyte isolation and cultivation which yield maximum numbers of viable cells. These procedures facilitated the characterization of selected biochemical and functional properties of the human monocyte during its in vitro maturation.

Human monocytes were isolated from blood with either albumin or Ficoll-Hypaque gradients. The albumin gradient, a modification of the method of Bennett and Cohn (22), yielded 15% more monocytes than did Ficoll-Hypaque but no significant differences were noted in either cell morphology or in vitro cell survival. This monocyte yield (74%) equalled the maximum previously reported for other albumin gradients (30-75%) (10, 11) and exceeds the estimated 50% yield by the method of Einstein et al. (6).

The lack of suitable conditions for the culture of human monocytes in vitro has been the major obstacle to the study of these cells. Large volumes of blood (100-400 ml) have been required which have restricted studies to either freshly explanted monocytes or to the small subpopulation of cells which survive for longer periods in vitro. For example, in one study where in vitro cell survival was quantitated, 90% of adherent monocytes were lost during the first 72 h (8).

In the present report, up to 50% of monocytes remained adherent after 1 wk of *in vitro* cultivation in plastic wells with NT medium supplemented with fresh autologous serum. The major factors contributing to enhanced cell survival were the choice of culture medium and the use of plastic culture wells. Previous studies have employed a variety of other media including 199, McCoy's, RPMI, and Dulbecco's (15, 23-25), supplemented with varying concentrations of either heat-inactivated autologous serum, pooled AB serum, fetal bovine serum, newborn calf serum, or horse serum (6, 15, 24, 26, 27). Although superior to heterologous serum, heated (56°C; 1/2-3 h) autologous serum markedly reduced the number of adherent cells, an effect which persisted in dialyzed, absorbed, and gamma globulin-depleted serum. The cultivation of monocytes on glass substrates also contributed to poor cell survival.

The cell monolayers contained 90-95% monocytes by multiple criteria including morphology, peroxidase cytochemistry, ingestion of latex beads, and presence of Fc and C3 receptors. The morphology of monocytes changed dramatically during cultivation. The cells were uniformly well spread initially but retracted their plasma membrane and reduced their diameters by 50% during the 1st day in culture, returning to original diameters by days 2-3, and then doubling their size by the end of the 1st week. This contrasts sharply with mouse peritoneal macrophages which are not well spread initially, but progressively increase their diameter during cultivation. Bianco et al. have also reported rapid spreading of adherent resident mouse peritoneal macrophages after exposure to activated complement and coagulation factors (28). The early changes in human monocyte spreading may be related to exposure to spreading factors generated during the process of isolation and initial cell cultivation.

Myeloperoxidase activity was progressively lost during *in vitro* cultivation and was virtually undetectable by cytochemical techniques after 4 days, as others have reported (15, 29). This was confirmed by the biochemical assay for myeloperoxidase activity. Lysozyme secretion continued at a linear rate throughout 1 wk of cell cultivation. Since the major loss of cells occurred during the 1st day *in vitro*, the secretory rate on a per cell basis was actually increased twofold on subsequent days. Lysozyme secretion expressed per milligram cell protein or per number of monocytes initially present in culture was three to sixfold greater than reported by Gordon et al. (15). This probably reflects increased cell-survival with the present culture methods.

The most striking biochemical change in the monocyte was an 11-fold increase in the activity of the plasma membrane associated ectoenzyme 5'-nucleotidase. The increase in 5'-nucleotidase activity was not related to the apparent surface area of the monocyte. There was no significant contribution of acid or alkaline phosphatase to this activity since inhibitors of these enzymes did not reduce total activity. Furthermore, the assay reaction was performed at pH 8.5 which precludes acid phosphatase activity and monocytes lack alkaline phosphatase (30). There was also no significant contribution of other cell types to this activity. Polymorphonuclear leukocytes have little apparent enzyme (31) and would not survive several days *in vitro*. Lymphocyte 5'-nucleotidase activity was only 10% of that found in monocytes per milligram of cell protein and this contribution would be negligible since cultures contained few, if any,

lymphocytes. The presence of other cell types was excluded by the morphologic and phagocytic criteria for monocyte identification.

The increase in 5'-nucleotidase activity could not be attributed to the loss of an enzyme inhibitor or the appearance of an activator as determined by mixing experiments employing cell lysates with negligible and maximal activity. The observation that activity did not increase in cells cultured in serum-free medium and that increased enzyme levels were not maintained in the presence of cycloheximide suggests either decreased synthesis of 5'-nucleotidase or an increased rate of enzyme degradation under these conditions. The location of 5'-nucleotidase activity within human monocytes was not determined but the kinetics of the response to a phagocytic pulse paralleled that of the mouse peritoneal macrophage (32, 33). In the mouse, 5'-nucleotidase is a constituent of the plasma membrane and is transiently decreased in activity after latex ingestion with the associated interiorization of membrane and rapid intracellular enzyme inactivation in the phagolysosome (33).

Recent interest in mononuclear cell 5'-nucleotidase activity has been related to its value as a plasma membrane marker in cell fractionation studies (34) and the absence of 5'-nucleotidase activity in lymphocytes from patients with chronic lymphocytic leukemia (35). 5'-nucleotidase activity is also markedly reduced in inflammatory mouse peritoneal macrophages (36). Resident macrophages have levels of enzyme activity (58 nmol AMP hydrolyzed/min per mg protein) comparable to cultivated human monocytes, whereas thioglycollate-stimulated macrophages have less activity (0.7 nmol/min per mg protein) than freshly-explanted monocytes. Edelson and Cohn have suggested that inflammatory macrophages have an elevated rate of enzyme degradation rather than decreased synthesis. The present studies do not distinguish between these potential mechanisms for the progressive increase in 5'-nucleotidase in cultured human monocytes. The regulators of cell 5'-nucleotidase activity and the physiologic role of this enzyme in mononuclear cells are at present unknown.

Monocytes and macrophages have traditionally been appreciated only for their phagocytic and digestive ability. However they are now recognized as major secretory cells (complement [6], neutral proteases [37], lysozyme [15], prostaglandins [38], endogenous pyrogen [39, 40]), and mediators of host defense against a variety of obligate intracellular micro-organisms (1, 24, 26, 41-45). The methods described herein for the isolation and cultivation of human monocytes make it possible to examine monocyte function in patients with specific disease states and to undertake the longitudinal study of cells *in vitro*. The present studies of monocyte 5'-nucleotidase activity demonstrate the need to further characterize the biochemical and physiologic changes which are associated with the cell differentiation process.

Summary

Human monocytes were isolated from peripheral blood by means of albumin or Ficoll-Hypaque gradients and subsequent adherence to plastic and glass surfaces. Cell cultures contained 90-95% monocytes by multiple criteria including morphology, peroxidase cytochemistry, ingestion of latex beads, and presence of Fc and complement receptors. The monocyte yield from albumin

gradients was 74% of the cells contained in whole blood and greater than that obtained with Ficoll-Hypaque. Determinations of cell number and protein indicated that approximately 50% of the initially adherent monocytes were present after 1 wk of cultivation in plastic wells containing Neuman-Tytell medium supplemented with fresh autologous serum. Monocytes have been cultured for up to 3 mo under these conditions. The morphology of monocytes changed dramatically during cultivation. The initially well spread cells retracted their plasma membranes during the 1st day in culture, returned to original diameter after 2-3 days, and doubled their size after 1 wk. The use of heated autologous serum resulted in severe cell clumping and poor viability.

Lysozyme secretion was constant after the 1st day of monocyte cultivation whereas myeloperoxidase activity was virtually undetectable after 4 days. 5'-nucleotidase activity increased 11-fold during this period. The increased 5'-nucleotidase activity was the result of endogenous synthesis and not related to either activation of latent enzyme nor to loss of enzyme inhibitors. Enzyme activity did not increase in cells cultured in serum-free medium and was reversibly depressed in monocytes phagocytizing latex particles.

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