ESTABLISHMENT OF LONG-TERM MONOCYTE SUSPENSION CULTURES FROM NORMAL HUMAN PERIPHERAL BLOOD

By S. ZAKI SALAHUDDIN, PHILLIP D. MARKHAM, and ROBERT C. GALLO*

From the Laboratory of Tumor Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205; and Litton Bionetics, Inc., Kensington, Maryland 20795

A detailed characterization of normal human monocyte growth, maturation, and function is limited by difficulties in obtaining sufficient quantities of relatively pure populations of monocyte-macrophages in different stages of maturation. Attempts to culture fresh normal human peripheral blood monocytes for prolonged periods have met with varying results as to length of culture life and properties of the cells involved. These attempts usually result in the development of cultures of adherent monocytoid-macrophage cells exhibiting little or no cell division, which persist for varying lengths of time in culture (1). A few cell lines have been established from leukemic donors which exhibit morphological, cytochemical, and functional properties attributable to cells of the monocyte-macrophage lineage. These include cells classified as histiocytic cell types (2–4) and a recently described monocytoid cell line established from a patient with acute monocytic leukemia (5).

Efforts have been made in our laboratory to develop technology for the long-term growth of different classes of human leukocytes in liquid suspension culture, and progress in the growth of normal mature T lymphocytes (6–8), neoplastic mature T lymphocytes (9), B lymphoblasts (10), and myeloid cells of leukemic origin (11, 12) has been made. Much of this work has involved use of specific growth factors, e.g., T cell growth factor (TCGF)[1] (6–8) for T cells. In addition, we have recently described methods using a combination of cell separation and specific culture procedures not requiring addition of exogenous growth factors which routinely support the in vitro replication of normal myeloid-monocytoid cells established from fetal cord blood leukocytes (13). The use of these new procedures to initiate long-term, nonlymphoid cultures from adult human peripheral blood (nine donors) or bone marrow (six donors) resulted in only short-term cell proliferation until leukocytes from normal human donors seronegative for antibodies against Epstein-Barr virus viral capsid antigen (EBV-VCA) and Epstein-Barr virus nuclear antigen (EBNA) were used. In these instances, long-term (~5 mo) cultures composed of replicating monocytes-

* To whom correspondence should be addressed at the Laboratory of Tumor Cell Biology, National Cancer Institute, Bethesda, Md. 20205.

[1] *Abbreviations used in this paper:* CM, conditioned media; CSF, colony-stimulating factor; DMSO, dimethyl sulfoxide; EBNA, Epstein-Barr virus nuclear antigen; EBV, Epstein-Barr virus; EBV-VCA, EBV viral capsid antigen; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HLA-DR, major histocompatibility antigen-DR locus; LAF, lymphocyte activating factor; LPS, lipopolysaccharide; NBT, nitroblue tetrazolium; OKM-1, monoclonal antibody reactive with human peripheral blood monocytes; PBS, Dulbecco's phosphate-buffered saline; PG, prostaglandin; PGE, prostaglandin E; PHA, phytohemagglutinin; SIg, cell surface bound immunoglobulin; TCGF, T cell growth factor; TPA, tetradecamylphorbal acetate.

macrophages in suspension and adherent nonreplicating macrophages were consistently and reproducibly obtained from three EBV seronegative donors. This report describes the establishment and partial characterization of these cultures. In addition to the practical advantages of these systems, the demonstration that committed normal hematopoietic cells, e.g., T lymphocytes (6, 7), monocytes, and myeloid cells (13), can proliferate for extended periods indicates that readily recognizable cells can have long self-replicative capacities, a property often attributed only to an unidentifiable, so-called "stem" cell.

Materials and Methods

Preparation and Culture of Leukocytes. Freshly drawn heparinized blood and bone marrow samples were allowed to settle at unit gravity for 30 min. The buffy coat leukocytes were then removed and layered over lymphocyte separation medium (Litton Bionetics, Inc., Kensington, MD) and centrifuged for 25 min at 600 *g*. Mixed populations of mononuclear leukocytes were recovered from the interphase band, rinsed with RPMI 1640 (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) or alpha medium (Flow Laboratories, Rockville, MD), resuspended in media ($0.5–1.0 \times 10^7$/ml), and 4 ml was layered over each preformed supplemented Percoll gradient (13). Materials for gradients were dissolved in Dulbecco's phosphate-buffered saline (PBS) at 37°C and filtered (0.45-μm) before use. Each of four gradient fractions was prepared before use by mixing equal volumes of Percoll (20% vol/vol) with; (*a*) Dextran 2000 (3% wt/vol), (*b*) Dextran T70 (12% wt/vol), (*c*) Ficoll 400 (20% wt/vol), and (*d*)Hypaque-M 90% (60% vol/vol). Gradients were prepared in 15-ml plastic centrifuge tubes (25310; Corning Glass Works, Science Products Div., Corning, NY) by layering 2 ml of each fraction in (*d*) to (*a*) order. Centrifugation was for 20 min at 700 *g* in an International Electron-Clinical Laboratory (Needham, MA) table top centrifuge at room temperature. Leukocytes were recovered from the appropriate interphase, and after a media rinse, were quantitated for viable cells using trypan blue dye exclusion. Final leukocyte preparations containing 95% viable cells were initially cultured at 2.5×10^5 leukocytes/ml in 5×10^{-7} M hydrocortisone and 2.5 μg/ml vitamin D_3 supplemented RPMI 1640 or alpha medium containing 20% FBS as described previously (13). Cultures were incubated with gentle, continuous rotation at 37°C in a 5% CO_2 atmosphere and were refed weekly by a total change of medium. Once cultures were stabilized, they were seeded at $2–3 \times 10^4$ cells/ml.

Characterization of Leukocytes

MORPHOLOGY. Standard differential counts were performed on Wright-Giemsa-stained, cytocentrifuge-prepared cells to determine the gross morphology of the cell population and their state of differentiation. Myeloid cells through the metamyelocyte stage were classified as immature.

ELECTRON MICROSCOPY. The cells were fixed for 1 h at room temperature in a solution containing 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.2. After fixation, the cells were washed several times in a 0.2 M sodium cacodylate buffer, pH 7.4, post-fixed for 45 min in Dalton's chromeosmium, pelleted, rinsed with distilled water, and stained for 1 h *en bloc* in a 1% aqueous solution of uranyl acetate. The pellet was dehydrated in ethanol and embedded in the ultra low viscosity epoxy resin NC-1010 (Polaron Instruments, Inc., Doylestown, PA). Thin sections were cut on an LKB Ultrotome III with a diamond knife, picked up on 300-mesh copper grids, and stained with uranyl acetate and lead citrate. The sections were examined in a Siemens Elmsikop 1A electron microscope (Siemens Corp., Iselin, NJ) at 80 kV.

CYTOCHEMISTRY. Published procedures were followed for the cytochemical evaluation of fresh and cultured cells. The tests performed included stains for naphthyl ASD chloracetate esterase (14), α-naphthyl acetate esterase (14), acid phosphatase (15), and myeloperoxidase (16).

NITROBLUE TETRAZOLIUM REDUCTION. The nitroblue tetrazolium (NBT) reduction test was performed on suspension and adherent cells with or without the addition of freshly diluted TPA, as previously described (13, 17). Cells were incubated for 25 min at 37°C in media

containing 0.2% NBT and the desired concentration of tetradecamylphorbal acetate (TPA). Cells were rinsed twice with media, pelleted on microscope slides using Shandon-Elliot cytospin, and stained with Wright-Giemsa. Cells with dark formazan deposits were scored positive.

PHAGOCYTOSIS. Suspension cells were mixed with either sterile carbonyl iron (Technicon Instruments Corp., Tarrytown, NY) at a concentration of 10% (vol/vol) or with formalin-fixed *Candida albicans* at a concentration of 4×10^6/ml with or without the addition of freshly diluted TPA, and incubated at 37°C for 25 min. After incubation, cells were rinsed twice with media, and cytocentrifuge smears prepared. These were Wright-Giemsa stained before evaluation. Adherent cells on the plastic surface of culture flasks were treated with either carbonyl iron or *Candida albicans*, rinsed, and evaluated directly under an inverted microscope after Wright-Giemsa staining.

CYTOGENETICS. Chromosome preparations were made according to standard procedures (18). In brief, cells grown before confluency were treated with 0.02 μg/ml of colcemid for 1–2 h at 37°C. After washing with PBS, the cell pellet was resuspended in 0.075 M KCl and allowed to stand at room temperature for 20 min. The cells were then fixed by three changes of a fresh mixture of methanol and glacial acetic acid in 3:1 ratio. After the last change of the fixatives, the cells were deposited on glass microscope slides and air-dried overnight. The slides were banded with trypsin and stained in 2% Giemsa stain in Sorensen's buffer, pH 6.8, after ~2 wk of aging.

DETECTION OF EBV ANTIGENS AND ANTIBODIES. Serum from donors were tested for the presence of antibodies reactive against EBNA and EBV-VCA and cells tested directly for these antigens by published procedures (19).

ROSETTE FORMATION. Leukocytes were tested for their ability to form rosettes with either sheep or bovine erythrocytes either directly (E rosette) after treatment of erythrocytes with anti-sheep or bovine erythrocytes serum (EA-rosettes) or addition of C_5 deficient mouse complement to the EA preparation (EAC-rosettes) by published procedures (20).

SURFACE-BOUND IMMUNOGLOBULIN. The presence of cell surface-bound Ig (SIg) was determined by direct procedures using fluorescence-labeled $F(ab')_2$ fragments of anti-human IgG, A, and M.

HLA-DR. Histocompatibility antigens locus D were detected on cells using a murine monoclonal antiserum directed against HLA-DR structural components (21) (Becton, Dickinson & Co., Sunnyvale, CA). Reacting cells were identified using fluorescein isothiocyanate (FITC)-labeled anti-murine IgG, $F(ab')_2$-specific serum.

OKM-1. Murine monoclonal antiserum, reactive with peripheral blood monocytes (22) (Ortho Pharmaceutical Corp., Raritan, NJ) was used to detect monocyte-macrophages. Reacting cells were identified using FITC-labeled anti-murine IgG, $F(ab')_2$-specific serum.

COLONY GROWTH IN SEMISOLID MEDIA. Cultured leukocytes were tested for their ability to grow as colonies in 0.3% agarose or 0.8% methylcellulose with or without addition of colony-stimulating factor (CSF). Conditioned media from a SV-40 transformed human placental cell line (23) was used as a source of exogenous CSF. Cells (1×10^5) mixed with methylcellulose (0.8%) or agarose (0.3%), fetal bovine serum (20%), and conditioned media containing CSF (0, 5, 10 or 20%) in a final volume of 1 ml were added to a 35 × 10-mm culture dish and incubated for 14 d at 37°C in a humid, 5% CO_2 atmosphere. Assays were routinely performed in triplicate. Dishes were observed at days 7 and 14 for formation of colonies (consisting of ≥50 cells) or clusters (usually <20 cells).

Testing for the Release of Biologically Active Materials

CSF. Conditioned medium (CM) from cells in culture for various lengths of time was tested for CSF activity as previously described (13). 1×10^5 fresh human bone marrow leukocytes were mixed with either methylcellulose (0.8%) or agarose (0.3%), FBS (20%), and varying concentrations of CM (5–30%). 1 ml of this mixture was placed into 35 × 10-mm petri dishes, incubated in a humid atmosphere at 37°C, 5% CO_2, and observed at days 7 and 14 for colony (>50 cells) and cluster (<20 cells) formation.

PROSTAGLANDIN DETERMINATION. Measurement of production of prostaglandin (PG) in the culture media was performed by a modification of published radioimmunoassay procedures (24). For the purpose of this study, a ^{3}H-prostaglandin E (PGE) kit, which detects PGE_1 and E_2, was used (RIA Kit, CA-501; Clinical Assays Inc., Div. of Travenol Laboratories, Cambridge, MA).

LYSOZYME PRODUCTION. Media from 3-, 8-, and 12-d cultures seeded at 2×10^5 cells/ml were assayed for the presence of lysozyme by the radial diffusion lysoplate procedure (25). Dr. Elliott F. Osserman (Institute for Cancer Research, Columbia University College of Physicians and Surgeons, New York) graciously performed some determinations, and others were conducted using lysozyme test kits (Kallestad Laboratories, Inc., Austin, TX). Purified human lysozyme was used as the standard and the specificity of these reactions was determined using antisera prepared against purified rat and human lysozyme.

LYMPHOCYTE-ACTIVATING FACTOR (LAF). Culture fluids were tested at various times (24, 48, and 74 h) following plating of 2×10^4 cells/ml. These cultures were tested both unstimulated and after stimulation with lipopolysaccharide (LPS) and phytohemagglutinin (PHA), either individually or in combination (26).

TCGF. CM from cultured cells were tested for TCGF activity in a short-term thymidine uptake assay and for its ability to support the growth of activated T lymphocytes (8).

Results

Establishment of Cultures. Techniques that were recently developed for the establishment of long-term replicating suspension cultures of fresh human myeloid-monocytoid cells from fetal cord blood donors (13) were used in an attempt to initiate cultures from adult peripheral blood and bone marrow aspirates. A culture of slowly replicating, nonlymphoid suspension cells developed in only 1 instance out of 10 attempts with normal adult peripheral blood samples in a preliminary survey. Closer characterization demonstrated that this donor was seronegative for antibodies directed against EBNA- and EBV-VCA. Repeated samples (eight over a period of ~18 mo) from this same donor consistently developed into long-term cultures using these techniques. Subsequently, leukocytes prepared from two additional unrelated EBV-seronegative donors also responded to these conditions and developed into slowly replicating suspension cultures. DB is a 33-yr-old male, MB a 20-yr-old male, and CB a 24-yr-old female. None have an unusual history of illness or use of medication.

Leukocytes prepared from bone marrow aspirates from six EBV-seropositive donors and one EBV-seronegative donor failed to develop into long-term, nonlymphoid cultures using the conditions described, although a high proportion (50%) of the bone marrow samples from EBV-seropositive donors did convert into B lymphoblast cultures. The incidence of successful establishment of these suspension cultures is summarized in Table I, and an example of the growth characteristics of cultures initiated from the three EBV-seronegative donors is shown in Fig. 1. All cultures behaved in a similar fashion, continuing to replicate slowly for ~5 mo.

Description of Cultures. Newly initiated cultures containined a variety of cell types, including myeloid, monocytoid, and lymphoid elements. During the first few weeks, both neutrophils and monocyte-macrophages predominated in the culture. When EBV-seronegative adult peripheral blood leukocytes are used, the neutrophilic cells rapidly disappear and monocytes and macrophages dominate the cultures. The differential cell count of cells from three EBV-seronegative donors at different times in culture is given in Table II. In addition to the gradual, complete conversion to a pure population of monocytoid cells, a small percentage of eosinophils persist in culture for several passages.

During the early stages in culture (2nd wk), adherent cells begin to appear attached to the surface of the culture flask, and these cells persist throughout the life of the culture at ~20% of the total cell population. These adherent, nondividing cells appear to be macrophages. The more immature replicating suspension cells, recognizable as

TABLE I

Long-Term Leukocyte (Nonlymphoid) Cultures from Normal Adult Cells

Specimen source*	Serum antibodies against EBNA and EBV-VCA‡	Number replicating/number initiated
Peripheral blood	Seropositive	0/9
	Seronegative	3/3§
Bone marrow	Seropositive	0/6‖
	Seronegative	0/1

* Leukocytes from normal adult human peripheral blood or bone marrow were collected and separated on discontinuous Percoll gradients and cultured in hydrocortisone and vitamin D_3-supplemented media, as described in Materials and Methods.

‡ Serum from donors were screened for antibodies reacting against EBNA and EBF-VCA, as described in Materials and Methods.

§ Replicating long-term cultures were repeatedly established from one of the EBV-seronegative donors (DB) in eight independent attempts.

‖ Three of the cultures initiated from the bone marrow aspirates eventually developed into B lymphoblastoid cultures.

monocytoid cells, form clusters of 10–12 cells and sometimes loose clumps of 50–100 cells. The larger clumps develop necrotic centers, which adversely affect all the cells in the clump if they are not routinely broken up. These cells begin as smaller cells (10–15-μm Diam) and as they mature they grow larger, reaching a size of ~25-μm Diam. Single cells are also present and are generally the nonreplicating, more mature cells (20–25 μm Diam), which proceed to adhere to the culture flask surface. If cultures are allowed to remain static, most of the monocytes in culture will eventually adhere to the plastic surface of the culture vessel. This significantly reduces their growth rate and accelerates their maturation into macrophage-appearing cells. The average culture doubling time is ~150 h, and the saturation density of these cultures ~1.5 × 10^5 cells/ml. Studies that further define these cells as monocyte-macrophages are described below. Unless specified otherwise, the assays were limited to the growing cells in suspension and do not include the adherent cells.

Morphological Characterization of Cells. Wright-Giemsa-stained cytospin smears of the suspension cells allowed recognition of several distinguishable monocyte-macrophage cell types, which we have separated into four stages for descriptive purposes. These include (*a*) stage 1: immature monoblastic cells, ~10–15 μm Diam, a high nuclear/cytoplasmic ratio (1:1), a large, immature nucleus often containing distinct nucleoli, and a smooth basophilic cytoplasm containing few or no granules or vacuoles; (*b*) stage 2: slightly larger monocytic cells having a reduction in nuclear/cytoplasmic ratio (1:2), the beginning of nuclear pyknosis, disappearance of nucleoli, reduced basophilic cytoplasm, and emergence of vacuolization; (*c*) stage 3: monocyte-macrophage cells, ~20–25 μm Diam, a low nuclear/cytoplasmic ratio (1:3), progressively higher nuclear pyknosis with a clear and often (bi- or multinucleate) eccentric nucleus, cytoplasm containing a number of irregular granules and vacuoles; (*d*) stage 4: macrophages, a stronger tendency to adhere to culture flask surfaces, low nucleus/cytoplasmic ratio with characteristics similar to stage 3 cells, a clear, highly vacuolized cytoplasm containing phagocytic vesicles, and an irregular cellular margin with numerous filaments. All four monocyte stages can usually be found in any given culture, however, mitotic figures are primarily seen in stage 1 and 2 cells. A noticeable

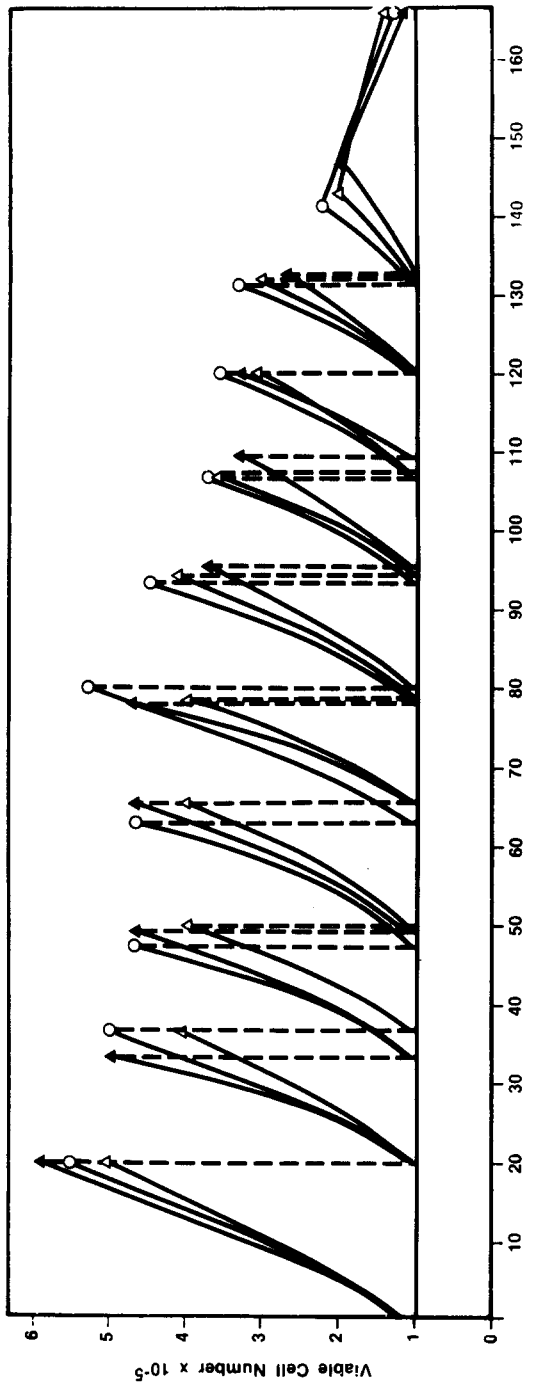

FIG. 1. Growth curves for leukocytes from the peripheral blood of three normal EBV-seronegative donors. Peripheral blood was separated and cultured as described in Materials and Methods. Dotted lines indicate a subculturing of cells into 5 ml fresh medium/T-25 flask. ○, donor DB (the average of eight separate specimens); △, donor CB; ▲, donor MB.

TABLE II

*Morphological Classification of Long-Term Leukocyte Cultures Established from Three Normal Donors (DB, MB, CB)**

Sample treatment	Days in culture	Immature myeloid			Neutrophil			Eosinophil			Monocyte			Lymphocyte		
		DB	MB	CB	DB	MB	CB	DB	MB	CB	DB	MB	CB	DB	MB	CB
		%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Buffy Coat		0	11.0	11	6	61.5	52	65	1.5	2	0	3.5	4	6	22.0	30
Percoll-Fraction 3	0	31.5	37	31	34.5	13	26	1.0	0	2	14.0	9	17	19.0	41	23
	35	33.5	33	14	25.0	10	44	2.5	6	2	(35.0	35	39	5.2	16	1
		(30–40)			(20–32)			(1–5)			31–41			(2–10)		
	65	1.0			0.5			2.5			95.0			0.5		
		(0–3)			(0–2)			(1–5)			(90–99)			(0–2)		
	95	0.5	3	0		0	0	1.5	0	4	98.0	97	96	0	0	0
		(0–1)						(0–5)			(95–100)					
	100	0	0	0	0	0	0	0	1	0	100.0	99	10	0	0	0
	150	0			0			0			100.0		0			

* Cell classification was determined by differential counts based on cell morphology. DB, MB, and CB are samples from three untreated EBV-seronegative donors. Immature myeloid cells consist of cells at blast through metamyelocyte stage (cultures routinely contain ≤1% blasts). Percentages do not include a low number (<5%) of not easily identifiable cells and do not include adherent macrophages, which constituted ~20% of the total cell number in a given culture flask. Percentages for donor DB are the average and range (in parentheses) of four independently established long-term cultures.

shift to a higher proportion of mature cell types is seen in the later stages of culture life and cultures eventually terminate as non-dividing macrophages. An example of the light microscopic appearance of a representative culture (DB) demonstrating the various monocyte cell stages is illustrated in Fig. 2.

Electron microscopic studies further revealed the monocyte-macrophage characteristics of these cells. The less mature monocyte-macrophage shown in Fig. 3 A had a rounded, relatively immature nucleus containing a distinct nucleolus and normal chromatin distribution. The cytoplasm contains a normal rough endoplasmic reticulum, mitochondria, and numerous distinct lysosomal bodies, vacuoles, and phagosomes. The more mature cell shown in Figure 3 B has a mature nucleus without a detectable nucleolus. The cytoplasm contains fewer lysozymal bodies, numerous vacuoles and unfolding of the cytoplasmic membrane.

Cytochemical and Immunological Characterization. The monocyte-macrophage charac-

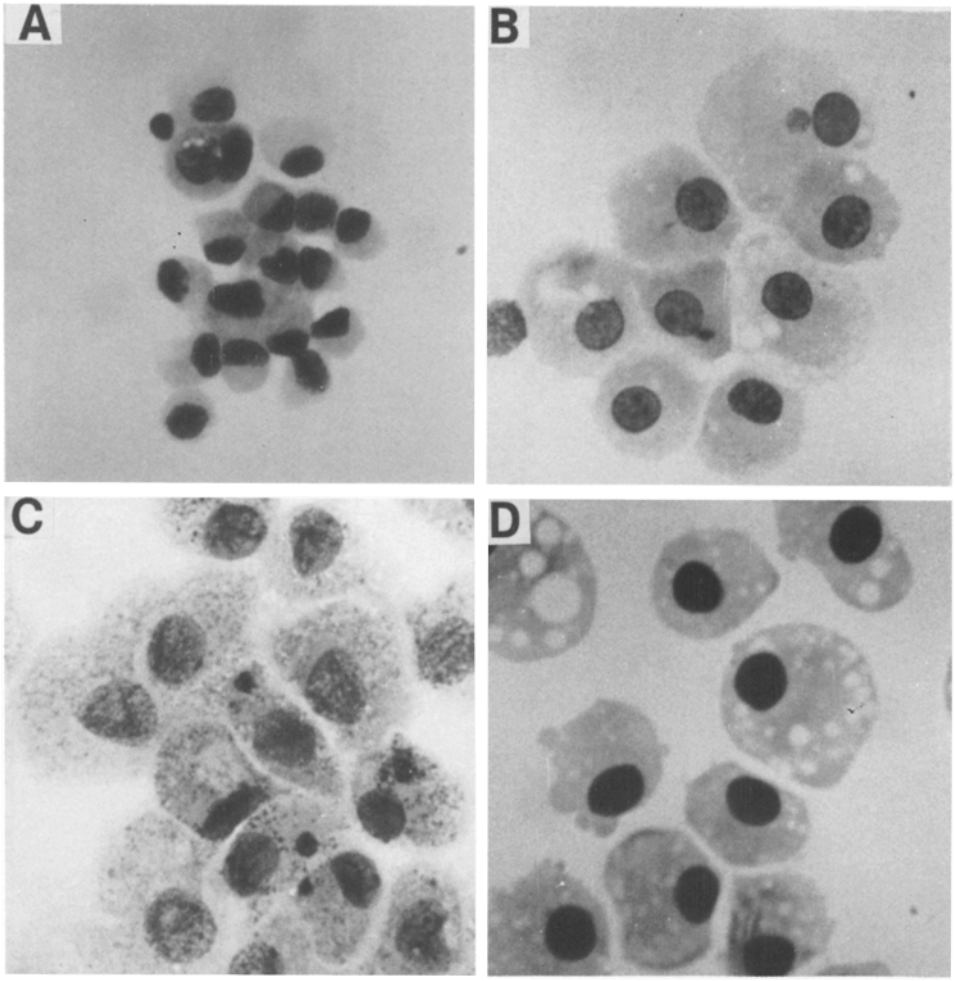

FIG. 2. Light microscopic appearance of cultured leukocytes from donor DB. Cytospin preparations of suspension cells ~80 d in culture were stained with Wright-Giemsa. Panel A, stage 1 cells, × 1,000; B, stage of 2 cells, × 1,000; C, stage 3 cells, × 1,000; D, stage 4 cells, × 1,000.

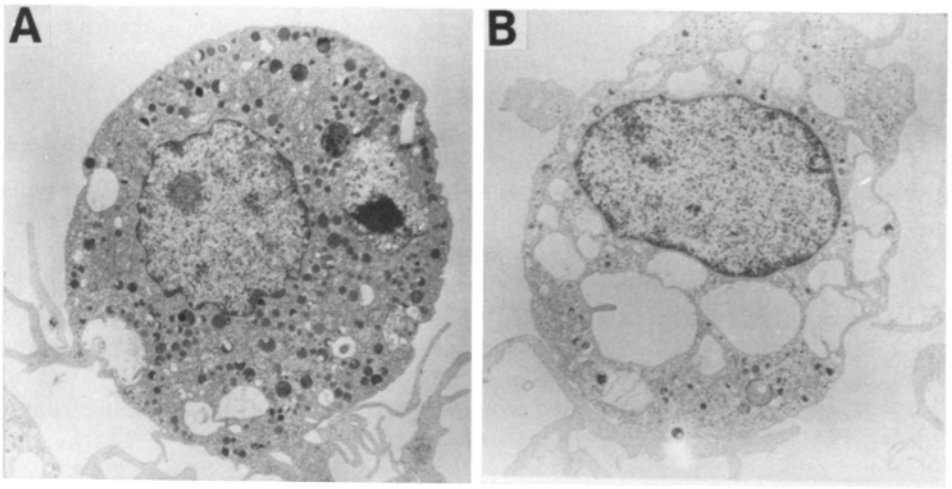

FIG. 3. Electron microscopic appearance of leukocytes cultured from donor DB. Cells from a 80-d culture were fixed, embedded, stained, and sectioned as described in Materials and Methods. A, immature monocyte; B, mature monocyte.

TABLE III

*Cytochemical Characterization of Long-Term Leukocyte Cultures Established from Three Normal EBV-Seronegative Donors (DB, MB, CB)**

Days in culture	Cytochemistry									
	Chloracetate esterase			Nonspecific esterase			Myelo-peroxidase			Acid phos-pha-tase
	DB	MB	CB	DB	MB	CB	DB	MB	CB	DB
	%	%	%	%	%	%	%	%	%	%
20	41 (20–62)		22	45 (11–80)		46	46	(7–71)	19	
35	0	11		15 (10–18)	52		11	18 (1–22)		
65	0	0	0	50 (42–60)	81	69	0	0	0	40
95	2			86 (79–92)			0			50
110	0	0	0	85 (68–98)	68	81	0	0	0	

* Cells were prepared and stained for specific enzymes as described in Materials and Methods. Percentages indicate the percent positive of at least 200 cells screened for the indicated reactions. For donor DB, the average and range (in parenthesis) of multiple (2–6) independent cultures tested at the indicated passage level is given.

teristics of these cells were further verified in assays for cytochemical, immunological, and functional markers attributable to cells of this lineage. The results given in Table III demonstrate that long-term cultures are positive for α-naphthyl acetate esterase (nonspecific esterase) and acid phosphatase and negative for chloracetate esterase and myeloperoxidase.

TABLE IV
*Immunological Characteristics of Monocyte Culture DB**

Days in culture	Rosette formation			Surface immunofluorescence			EBNA/ VCA
	E	EA	EAC	SIg	OKM-1	HLA-DR	
	%	%	%	%	%	%	%
65	0	75	12	0	92	90	0
95	0	80	20	0	99	90	0

* Cultured cells from donor DB were prepared and tested for the indicated reactivity as described in Materials and Methods. Numbers indicate the percentage of cell population giving a positive reaction (>200 cells observed).

Several surface characteristics of these cultures are summarized in Table IV. These monocytic cells form rosettes (~80%) with antibody-treated bovine erythrocytes (EA-positive) and, to a lesser extent (12–20%) with complement treated EA cells (EAC-positive). Also, murine monoclonal antisera specific for human peripheral blood monocytes and macrophages (OKM-1) and sera specific for HLA-DR structural composition both react with a high proportion (90–100%) of these cells. The nonlymphoid nature of these cells is demonstrated by their failure to form spontaneous rosettes with sheep erythrocytes and their lack of detectable sIg or antigens related to EBV (Table IV).

Induction of Functional Maturation. Functional characterization of the replicating cultures demonstrated that unstimulated cells have a limited capacity to reduce NBT (superoxide reaction) or to phagocytize iron filings or fixed *Candida albicans* (see Fig. 4, 0 μg/ml TPA). These activities were not appreciably increased by pretreatment with concentrations of TPA (100 ng/ml for 25 min) or dimethyl sulfoxide (DMSO) (1.2% for 6 d, not shown), i.e., conditions found effective for the leukemic promyelocyte cell line, HL-60 (27) and a recently established myelo-monocytic leukemia cell line, HL92 (unpublished observation). However, as illustrated in Fig. 4, when a lower TPA concentration (50 ng/ml) was used, >50% of the monocytoid cells from donor DB were induced for both superoxide production and phagocytosis. The adherent cells in these cultures were phagocytic without stimulation by TPA (data not shown).

Liberation of Biological Agents. Several of the monocyte-macrophage cultures, six from donor DB and one from MB, were sampled at various times after subculturing for several biological modifiers reportedly released by normal and leukemic monocytes and/or macrophages (28, 29), e.g., lysozyme, CSF, PGE, and LAF, and for TCGF. As shown in Table V, all cultures tested were found to release lysozyme, CSF, and PGE into culture supernatant fluids. However, no detectable levels of LAF or TCGF were found in any of the cultures tested with or without mitogen stimulation (not shown). The lysozyme liberated by these cultures were neutralized by rabbit anti-human lysozyme sera, but not by rabbit anti-rat lysozyme sera. In CSF assays, a high proportion (~50%) of the colonies formed by normal human bone marrow leukocytes in the presence of DB or MB CM consisted of esoinophils with the remainder being composed primarily of neutrophils or macrophages.

Other Characteristics. Cells at different passage levels from several of the DB and MB cultures were tested for their ability to grow as colonies in 0.33% agarose or 0.8% methylcellulose with or without addition of exogenous CSF. Colony formation occurred only in rare instances, even in the presence of exogenous CSF, using

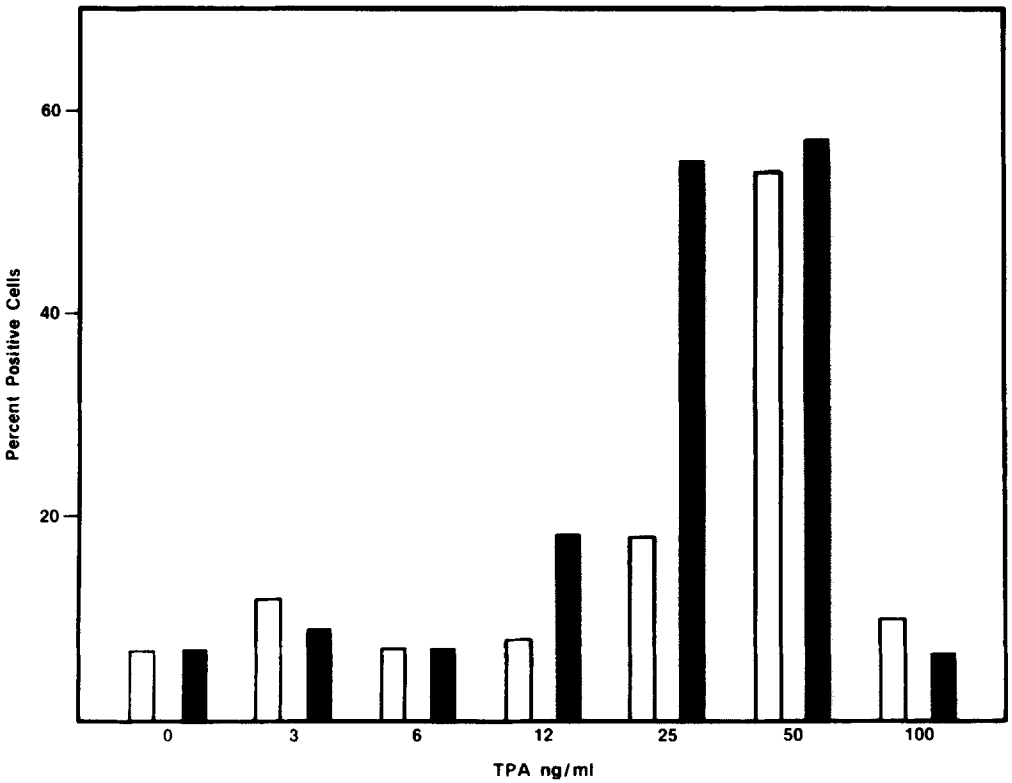

FIG. 4. The effect of TPA on the ability of monocytes from donor DB to reduce NBT and to phagocytize *Candida albicans*. Monocytes from donor DB ~80 d in culture were treated with the indicated concentrations of TPA and tested for NBT reduction (□) and phagocytosis (■) of *Candida albicans* as described in Materials and Methods.

conditions where fresh human bone marrow leukocytes form 50–100 colonies/10^5 cells in the presence of added CSF. Detailed chromosome analysis of these cultures using trypsin-Giemsa banding techniques showed that they consisted of normal diploid cells with no detectable chromosomal abnormalities (not shown).

Discussion

The conditions described here permit the culturing of monocytic cells from the peripheral blood of normal donors that are apparently in different stages of maturation, and which replicate and differentiate over a long period of time (~5 mo). Previously reported monocyte-macrophage cultures from normal donors were consistently composed of adherent, nondividing cells in the terminal stages of maturation (reviewed in 1). Leukocytes prepared from normal human EBV-seronegative donors by cell separation procedures and cultured using special growth conditions (13) developed into long-term, slowly replicating suspension cultures which exhibit many morphological, cytochemical, and biological properties, consistent with those reported for fresh normal and cultured leukemic monocytes and macrophages. In addition to morphological and ultrastructural features, other characteristics of monocytes and macrophages exhibited by these cultures included: (*a*) the presence of α-naphthyl acetate esterase (nonspecific esterase) and acid phosphatase; (*b*) reactivity with murine

TABLE V

*Biological Mediators Liberated by Normal Human Monocyte-Macrophage Cultures**

Source of supernatant fluids	Day post-subculture	Activity tested		
		CSF	PGE	Lysozme
		colonies/ 10^5 cells	*ng/ml*	*μg/ml*
DB-I	3	60		2.6
	8	24	30	1
	12			1.8
DB-II	3	40		
	8	26	49	
DB-IV	3	36		
	8	0	52	
DB-V	3	60		
	8	28	39	
DB-VII	3	52		
	8	38	42	
DB-VIII	3	38		
	8	28	45	
MB-I	3	80		3.0
	8		24	1
	12			1.8
Other cultures‡				
3A	5	60		
NuS-2	5	0	19	
HL-60	5	0	0.6	2.3
Culture media		0	0.5	1

* Samples prepared from the long-term monocyte-macrophage cultures from donors DB and MB were assayed for CSF, PGE, and lysozyme as described in Materials and Methods.

‡ 3A, human placental monolayer cells, transformed by SV-40 (22); NuS-2, monocyte cell line established from a nude mouse spleen (S. L. Salahuddin, unpublished result); HL-60, human promyelocyte cell line (9).

monoclonal antibody specific for fresh human peripheral blood monocytes (OKM-1); (*c*) the presence of receptors for the Fc portion of Ig (EA rosette-positive), and for complement (EAC rosette-positive); (*d*) release of lysozyme, CSF, and PGE; (*e*) the ability to phagocytize yeast or iron particles and to generate superoxide (NBT reduction) after induction by TPA; and (*f*) lack of lymphoid markers. Release of LAF, another macrophage product (26), and TCGF, produced by T lymphocytes (6, 7), were not detected with or without mitogen stimulation in either the immature suspension cells or in the mature adherent cell population.

It is not known why leukocytes prepared from EBV seronegative donors respond to our cell preparation and culture techniques and develop into long-term, replicating, monocytic cultures, whereas those from EBV-seropositive donors have, so far, been

negative. This observation may have relevance to an earlier report describing the growth of monocyte-macrophage cells from only 1 in 14 normal donors (3). Very little has been published regarding cellular differences between EBV-seropositive and seronegative donors. Most studies (30–32) concerning these donors have centered around possible differences in B and T lymphoid cells related to their content, expression, and susceptibility to EBV and the ability of activated cytotoxic T lymphocytes to immunologically regulate EBV-induced B lymphoblast growth. It is probable that undefined hematological differences do exist between EBV-seronegative and seropositive donors, because in our experience, sources of blood leukocytes with a hematological picture markedly different from that of normal adult blood can respond to our cell separation and culture conditions as long-term myeloid or monocyte-macrophage cultures, e.g., fetal cord blood (13) and some leukemic blood (S. Z. Salahudin, personal observation).

Careful observation of the cultures established from EBV-seronegative donors demonstrates an apparent, gradual shift in the maturity of monocytic cells, evidenced by changes in nuclear and cytoplasmic characteristics, with time in culture. This process culminates in the most mature stage, i.e., adherent, nonreplicating macrophages. The immature nature of the monocytes in suspension is also suggested by the need for TPA induction before these cells became phagocytic, possibly analogous to similar observations with some leukemic and normal cultures (33–35). The mature adherent cell population (macrophage) in these cultures is phagocytic without special treatment.

Several biological activities are liberated by the monocytic cultures, e.g., lysozyme, CSF, and PGE (Table V). It was found that CM from these cultures is able to support predominantly neutrophil and eosinophil colony growth from fresh human bone marrow leukocytes with very few monocyte-macrophage colonies being formed. This may be due to the presence of PGE which selectively inhibits in vitro monocyte-macrophage colony formation (36, 37). We have observed that maximum levels of either CSF or PGE are produced at different times after subculturing of the cultures. If the interaction of CSF and PGE, with each other and with other biological components, is critical to the regulation of granulopoiesis and cellular immunity as has been suggested (28, 36), this culture system could prove of value as a detailed study of this model.

It is possible that these long-term monocyte cultures also release an activity specifically supporting the growth of eosinophils. Other sources of CSF have been reported to produce an eosinophil-stimulating activity (37, 38) and its presene in these cultures is suggested by: (*a*) the persistence of eosinophils at low levels, even after several months in vitro (Table II); and (*b*) the high proportion (50%) of eosinophilic colonies in semi-solid medium produced by fresh bone marrow leukocytes in the presence of CM from these cultures.

Monocyte-macrophages are intricately involved in the regulation of many cellular and humoral immunological responses (29, 39), and the observations made in this report may prove valuable in the study of monocyte involvement in these processes. Relatively pure populations of replicating monocytes and macrophages at different levels of maturation can be obtained using the techniques described and can be used for monocyte-macrophage characterization, comparisons with cells from leukemic sources, and detailed studies of cellular interactions. Our experience with these

cultures also supports previous observations that self-replicative capability is not necessarily restricted to unidentifiable stem cells, but can occur under appropriate conditions in committed hematopoietic cells as well, e.g., T lymphocytes (6, 7), myeloid cells, and monocytes (13).

Summary

The long-term suspension growth of normal, immature myeloid cells from fresh human cord blood was recently reported and required cells separated on supplemented discontinuous Percoll gradients, growth in media containing hydrocortisone and vitamin D_3, and gentle, continuous agitation (13). When normal adult bone marrow (six donors) or blood from Epstein-Barr virus (EBV)-seropositive donors (nine donors) was used as a source of fresh human leukocytes, only short-term proliferation of myeloid cells was achieved with the same techniques. However, when leukocytes prepared from EBV seronegative normal adult peripheral blood were used, pure populations of monocytes and macrophages that replicate slowly in liquid suspension culture for >5 mo were repeatedly obtained from three independent donors. These cultures consists of several morphologically distinguishable monocytic cell types, including an ~20% adherent macrophage population. The monocytic nature of these cultures was confirmed by cytochemical, immunological, and functional criteria. These monocytes retain a normal chromosome pattern and can be induced to differentiate to phagocytic cells by treatment with tetradecanylphorbal acetate. Eventually, the cultures terminate as nonreplicating mature macrophages. These liquid suspension cultures should be a valuable resource for morphological, biochemical, and functional studies of developing monocyte-macrophages and their interaction with other cell types in normal and various pathological situations.

The authors wish to thank Dr. E. Osserman for assistance with lysozyme analysis, Dr. K. Smith for assistance in LAF analysis, and Dr. C. Tai, Bio-Tech Research Lab, Inc., Rockville, MD for chromosomal analysis.

Received for publication 22 January 1982 and in revised form 15 March 1982.

References

1. Zuckerman, S. H., S. K. Ackerman, and S. D. Douglas. 1979. Long-term human peripheral blood monocyte cultures: establishment, metabolism and morphology of primary human monocyte-macrophage cell cultures. *Immunology.* **38:**401.
2. Epstein, A. L., R. Levey, H. Kim, W. Henle, and H. S. Kaplan. 1978. Biology of the human malignant lymphomas. IV. Functional characterization of ten diffuse histiocytic lymphoma cell lines. *Cancer.* **42:**2379.
3. DiPersio, J. F., J. K. Brennan, M. A. Lichtman, and B. L. Speiser. 1978. Human cell lines that elaborate colony-stimulating activity for the marrow cells of man and other species. *Blood.* **51:**507.
4. Sundstrom, C., and K. Nilsson. 1976. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int. J. Cancer.* **17:**565.
5. Tsuchiya, S., M. Yamabe, Y. Yamaguchi, Y. Kobayashi, T. Konno, and K. Tada. 1980. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int. J. Cancer.* **26:**171.
6. Morgan, D. A., F. W. Ruscetti, and R. C. Gallo. 1976. Selective *in vitro* growth of T-lymphocytes from normal human bone marrow. *Science (Wash. D.C.).* **193:**1007.

7. Ruscetti, F. W., D. A. Morgan, and R. C. Gallo. 1977. Functional and morphological characterization of human T-cells continuously grown *in vitro*. *J. Immunol.* **119:**131.
8. Meir, J. W., and R. C. Gallo. 1980. Purification and some characteristics of human T-cell growth factor (TCGF) from PHA-stimulated lymphocyte conditioned media. *Proc. Natl. Acad. Sci. U. S. A.* **77:**6134.
9. Poiesz, B. J., F. W. Ruscetti, J. W. Mier, A. M. Woods, and R. C. Gallo. 1980. T-cell lines established from T-lymphocyte neoplasias by direct response to T-cell growth factor. *Proc. Natl. Acad. Sci., U. S. A.* **77:**6815.
10. Markham, P. D., F. Ruscetti, Z. Salahuddin, R. E. Gallagher, and R. C. Gallo. 1979. Enhanced induction of growth of B-lymphoblasts from fresh human blood by primate type-C retroviruses (gibbon ape leukemia virus and simian sarcoma virus). *Int. J. Cancer.* **23:**148.
11. Gallagher, R. E., F. Ruscetti, S. Collins, and R. Gallo. 1975. Growth and differentiation of human myelogenous leukemia cells in conditioned medium from human embryo cultured cells. *In* Advances in Comparative Leukemia Research. P. Bentvelzen, editor. Elsevier/ North-Holland Biomedical Press. Amsterdam. 303–306.
12. Collins, S. J., R. C. Gallo, and R. E. Gallagher. 1977. Continuous growth and differentiation of human myeloid leukemic cells in suspension culture. *Nature (Lond.).* **270:**347.
13. Salahuddin, S. Z., P. D. Markham, F. W. Ruscetti, and R. C. Gallo. 1981. Long-term suspension cultures of human cord blood myeloid cells. *Blood.* **58:**931.
14. Yam, L. T., C. Y. Li, and W. W. Crosby. 1971. Cytochemical identification of monocyte and granulocytes. *Am. J. Pathol.* **55:**283.
15. Goldberg, A. F. 1964. Acid phosphatase activity in Auer rods. *Blood.* **24:**305.
16. Kaplow, L. A. 1965. Simplified myeloperoxidase stain using benzidine dihydrochloride. *Blood* **26:**215.
17. Segal, A. W. 1974. Nitroblue-tetrazolium tests. *Lancet.* **II:**1248.
18. Lavappa, K. S. 1978. Trypsin-Giemsa binding procedures for chrormosome preparations from cultured mammalian cells. *TCA (Tissue Cult. Assoc.) Man.* **4:**761.
19. Reedman, B. M., and G. Klein. 1973. Cellular localization of an Epstein-Barr virus (EBV) associated complement-fixing antigen in producer and non-producer lymphoblastoid cell lines. *Int. J. Cancer.* **11:**499.
20. Jondal, M., and G. Klein. 1973. Surface markers on human B and T lymphocytes. II. Presence of Epstein-Barr virus receptors on B-lymphocytes. *J. Exp. Med.* **138:**1365.
21. Grumet, F. C., D. J. Charron, B. M. Fendly, R. Levy, and D. B. Ness. 1980. HLA-DR epitope region definition by use of monoclonal antibody probes. *J. Immunol.* **125:**2785.
22. Breard, J., E. I. Reinherz, P. C. Kung, G. Goldstein, and S. F. Schlossman. 1980. A monoclonal antibody reactive with human peripheral blood monocytes. *J. Immunol.* **124:**1943.
23. Ruscetti, F. W., J. Y. Chou, and R. C. Gallo. 1982. Human trophoblasts: cellular source of colony-stimulating activity in placental tissue. *Blood.* In press.
24. Levine, L., R. M. G. Cernosek, and H. Vuanakis. 1971. Specificities of Prostaglandins B_1, $F_{1\alpha}$, and $F_{2\alpha}$ antigen-antibody reactions. *J. Biol. Chem.* **246:**6782.
25. Osserman, E. F., and D. P. Lawlor. 1966. Serum and urinary lysozyme (muramidase) in monocyte and monomyelocytic leukemia. *J. Exp. Med.* **124:**921.
26. Oppenheim, J. J., S. B. Mizel, and M. S. Meltzer. 1978. Comparison of lymphocyte and mononuclear phagocyte derived mitogenic amplification factors. *In* Biology of the Lymphokines. S. Cohen, E. Pick, and J. J. Oppenheim, editors. Academic Press, Inc., New York. 291–302.
27. Collins, S. J., F. W. Ruscetti, R. E. Gallagher, and R. C. Gallo. 1979. Normal functional characteristics of cultured promyelocytic leukemia cells (HL-60) after induction of differentiation by dimethylsulfoxide. *J. Exp. Med.* **149:**969.

28. Kurland, J. J., H. E. Broxmeyer, L. M. Pelus, R. S. Bockman, and M. A. S. Moore. 1978. Role for monocyte-macrophage-derived colony stimulating factor and prostaglandin E in the positive and negative feedback control of myeloid stem cell proliferation. *Blood.* **52:**388.
29. Nathan, C. F., H. W. Murray, and Z. A. Cohn. 1980. The macrophage as an effector cell. *New Eng. J. Med.* **303:**622.
30. Gervais, F., A. Willis, M. Leyritz, A. Lebrun, and J. H. Joncas. 1981. Relative lack of Epstein-Barr virus (EBV) receptors on B cells from persistently EBV seronegative adults. *J. Immunol.* **126:**897.
31. Thorley-Lawson, N. A., L. Chess, and J. L. Strominger. 1977. Suppression of *in vitro* Epstein-Barr virus infection: A new role for adult human T-lymphocytes. *J. Exp. Med.* **146:**495.
32. Shope, T. C., and J. Kaplan. 1979. Inhibition of the *in vitro* outgrowth of Epstein-Barr virus-infected lymphocytes by T_G lymphocytes. *J. Immunol.* **123:**2150.
33. Maio, R. M., A. H. Fieldsteel, and D. W. Dodge. 1978. Opposing effects of tumor promoters on erythroid differentiation. *Nature (Lond.).* **274:**271.
34. Rovera, G., D. Santoli, and C. Damsky. 1979. Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with a phorbol diester. *Proc. Natl. Acad. Sci. U. S. A.* **76:**2779.
35. Greenberger, J. S., P. E. Newberger, and M. Sukukeeny. 1980. Phorbol myristate stimulates macrophage differentiation and replication and alters granulopoiesis and leukemogenesis in long-term bone marrow culture. *Blood.* **56:**368.
36. Pelus, L. M., H. E. Broxmeyer, J. I. Kurland, and M. A. S. Moore. 1979. Regulation of macrophage and granulocyte proliferation. *J. Exp. Med.* **150:**277.
37. Burgess, A. W., and D. Metcalf. 1980. The nature and action of granulocyte-macrophage colony stimulating factors. *Blood.* **56:**947.
38. Lusis, A. J., D. H. Quon, and D. W. Golde. 1981. Purification and characterization of a human T-lymphocyte-derived granulocyte-macrophage colony-stimulating factor. *Blood.* **57:**13.
39. Moller, G. 1978. Role of macrophages in the immune response. *Immunol. Rev.* **40:**3.