IDENTIFICATION AND CHARACTERIZATION OF THE MONOBLAST IN MONONUCLEAR PHAGOCYTE COLONIES GROWN IN VITRO*

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Mononuclear phagocytes originate in the bone marrow, where dividing promonocytes form monocytes. The monocytes leave the bone marrow and are transported via the circulation to the tissues, in which they become macrophages. This pathway is followed by the mononuclear phagocytes in the normal steady state (1, 2) as well as in acute and chronic inflammations (1, 3–10), although in some forms of chronic inflammation local proliferation may occur in the tissues (11, 12).

The promonocyte is the most immature cell of the mononuclear phagocyte system to have been fully characterized so far (2, 13, 14), but it is unlikely that this cell is the direct descendant of the stem cell. The available evidence suggests that at least one other type of cell occurs between the stem cell and the promonocyte (2, 15). It is not yet known whether this precursor of the promonocyte is also a mononuclear phagocyte. Some authors think that the mononuclear phagocytes in the bone marrow derive from immature granulocytes at some stage (16–18), but direct proof is lacking. This problem led us to investigate the origin of the bone marrow promonocytes.

Since promonocytes constitute only about 0.25% of the nucleated bone marrow cells (13), promonocyte precursors can be expected to occur in low numbers and therefore cytological preparations will not be very informative. The available methods for the study of the bone marrow monocyte and promonocyte (2, 13), which are based on the common property of mononuclear phagocytes to adhere to glass (19, 20), have not led to the identification of any type of cell preceding the promonocyte.

Another way to study immature bone marrow cells is the recently described technique by which in the presence of a colony-stimulating factor mononuclear phagocyte and granulocyte colonies are grown in vitro (21–23). This kind of culture, in which each colony develops from a single immature bone marrow cell (18, 21, 24–26), would be suitable for the investigation of the immature proliferating mononuclear phagocytes, but study of the characteristics of the cells in these colonies is hampered by the agar or methyl cellulose used as support for the cells.

For the present study, therefore, this method was modified such that leukocyte colonies develop in a liquid medium on a glass surface. The cells adhering to the glass surface (e.g., mononuclear phagocytes) are then directly accessible for observation and characterization.

The aim of the present study was to identify and characterize the promonocyte precursor in the mononuclear phagocyte colony. The findings concerning the morphology, cytochemistry, functional characteristics, and proliferative capac-

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ity of the immature and mature cells of the mononuclear phagocyte colonies are described and compared with those of the cells of the granulocyte colonies occurring in these cultures.

Materials and Methods

Animals. The study was done in specific pathogen-free male Swiss mice (Central Institute for the Breeding of Laboratory Animals TNO, Bilthoven, The Netherlands) weighing between 25 and 30 g.

Bone Marrow Cell Cultures. The femur was isolated and removed intact from the hind limb. After the bone was cleared of adherent muscle, it was cut at both ends in the region of the metaphysis. The bone marrow was expressed by flushing the shaft from the proximal side with 1 ml culture medium. The bone marrow of three mice was collected in a Falcon tube (Falcon Plastics, Div. of BioQuest, Cockeysville, Md.) and dispersed by repeated gentle aspiration in a pipette. The nucleated bone marrow cells were counted in a hemocytometer. About 5×10^4 nucleated bone marrow cells, suspended in 2 ml Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N. Y.) containing 20% horse serum (Flow Laboratories, Ayrshire, Scotland) and 20% conditioned medium, were incubated in Leighton tubes provided with a flying cover slip (10 × 35 mm) and held at 37°C in a water-saturated atmosphere of 10% CO_2 in air. The conditioned medium was prepared by incubating embryonic mouse fibroblasts with Waymouth medium (MB 752/1; GIBCO) containing 5% newborn calf serum (GIBCO). The preparation of this conditioned medium has been described in detail elsewhere.[1] Batches of conditioned medium of about the same strength (mean effective dose at a dilution of 1:70) were used in all of the experiments. With this dilution of the conditioned medium the maximal number of colonies is formed in the bone marrow cultures. Unless stated otherwise, the cultures were terminated after 4 days of incubation by gentle removal of the cover slip from the Leighton tube.

Peritoneal Cell Cultures. The technique for harvesting and culturing peritoneal cells has been described in detail elsewhere (1). About 1×10^6 peritoneal cells suspended in medium 199 (GIBCO) containing 20% newborn calf serum, were incubated in a Leighton tube with a flying cover slip for 24 h at 37°C in a humidified atmosphere of 10% CO_2 in air. After 2 and 24 h of incubation the cover slip was washed firmly with medium 199 and removed after the last washing.

Light, Phase-Contrast, and Fluorescence Microscopy. For light microscopy the cover slip was rapidly air dried, then, unless stated otherwise, fixed in absolute methanol for 10 min, and stained with Giemsa's stain for 15 min. Photographs were taken with a Zeiss photomicroscope (Carl Zeiss, Oberkochen, West Germany).

For phase-contrast microscopy the preparation was fixed in 2% glutaraldehyde in buffered saline (Hemagglutination buffer; Difco Laboratories, Detroit, Mich.) at pH 7.2 for 30 min at 4°C. Phase-contrast microscopy of the colonies during incubation in the Leighton tube (unfixed preparations) was done with an inverted phase-contrast microscope (Carl Zeiss, (27). The colonies were photographed through the Leighton tube with a Polaroid camera (Polaroid Corporation, Cambridge, Mass). Immunofluorescence was done with a fluorescence microscope (E. Leitz, Wetzler, West Germany).

Cytochemistry

PEROXIDASE. Peroxidase activity was determined according to Kaplow (28) using benzidine dihydrochloride (Fluka, A. G., Buchs, Switzerland) as substrate at pH 6.0. With 0.02% (vol/vol) hydrogen peroxide, as used in Kaplow's original method, the granulocytic cells were positive, but the mononuclear phagocytes of the colonies were usually negative. Peroxidase activity could, however, be demonstrated in the mononuclear phagocytes by using lower concentrations of hydrogen peroxide. A concentration of 0.002% (vol/vol) proved to be optimal and was used throughout this study.

ESTERASE. Esterase activity was investigated according to Ornstein and Ansley (references 29 and 30 and personal communication) the cover slip being incubated for 25 min at room temperature with α-naphthyl butyrate (to be called esterase 1) (Sigma Chemical Co., St. Louis, Mo.) at pH

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[1] Goud, Th. J. L. M., C. Schotte, and R. van Furth. Studies on the colony-stimulating activity of medium conditioned by embryonic mouse fibroblasts. Manuscript submitted for publication.

6.0 or with N-acetyl DL-alanyl 1-naphthylester (to be called esterase 2) (Fox Chemical, Los Angeles, Calif.) at pH 7.0.

Under these conditions incubation with α-naphthyl butyrate gives a distinct diffuse cytoplasmic staining of the mononuclear phagocytes; some lymphocytes may show a few inconspicuous granules; and the polymorphonuclear leukocytes are negative (29, 30). Incubation with N-acetyl DL-alanyl 1-naphthylester gives a granular staining of the polymorphonuclear leukocytes; mononuclear phagocytes are negative (31).

ACID PHOSPHATASE. Acid phosphatase activity was assayed according to Barka and Anderson (32) with sodium naphthol AS-BI phosphate (Sigma Chemical Co.) as substrate at pH 5.0, but in unfixed preparations.

β-GLUCURONIDASE. β-glucuronidase activity was investigated according to Hayashi et al. (33) with the modifications of Lorbacher et al. (34), using naphthol AS-BI β-D-glucuronic acid (Sigma Chemical Co.) as substrate at pH 5.0.

The presence of lysozyme in colony cells was demonstrated by the immunofluorescence method according to Glynn and Parkman (35), the fixed cells (fixation in 5% acetic acid in 98% ethanol for 20 min at −20°C) being incubated with rabbit antilysozyme serum (dilution 1:16) for 30 min at room temperature. After washing of the cells with phosphate-buffered saline (pH 7.8), the binding of antilysozyme to the cells was visualized by incubation with fluorescein isothiocyanate-conjugated goat antirabbit globulin (dilution 1:20) (Nordic Diagnostics, Tilburg, The Netherlands) for 30 min at room temperature, followed by washing with phosphate-buffered saline.

Antilysozyme serum was prepared in rabbits by repeated intramuscular immunization with rat lysozyme (kindly supplied by E. F. Ossermann, Columbia College of Physicians and Surgeons, New York) together with Freund's complete adjuvant (Difco Laboratories). This lysozyme preparation was free of mouse serum proteins, as determined by immunoelectrophoresis with goat antimouse plasma protein serum (Nordic Diagnostics). The prepared rabbit antilysozyme serum contained no antibodies against mouse serum proteins, and showed one precipitation line with lysozyme as antigen in the immunoelectrophoretic pattern. Furthermore, the specificity of the antilysozyme serum was demonstrated by immunofluorescence: mouse peritoneal macrophages, which contain and synthesize lysozyme (35–37), showed a diffuse positive staining, and embryonic mouse fibroblasts, which do not synthesize lysozyme (27), were negative.

In the control preparations of the cytochemical-staining reactions the substrate was omitted from the incubation fluid; for the immunofluorescence controls the incubation was performed with normal rabbit serum instead of rabbit antilysozyme serum. All controls were negative.

Function Studies

PHAGOCYTOSIS. Phagocytosis was investigated by replacing the medium with 1 ml of Dulbecco's modified Eagle's medium containing 10% newborn calf serum and 1×10^6 or 1×10^7 *Staphylococcus albus* (13). The cover slip was incubated for 1 h at 37°C and then washed thoroughly with Dulbecco's modified Eagle's medium. Phagocytosis was also studied using 5×10^8 latex particles (0.81 μm; Difco Laboratories) suspended in 1 ml Dulbecco's modified Eagle's medium containing 10% newborn calf serum, incubated for 1 h at 37°C, and then washed vigorously with Dulbecco's modified Eagle's medium. The phagocytosis of antibody-coated red cells is described below.

PINOCYTOSIS. Pinocytosis was studied by substituting the medium with 1 ml culture medium containing 10, 25, or 100 μg dextran sulphate (mol wt 500,000; Pharmacia Fine Chemicals, Inc., Uppsala, Sweden). After another 24 h of incubation at 37°C, the cover slip was washed vigorously with Dulbecco's modified Eagle's medium, fixed in absolute methanol for 10 min, and stained for 8 min with 2.5% (vol/vol) Giemsa stain in water, which stains the endocytized dextran sulphate metachromatically (38).

Receptors at the Cell Surface

IGG RECEPTORS. The presence of IgG receptors was studied with IgG-coated sheep red blood cells according to Uhr (39) with the modifications of Gordon and Cohn (40).

Equal volumes of 2% (vol/vol) sheep red cells and 10% (vol/vol) inactivated mouse antisheep red cell serum (prepared by repeated intravenous immunization of mice with sheep red cells; hemagglutination titer of the antiserum: 1:1,500) in medium 199 were added and then incubated for 30 min at 37°C, after which the coated red cells were washed twice with medium 199.

Rosette formation was studied by removing the medium from the cover slip, which was then

washed three times with medium 199 to remove traces of serum. After incubation for 15 min at room temperature in 1 ml medium 199 containing 0.2% (vol/vol) IgG-coated red cells, the cover slip was firmly washed eight times with medium 199. Phagocytosis was studied by two-step incubation: first the cover slip was incubated as described for rosette formation and then again in medium 199 containing 10% inactivated fetal bovine serum (Flow Laboratories) for 1 h at 37°C, followed by washing with medium 199.

IgM RECEPTORS. The presence of IgM receptors was studied with IgM-coated sheep red blood cells. Equal volumes of 2% (vol/vol) sheep red cells and 10% (vol/vol) rabbit antisheep red cell IgM (Cordis Laboratories, Miami, Fla.) in medium 199 were added, incubated for 15 min at 37°C, and then washed twice with medium 199. Rosette formation was studied by removing the medium from the cover slip, which was then washed three times with medium 199 and incubated with 0.25% (vol/vol) IgM-coated red cells in 1 ml of medium 199 for 1 h at 37°C after which the cover slip was firmly washed with medium 199.

COMPLEMENT RECEPTORS. The presence of complement (C) receptors was studied with sheep red blood cells coated with IgM and C. After coating of the red cells with IgM, as described above, equal volumes of 2.5% (vol/vol) coated red cells in medium 199 and fresh noninactivated mouse serum as source of C were added, incubated for 10 min at 37°C, and then washed twice with medium 199.

Rosette formation was studied according to the procedure described for IgM receptors, except that double-coated red cells were used and incubation was performed at 4°C. The procedure used to study phagocytosis was the same as that for rosette formation; the incubation was performed at 37°C.

[^{3}H]Thymidine Labeling. To study DNA synthesis, the culture medium was replaced by medium containing 0.1 μCi/ml [^{3}H]thymidine (sp act 6.7 Ci/mmol, New England Nuclear, Boston, Mass.). Autoradiography was performed with Ilford Nuclear Research Emulsion K 5 in gel form (Ilford Ltd., Essex, England) (1); the exposure time was 10 days. Cells containing less than three grains over the nucleus were considered negative.

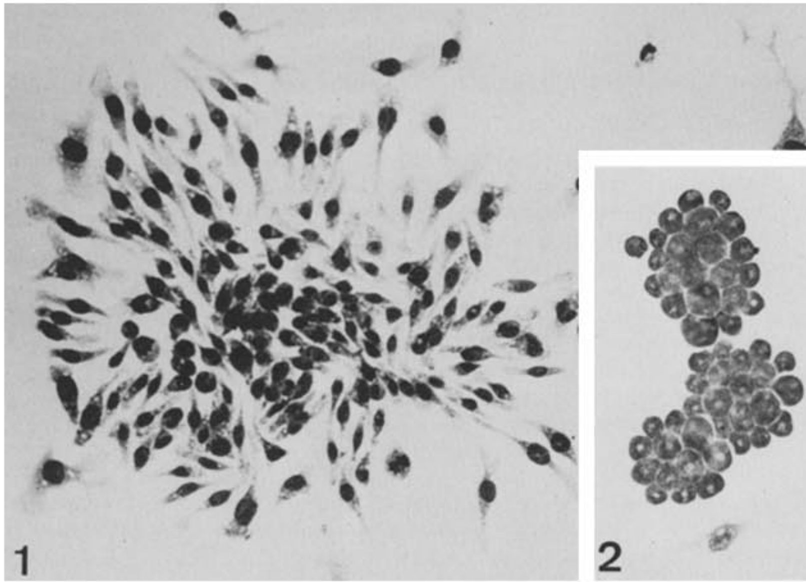
Cell Counts. In this study a group of four or more cells was considered a colony. To determine the characteristics of the component cells at least 200 cells of each cell type were examined per time point in preparations deriving from at least two experiments.

Results

Structure of the Colonies. The liquid cultures of bone marrow cells show two kinds of colonies, one consisting of granulocytic cells and the other of mononuclear phagocytes. The distinction between the cells of these two kinds of colonies is discussed below.

The structure of the granulocyte colonies is characterized by the close proximity of the cells, some of which lie on top of other cells (Fig. 2). In the mononuclear phagocyte colonies, on the contrary, the cells form a monolayer on the glass surface and show more separation; the round cells are located more centrally, and the elongated cells swarm toward the periphery (Fig. 1). Only in the later stages of incubation (after day 4) is some crowding of cells seen in the center of these colonies. The ratio of granulocyte to mononuclear phagocyte colonies is of the order of 1 to 2.

The colonies on the cover slip are well separated, provided that no more than 5 $\times 10^4$ bone marrow cells are plated per Leighton tube. All colonies are composed exclusively of either mononuclear phagocytes or granulocytic cells. Mixed colonies consisting of both types of cells were never observed. The mononuclear phagocyte colonies occasionally showed a few granulocytes, but these cells had probably been introduced during the procedure used for the termination of incubation, since comparison of the micrographs of the same colonies in the Leighton tube taken just before termination and in the Giemsa-stained prepara-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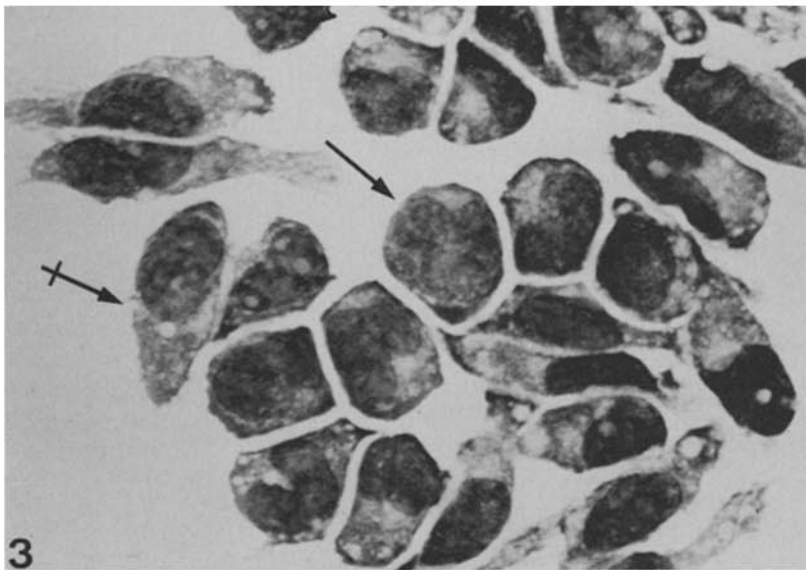
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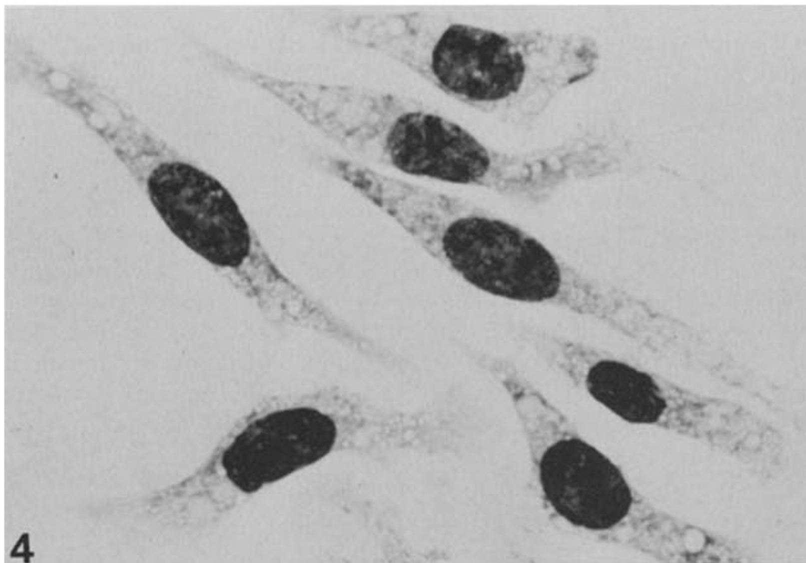
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tion, showed that these granulocytic cells were not present in the mononuclear phagocyte colonies before termination of the incubation.

Glass Adherence of the Cells of Mononuclear Phagocyte Colonies. Since the liquid culture does not contain agar or methyl cellulose as support for the cells, the question of whether the cells of the mononuclear phagocyte colonies remain stuck to the glass surface during incubation and during the termination of the culture had to be settled.

To this end, the growth of individual colonies was followed with the inverted phase-contrast microscope from the early time points up to the end of incubation. Observations were done at short (15 min) and long intervals (12 h), and some colonies were followed continuously during a period of a few hours. It was found that the mononuclear phagocytes do not leave the colony, even during divisions. Fusion of colonies was never observed, and the medium above the cover slip proved to be almost entirely devoid of cells.

To determine whether cells are lost from the colonies during the procedures applied at the termination of incubation (removal of the cover slip, air drying, and fixation) mononuclear phagocyte colonies were photographed on the cover slip in the Leighton tube just before termination and later in the Giemsa-stained preparation. Comparison of these photographs showed that the original structure of the mononuclear phagocyte colonies was preserved throughout. Cell counts of five colonies, done on these micrographs, showed that virtually no cells are lost from the mononuclear phagocyte colonies, and some colonies even showed a cell increase (Table I). Comparison of individual cells of a colony in the Giemsa-stained preparation and in the phase-contrast micrograph made it possible to localize the sites of cell increment (because of the large number of cells and crowding in the center this could not be done reliably for colonies 2 and 4). These new cells, which occurred in pairs, were small and had a loose nuclear chromatin structure, these features being characteristic for recent division (referred to in Table I as cells in postmitosis phase). These divisions had apparently occurred between the last observation in the phase-contrast microscope and the fixation of the preparation, an interval lasting on the average for 15–45 min. This finding once again demonstrates that dividing cells do not leave the mononuclear phagocyte colony, even during the disturbance accompanying termination of the incubation. Since all of the mononuclear phagocytes in the colonies remain stuck to the glass surface during incubation and the termination procedure, it may be concluded that the mononuclear phagocyte colonies in the fixed and stained preparations reliably represent the colonies as they have developed in the Leighton tube.

Morphology of the Cells of Mononuclear Phagocyte and Granulocyte Colo-

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FIG. 1. Mononuclear phagocyte colony on the 4th day of incubation. Note the loose, single-layered colony structure. Some crowding of cells is seen in the center. Giemsa stain. × 250.

FIG. 2. Granulocyte colony (broken in two parts or two colonies) on the 4th day of incubation. Note the tight structure of the colony. Most of the cells have a doughnut-shaped nucleus. Giemsa stain. × 400.

FIG. 3. Part of a mononuclear phagocyte colony showing monoblasts (↑) and promonocytes (↕). Giemsa stain. × 1,250.

FIG. 4. Part of a mononuclear phagocyte colony showing macrophages. Giemsa stain. × 1,250.

nies. When the cells of the mononuclear phagocyte colonies are characterized according to morphological criteria (cell size and shape, nuclear-to-cytoplasmic ratio, basophilia of the cytoplasm, and the number of granules and pinocytic vesicles), three types of cells can be distinguished: the macrophage and the promonocyte, already identified and characterized (13, 41), and a third, more immature cell type, which was not recognized before and will be called the monoblast (Figs. 3 and 4, and Table II). All three of these cell types occur in the majority of the 4-day old colonies.

The macrophage, which is the largest cell in these colonies, is well stretched and markedly elongated on the glass surface, usually showing two and sometimes three or four pseudopods (Fig. 4). The nuclear-to-cytoplasmic ratio is lower than 1. The light grayish-blue cytoplasm contains numerous small vesicles, and in the phase-contrast microscope a number of dense granules are seen, mainly situated around the nucleus. There are a number of mitochondria, especially in the pseudopods.

TABLE I

*Number of Cells in Mononuclear Phagocyte Colonies before and after Termination of the Culture**

Colony	No. of cells counted per colony before termination	After termination		
		No. of cells counted per colony	No. of cells in postmitosis phase‡	Corrected no. of cells per colony§
1	89	91	10	86
2	192	199		
3	88	89	2	88
4	119	114		
5	54	54	0	54

* Incubation terminated on day 4; counts made before termination in phase-contrast micrographs, after termination in Giemsa-stained preparations.
‡ Cells with characteristics indicating recent division which had apparently occurred in the interval between the phase-contrast micrograph and the fixation of the cells.
§ No. of cells per colony in Giemsa-stained preparations minus half the no. of cells in postmitosis phase.

TABLE II

Morphological Characteristics of Cells of Mononuclear Phagocyte Colonies

	Cell			Nucleus		Cytoplasm	
	Size*	Shape	Surface ruffling‡	Shape	Nuclear-to-cytoplasmic ratio	Basophilia‡	Granules and vesicles‡
	μm						
Monoblasts	10 × 12	Round	(+)	Round or indented	>1	++	(+)
Promonocytes	13 × 34	Slightly stretched	+	Round or indented	1	+	+
Macrophages	17 × 69	Extremely elongated	++	Oval or indented	<1	(+)	++

* Means of smallest and largest diameters of the cell.
‡ Increasing degree indicated in the sequence (+), +, and ++.

The promonocyte is a more immature, smaller cell and shows less stretching; this cell usually has only one pseudopod (Fig. 3). The nuclear-to-cytoplasmic ratio is about 1. Some vesicles are present in the basophilic cytoplasm, and phase-contrast microscopy shows a few dense granules situated mainly around the nucleus.

The monoblast is a round, not stretched cell, which is a little smaller than the promonocyte (Fig. 3). Its surface is slightly ruffled. The nucleus is round or slightly indented, has a fine dense chromatin structure, and is surrounded by a small rim of strongly basophilic cytoplasm sometimes showing a few vesicles. In the phase-contrast microscope only a few granules are seen. All these features indicate that the monoblast is a more immature cell type than the promonocyte (Table II).

The three types of mononuclear phagocytes can be distinguished throughout the incubation period, but the percentages of the different cell types in the colony change with time (Table III). In the first few days of incubation the mononuclear phagocyte colonies consist almost solely of monoblasts, although at this stage promonocytes and macrophages are already seen in some colonies; later on, the percentages of promonocytes and macrophages increase; and in the last part of the incubation period almost all of the cells in the mononuclear phagocyte colonies are macrophages.

In the granulocyte colonies different types of cells can be distinguished, including the immature myeloblasts, promyelocytes, and myelocytes, and more mature cells, namely stabs and neutrophil polymorphonuclears (Fig. 5). Eosinophilic and basophilic granulocytes were not seen. Typical features of the cells in these colonies are a smooth cell surface and the absence of stretching. The immature cells (9×11 μm) have a dark-blue cytoplasm and a round or doughnut-shaped nucleus. The more mature cells are smaller (7×8 μm) and have a lobulated nucleus and a light grayish-blue cytoplasm with tiny vesicles.

Cytochemical Characteristics of the Cells of Mononuclear Phagocyte and Granulocyte Colonies. The cytochemical studies showed for some enzymes very distinct differences between the cells of mononuclear phagocyte and granulocyte colonies.

Where Kaplow's (28) method is applied, the mononuclear phagocyte colony cells are negative for peroxidase and all cells of the granulocyte colonies are strongly positive. This finding is in contrast to expectation, since the majority of the bone marrow promonocytes and monocytes are known to be peroxidase positive (13, 14). Similar negative results were obtained with other cytochemical methods for demonstrating peroxidase activity (42–44). The same holds for other durations and methods of fixation (2.5% glutaraldehyde, absolute methanol, absolute ethanol, and formalin vapor), the omission of fixation, and incubation at different pH values of the substrate. Furthermore, inhibition of the reaction by penicillin (45), by the colony-stimulating factor, or by serum could also be excluded.

However, the use of a much lower concentration (0.002% vol/vol) of hydrogen peroxide than that recommended by Kaplow made it possible to demonstrate a weak granular peroxidase activity in about half of the mononuclear phagocyte colonies. In these positive colonies peroxidase-positive granules are seen in the

TABLE III
*Distribution Percentages of Cells of Mononuclear Phagocyte Colonies**

Duration of incubation	Monoblasts	Promonocytes	Macrophages
days	%	%	%
1	98	2	0
2	87	8	5
3	52	38	10
4	29	47	24
8	4	6	90

* Determined in Giemsa-stained preparations.

majority of the monoblasts and promonocytes, but in only a low percentage of the macrophages (Table IV).

With α-naphthyl butyrate as substrate (esterase 1) almost all mononuclear phagocytes are esterase positive, the diffuse cytoplasmic staining increasing in intensity from monoblast to macrophage; the cells of granulocyte colonies are negative with this substrate. However, when *N*-acetyl DL-alanyl 1-naphthylester is used as substrate (esterase 2) all mononuclear phagocytes are negative and the granulocytic cells show a strong granular staining (Table IV).

Nearly all of the mononuclear phagocytes in colonies are positive for acid phosphatase, showing a granular, mainly perinuclear, and a diffuse cytoplasmic staining which is more intense in macrophages than in the immature cells; all of the cells of the granulocyte colonies contain this enzyme too (Table IV). β-glucuronidase activity with a granular mainly perinuclear and a diffuse cytoplasmic localization occurs in almost all mononuclear phagocytes, the intensity in the immature and mature cells of the colony being about the same; all cells of the granulocyte colonies are also positive (Table IV).

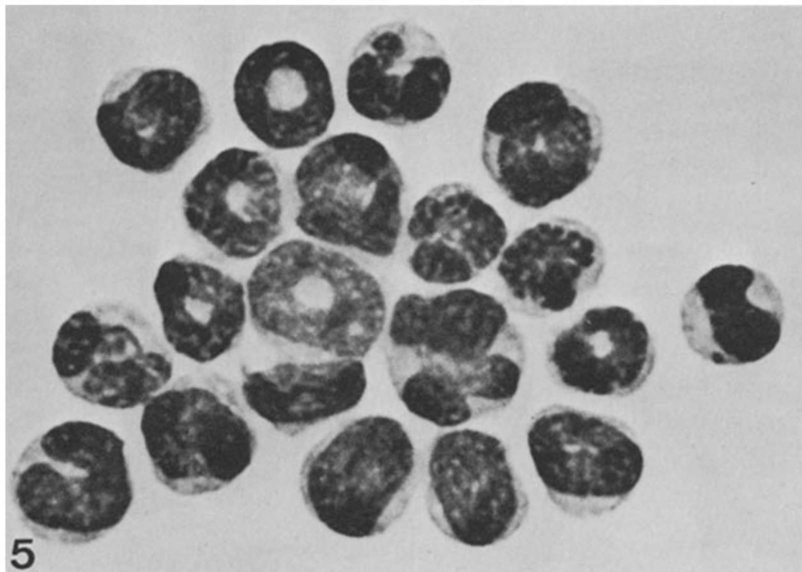
Throughout incubation (i.e., from day 2 until day 8) the percentages of monoblasts, promonocytes, and macrophages positive for esterase 1 (90–97%), acid phosphatase (89–99%), and β-glucuronidase (92–100%) remain constant, indicating that these cytochemical characteristics do not differ in cells of young and old mononuclear phagocyte colonies (Table IV). Lysozyme appears to be present in small amounts in monoblasts, promonocytes, and macrophages, the percentages of positive cells increasing in that sequence. All cells of granulocyte colonies contain a large amount of lysozyme.

For comparison, the various cytochemical reactions were also performed in peritoneal macrophages. Of these cells, 100% are positive for esterase 1, none for esterase 2, 1% for peroxidase, 100% for acid phosphatase, 100% for β-glucuronidase, and 95% for lysozyme. These results demonstrate the cytochemical similarity of macrophages in colonies and macrophages obtained directly from mice.

Functional Capacities of the Cells of Mononuclear Phagocyte and Granulocyte Colonies. The mononuclear phagocyte and granulocyte colonies also show a difference in functional capacities. Although most of the mononuclear phagocytes are able to pinocytize, the degree of activity of the immature and mature mononuclear phagocytes differs widely, the pinocytic index increasing strongly

in the sequence of monoblast, promonocyte, and macrophage. The amount of dextran sulphate endocytized by these cells also increases in the same sequence. When the colony cells are exposed to higher concentrations of dextran sulphate, the pinocytic activity of the immature mononuclear phagocytes continues to rise. The cells of granulocyte colonies do not show pinocytosis at any concentration of dextran sulphate (Table V).

The majority of the mononuclear phagocytes phagocytize *S. albus* in the



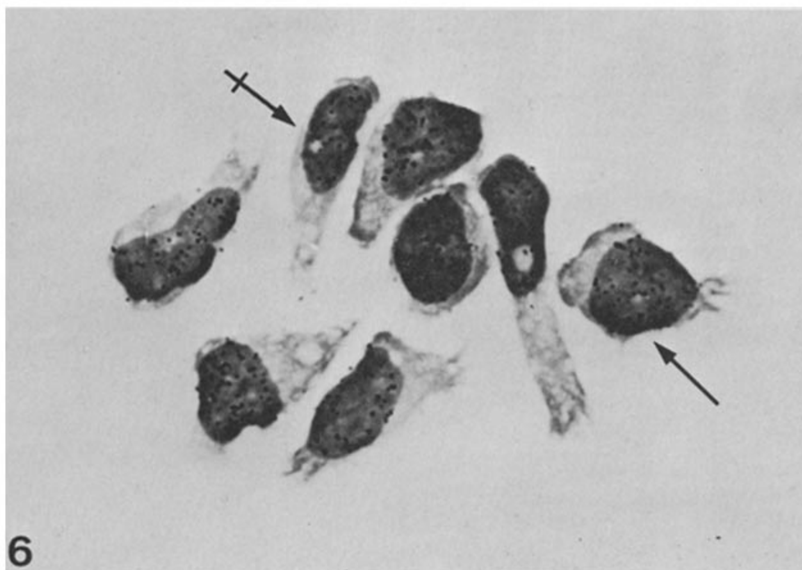


FIG. 5. Part of a granulocyte colony showing immature and mature cells. Giemsa stain. × 2,000.

FIG. 6. Part of a mononuclear phagocyte colony showing labeled monoblasts (↑) and promonocytes (‡). Giemsa stain, × 1,250.

TABLE IV

*Cytochemical Characteristics of Cells of Mononuclear Phagocyte and Granulocyte Colonies**

	Mononuclear phagocytes			Granulocytic cells
	Monoblasts	Promonocytes	Macrophages	
	%	%	%	%
Peroxidase‡	78	68	19	98
Esterase 1	91	90	93	0
Esterase 2	0	0	0	96
Acid phosphatase	89	97	99	94
β-glucuronidase	97	95	100	94
Lysozyme	43	55	78	98

* Percentage of positive cells determined on 4th day of incubation.

‡ These data pertain only to the mononuclear phagocyte colonies with peroxidase-positive cells; in 50% of the mononuclear phagocyte colonies all cells are negative. All granulocyte colonies are positive.

TABLE V

*Functional Characteristics of Cells of Mononuclear Phagocyte and Granulocyte Colonies**

	Mononuclear phagocytes			Granulocytic cells
	Monoblasts	Promonocytes	Macrophages	
	%	%	%	%
Pinocytosis				
Dextran sulphate				
10 μg/ml	13	50	92	0
25 μg/ml	15	67	99	0
100 μg/ml	21	76	97	0
Phagocytosis				
Bacteria				
1×10^6/ml	30	63	93	15
1×10^7/ml	26	64	95	36
Latex particles				
5×10^8/ml	47	88	100	10

* Percentage of positive cells determined on 4th day of incubation.

presence of serum, the phagocytic index and the number of bacteria ingested both increasing from monoblast to macrophage. The phagocytic indices of mononuclear phagocytes do not change when higher concentrations of bacteria are used (Table V). When the phagocytosis test is performed without serum, less than 1% of the monoblasts and promonocytes and less than 5% of the macrophages ingest these particles. Only a small percentage of the granulocytic cells shows phagocytosis, but the phagocytic index mounts with a higher concentration of bacteria.

When latex is used, the percentage of cells ingesting these particles is slightly

higher than for bacteria. Omission of serum in the latex phagocytosis experiments gives roughly similar phagocytic indices (Table V).

Throughout the incubation (day 2–day 8) neither the pinocytic activity of monoblasts (15–20%), promonocytes (67–71%), and macrophages (95–99%), nor the phagocytic indices for bacteria of monoblasts (20–30%), promonocytes (61–75%), and macrophages (92–99%) show any change. For comparison, the functional capacities of peritoneal macrophages were evaluated. The pinocytic activity of peritoneal macrophages is invariably high (100%) with each of the tested concentrations of dextran sulphate. The same applies to the phagocytic index of these cells for bacteria (98%) or latex particles (100%). Thus, the functional capacities of the macrophages of the colonies are similar to those of macrophages deriving directly from mice.

Receptors at the Surface of the Cells of Mononuclear Phagocyte and Granulocyte Colonies. The cells of mononuclear phagocyte and granulocyte colonies also differ with respect to the various receptors present at the cell surface. When incubated at 20°C in the presence of IgG-coated red cells almost all of the immature and mature cells of the mononuclear phagocyte colonies form rosettes comprising a few red cells around the monoblast, more red cells around the promonocyte, and numerous red cells around the macrophage. If after the rosette formation the cultures are further incubated at 37°C, the red cells are phagocytized by the mononuclear phagocytes. In the granulocyte colonies only a few of the cells form rosettes with only a few red cells, and phagocytosis of the red cells does not occur (Table VI). Cells of mononuclear phagocyte and granulocyte colonies incubated with IgM-coated red cells do not form rosettes, and phagocytosis is absent (Table VI).

A receptor for C was demonstrated on the cells of mononuclear phagocyte colonies by incubation at 4°C with IgM- and C-coated red cells. Both the percentage of positive cells and the number of red cells surrounding the mononuclear phagocytes increase markedly in the sequence of monoblast, promonocyte, and macrophage. Phagocytosis (tested at 37°C) is seen only in a small percentage of the cells. The cells of the granulocyte colonies do not form rosettes or ingest C-coated red cells (Table VI).

To permit comparison, the receptors at the surface of peritoneal macrophages were investigated. All of these cells (100%) are able to form rosettes and ingest IgG-coated red cells. With IgM-coated red cells neither rosette formation nor phagocytosis are seen. Almost all of these cells (89%) form rosettes with C-coated red cells, but ingestion is seen only in a small percentage (7%). The conclusion may be drawn that the macrophages of the colonies exhibit the same surface receptors as the macrophages obtained directly from mice. The cells of the mononuclear phagocyte colonies do not show rosette formation or phagocytosis when uncoated red cells or red cells preincubated with normal mouse serum are used instead of the antiserum.

[^{3}H]Thymidine Labeling of the Cells of Mononuclear Phagocyte Colonies. Since the colonies develop by proliferation, as shown by the observation of individual cells and colonies during incubation, it was necessary to determine which of the three cell types has the capacity to divide. Therefore, [^{3}H]thymidine was added to the cultures for a period of 8 h. Since this period is longer than the

TABLE VI

*Receptors at the Surface of Cells of Mononuclear Phagocyte and Granulocyte Colonies**

	Mononuclear phagocytes			Granulocytic cells
	Monoblasts	Promonocytes	Macrophages	
	%	%	%	%
IgG receptors				
Rosette formation	94	99	100	9
Phagocytosis	96	100	100	0
IgM receptors				
Rosette formation	0	0	0	0
Complement receptors				
Rosette formation	16	39	75	0
Phagocytosis	0	2	6	0

* Percentage of positive cells determined on 4th day of incubation.

sum of the duration of the G2, M, and G1 phases (27), all proliferating cells will have time to synthesize DNA during the incubation with [^{3}H]thymidine and thus be labeled in the autoradiographs (Fig. 6).

The results show that the labeling indices of monoblasts and promonocytes are high, indicating that almost all of these cells divide (Table VII). The labeling indices of the macrophages are low, indicating low proliferative activity; however, most probably the labeled macrophages are derived from divided labeled promonocytes.

Colony Formation by Promonocytes and Monoblasts. After the preceding experiments, which showed that both the monoblast and the promonocyte are proliferating cells, an attempt was made to determine whether these cells give rise to mononuclear phagocyte colonies.

First, promonocytes obtained directly from the bone marrow were investigated. Use was made of 6-h cultures, which permit selection of promonocytes and monocytes on the glass surface of the cover slip (13); monoblasts cannot be recognized among this population of mononuclear phagocytes. A suspension of about 1×10^6 nucleated bone marrow cells was incubated in a Leighton tube with a flying cover slip in the presence of conditioned medium. After 2 h of incubation the cover slip was vigorously washed, reincubated for 4 h, washed again, and then incubated for a period of 4 days. Only an occasional mononuclear phagocyte colony developed from the glass-adherent cells (a mean of 30 colonies per cover slip in three experiments). The nonadherent cells, obtained after the 2-h and 6-h incubations and replated in fresh Leighton tubes, gave rise to many granulocyte and mononuclear phagocyte colonies.

Since after 6 h of incubation each cover slip carried 2,000–4,000 promonocytes (2) and during the next 4 days of incubation only a small number of mononuclear phagocyte colonies (about 1 per 100 promonocytes) developed, it may be concluded that promonocytes are not capable of forming colonies. Furthermore, culture of supernates showed that the cells initiating the mononuclear phagocyte colonies adhere less firmly to the glass surface than do the promonocytes.

TABLE VII
*Labeling of Cells of Mononuclear Phagocyte Colonies**

Duration of incubation	Labeled cells		
	Monoblasts	Promonocytes	Macrophages
days	%	%	%
2	92	82	33
3	96	86	24
4	92	88	22

* Incubated for 8 h at 37°C in medium containing 0.1 μCi/ml [^{3}H]thymidine.

Next, the monoblast, the earliest and most immature cell recognized in the mononuclear phagocyte colony, was investigated. Cells of well-established colonies, immersed in Dulbecco's modified Eagle's medium, were detached from the cover slip by vigorous shaking of the Leighton tube. This was done after 4 days of incubation, because at that time all colony-forming cells are stimulated and have given rise to a progeny of at least four cells. The resulting cell suspension, studied in cytocentrifuge preparations, consists of many granulocytic cells and a number of immature mononuclear phagocytes (monoblasts and promonocytes).

When such cell suspensions are reincubated in the presence of conditioned medium in fresh Leighton tubes, numerous granulocyte and mononuclear phagocyte colonies develop. These secondary cultures did not show mixed colonies either. Since the preceding experiments showed that promonocytes do not form colonies in vitro, the most plausible conclusion seems to be that monoblasts initiated the mononuclear phagocyte colonies in the secondary cultures.

Discussion

The liquid culture technique described here is particularly suitable and reliable for the study of mononuclear phagocytes, because the cells of the mononuclear phagocyte colonies adhere to the cover slip and are therefore directly accessible for characterization. Since cells differing in maturity can be present at the same time in the same colony, this technique also provides a mean to study the immature cells of the mononuclear phagocyte cell line.

Of the three types of cells distinguished in the mononuclear phagocyte colonies, two have already been identified and characterized, namely the macrophage and the promonocyte; the third, a more immature type of cell, is here called the monoblast. Macrophages, promonocytes, and a more immature type of cell were also observed in an electron-microscopic study of mononuclear phagocyte colonies, in which occasional monocytes were also seen.[2] These three types of cells can be recognized throughout the incubation period, each exhibiting a typical and consistent set of properties, which justifies the distinction of three types of mononuclear phagocyte.

The macrophage is the most mature cell with a relatively low [^{3}H]thymidine-

[2] Fedorko, M. E., and R. van Furth. 1975. Ultrastructure of mouse mononuclear phagocytes in bone marrow colonies grown in vitro. Manuscript submitted for publication.

labeling index (22–33%). Most probably the macrophages do not synthesize DNA and labeled macrophages are cells originating from promonocytes which are labeled and have divided during the 8 h incubation with [^{3}H]thymidine. This cell is extremely elongated on the glass surface and its functional capacities are highly developed. In all of these respects and in their cytochemical characteristics the macrophages formed in mononuclear phagocyte colonies are similar to the macrophages isolated directly from animals (1, 35, 36, 41, 46–49).

The promonocyte is a less mature cell with a high labeling index after incubation with [^{3}H]thymidine (82–88%), indicating that almost all of these cells divide. The promonocyte shows less stretching than the macrophage. Its cytochemical characteristics and functional capacities are intermediate between those of the macrophage and the monoblast. Almost all of the characteristics of the promonocytes originating in mononuclear phagocyte colonies are similar to those seen in direct bone marrow preparations and 6-h cultures (2, 13, 14, and our unpublished observations). The difference in the intensity of peroxidase staining between promonocytes in colonies and in vivo might be based on scanty formation of the enzyme in vitro or continuous degranulation in the culture due to fusion of pinosomes with primary lysosomes, which would lead to fewer peroxidase-positive granules per cell. It is not yet explained why 50% of the colonies lack any demonstrable peroxidase activity. In an electron microscope study also, only a few peroxidase-positive granules were found in the promonocytes; some cells were entirely negative.[2]

The monoblast is the most immature cell seen in the mononuclear phagocyte colony. Its high labeling index with [^{3}H]thymidine (92–96%) indicates active proliferation. This round cell, which is smaller than the promonocyte, has nuclear chromatin with a fine dense structure. The almost round nucleus is surrounded by a small rim of strongly basophilic cytoplasm. Although the cell surface already shows the slight ruffling typical for mononuclear phagocytes, no pseudopods are present. The monoblast contains all of the enzymes (e.g. esterase, peroxidase, and lysozyme) shown to occur in the promonocyte and macrophage of the colony. C receptors are present in a small percentage of the monoblasts and IgG-receptors in almost all of them, but the number of receptor sites per cell is lower than in the other two cell types. The functional capacities of the monoblasts are also less developed than those of the promonocytes and macrophages. Although almost all monoblasts phagocytize antibody-coated red cells, only a small percentage phagocytizes bacteria or latex particles. Pinocytosis is also only seen in a small number of these cells, but the pinocytic index increases with increasing concentrations of dextran sulphate, which is known to stimulate pinocytosis (46).

The characteristics described above indicate a cell sequence monoblast-promonocyte-macrophage with signs of increasing maturity in this order. The distribution percentages of the three cell types during incubation are also indicative: in the first few days mononuclear phagocyte colonies consist almost entirely of monoblasts; later, promonocytes and macrophages appear; and still later, macrophages are the predominant cell type of the colonies, which shows that the monoblast is a younger cell type than the promonocyte (see Table III). This cell sequence is confirmed by the change in the cellular composition of individual colonies studied by phase-contrast microscopy: initially, the mononu-

clear phagocyte colony consists solely of monoblasts; promonocytes always appear later in the development of the colony (27). On this basis it may be concluded that the monoblast is the precursor of the promonocyte.

With respect to the question of which cell initiates the mononuclear phagocyte colony, there are three possibilities: either of the two dividing cells of the colony (the promonocyte and the monoblast) or a cell still more immature than the monoblast. It has been demonstrated that promonocytes selected in 6-h cultures do not give rise to colonies. Furthermore, transfer experiments with cells obtained from 4-day old cultures provided indications for the origin of mononuclear phagocyte colonies from monoblasts. The third possibility cannot be excluded but seems unlikely, since a cell type more immature than the monoblast—conceivably its precursor—has not been recognized at any stage of the developing mononuclear phagocyte colony. The supposition of its existence would imply a precursor of the monoblast that initiates the colony, does not multiply itself, but gives rise directly to monoblasts, which then proliferate for a number of cell generations (27). On the basis of these considerations, although definite proof is still lacking, we tend to conclude that the monoblast initiates the mononuclear phagocyte colony.

Cline and Sumner have described a blast cell preceding the promonocyte (50). This blast appears to be quite a different type of cell from the monoblast described here, since it is strongly peroxidase positive, lacks IgG receptors at the cell surface, and is unable to phagocytize, properties which our studies show to be characteristic for the cells of granulocyte colonies. However, these authors aspirated the cells from a suspension culture, which means that the kind of colony to which these blasts originally belonged is unknown. This uncertainty is avoided by the present technique, which provides a means to study the monoblast in the intact colony made up of immature and mature cells.

Besides the mononuclear phagocyte colonies also colonies of granulocytic cells are formed in these liquid cultures. A characteristic feature of these colonies is the tight structure. In addition to the typical morphology of the granulocytic cells, they are strongly positive for peroxidase and positive for esterase with *N*-acetyl DL-alanyl α-naphthylester as substrate. However, they are unable to pinocytize dextran sulphate and only a minority phagocytizes bacteria, latex particles, or antibody-coated red cells. IgG and C receptors could not be demonstrated at the surface of granulocytic cells, which are known to have a variable reactivity to antibody-coated red cells (51). On the basis of these criteria it is easy to distinguish between granulocyte and mononuclear phagocyte colonies. Mixed colonies consisting of both mononuclear phagocytes and granulocytic cells were never observed, nor colonies with transitional cell forms showing characteristics of both cell lines as described by Metcalf (52). This means that in the liquid culture system the colony-forming cell is already committed to form either granulocytic cells or mononuclear phagocytes. No evidence was found indicating the existence of a common progenitor of granulocytes and mononuclear phagocytes, which some authors suggest to be the myeloblast or the (pro)myelocyte (16–18). In their studies, however, both kinds of cell were distinguished mainly on the basis of morphological criteria.

From the results of the present study the conclusion may be drawn that the most immature cells of the mononuclear phagocyte and granulocyte cell lines

thus far identified, namely the monoblast and the myeloblast, have quite different characteristics. No evidence was found that mononuclear phagocytes derive at any stage from granulocytic cells.

Summary

A liquid culture technique for growing mononuclear phagocyte colonies on a glass surface is described. This useful and reliable technique made it possible to study immature mononuclear phagocytes.

In the mononuclear phagocyte colonies the cells grow separate from each other in a single layer. Three types of cells are recognized in these colonies, namely nondividing macrophages, and proliferating promonocytes and monoblasts. The macrophage and the promonocyte exhibit the typical characteristics previously demonstrated by the other methods, whereas the monoblast could only be fully characterized with the present liquid culture method. This proliferating cell (labeling index with [^{3}H]thymidine, 92–96%) is almost round (diameters, 10 × 12 μm), has only a small rim of strongly basophilic cytoplasm, almost devoid of granules, and shows a certain degree of ruffling of the cell surface. The monoblast is positive for esterase with α-naphthyl butyrate as substrate (91%), for peroxidase (78% in the peroxidase-positive colonies), and lysozyme (43%). The monoblast is able to pinocytize dextran sulphate (15–20%) and to phagocytize opsonized bacteria (20–30%), latex particles (47%), and IgG-coated red cells (96%). IgG receptors (94%) and complement receptors (16%) are present at the cell surface. In these respects the monoblast has the typical characteristics of the mononuclear phagocytes, but its properties show it to be a more immature cell type than the promonocyte. On the basis of these criteria and the sequence of appearance of the different cell types during incubation and during the development of the individual mononuclear phagocyte colony, monoblasts being present before promonocytes appear in the colony, it is concluded that the monoblast is the precursor of the promonocyte.

In these cultures granulocyte colonies are also formed, consisting of myeloblasts, (pro)myelocytes, stabs, and polymorphonuclear neutrophils. Besides the typically tight structure of this kind of colony, the granulocytic cells themselves are quite distinct from the mononuclear phagocytes by their morphology, cytochemical characteristics (e.g. all negative for esterase with α-naphthyl butyrate, but 96% positive with *N*-acetyl DL-alanyl 1-naphthylester), functional characteristics (pinocytic index 13–21%; phagocytic index; for opsonized bacteria 15–36%, for latex particles 10%, and for IgG-coated red cells 0%), and their very small number of IgG receptors and lack of complement receptors. On the basis of these criteria, these granulocytic cells are easily distinguished from the immature cells of the mononuclear phagocyte colonies.

The present study confirms the conclusion that the mononuclear phagocytes are a separate cell line, quite distinct from the granulocytic series, since even the most immature cells so far identified—the monoblast and the myeloblast—have quite different characteristics.

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