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Culture of Human Bone Marrow in the Teflon Culture Bag: Identification of the Human Monoblast

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The proliferation of human bone marrow cells was studied in a liquid culture system without colony-stimulating factor. Bone marrow cells suspended in medium containing horse serum and fetal calf serum were incubated in the Teflon culture bag. During the first week there was an increase in the number of blast cells and early cells of the granulocytic series, both of which showed a high ^3H -thymidine labeling index. The total number of mononuclear phagocytes increased during the first two weeks of culture. A number of characteristics of the cultured cells (α -naphthyl butyrate esterase, N-acetyl-DL-alanyl α -naphthyl esterase, Fc receptors, and phagocytosis) were determined. It was not feasible to recognize promonocytes and monoblasts with light microscopy, but with electron microscopy and the use of peroxidatic activity as marker, monoblasts and promonocytes were identified. The monoblast is a round cell with a few surface microextensions, a large nucleus, and few cytoplasmic granules. The nuclear envelope, the rough endoplasmic reticulum, and the granules show peroxidatic activity.

Key words: human bone marrow, bone marrow culture, monoblast, promonocyte, macrophage, granulocyte, peroxidase, Teflon culture bag

INTRODUCTION

For the culture of human bone marrow cells, most investigators use semisolid media, eg, agar and methylcellulose, for cell support; these studies have provided much information on the clonal growth of these cells [24]. However, when cells are grown in semisolid medium it is not easy to study morphological, cytochemical, and functional characteristics of the cells, and it is also difficult to obtain quantitative data on the cultured

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cells. A liquid culture technique, developed in our laboratory eliminated these problems for murine bone marrow, and the monoblast of the mouse could be identified and characterized with this technique [11,12].

This liquid culture system, in which the cells attach to a glass surface and the precursor cells of both the mononuclear phagocyte and granulocyte series proliferate, proved unsatisfactory for human bone marrow cells, because the human precursor cells adhere less well to a glass surface. The embryonic mouse fibroblast-conditioned medium, the source of the colony-stimulating factor used in the mouse system [14], was not very active for the human system (unpublished observations), and purified colony-stimulating factor did not produce a distinct effect on the proliferation of human marrow cells in a liquid culture system [13]. For rabbit bone marrow a culture method has been described in which the cells are incubated in a liquid medium containing 50% serum without conditioned medium [16], and we felt it worthwhile to assess these culture conditions for human bone marrow cells.

Recently, the results of the culture of murine bone marrow cells in the teflon film dish and the teflon culture bag were reported [19,20]. When cultured on a teflon membrane, even mature mononuclear phagocytes adhere very poorly, and a high percentage of the cells can easily be recovered for further study.

Compared with mononuclear phagocytes cultured on a glass surface, murine bone marrow mononuclear phagocytes cultured on a teflon surface show the same rate of proliferation and similar characteristics. An additional advantage of culture in the closed teflon culture bags (TCBs) is that the risk of contamination is minimized [20]. Encouraged by the good results obtained with long-term TCB culture of human monocytes [21] and of cells of patients with monocytic leukaemia (unpublished), we wished to know whether this system could be used for the culture of human bone marrow in order to identify the immature cells of the mononuclear phagocyte series. The present study concerns quantitative data on proliferation and the characteristics of cultured human mononuclear phagocytes.

MATERIALS AND METHODS

Bone Marrow Cultures

Aspiration samples of human bone marrow obtained from patients with a non-haematological disease and healthy volunteers, were collected in 80 U heparin. The cells were suspended in Medium 199 (Microbiological Associates, Walkersville, MD), as described earlier [12] and centrifuged on a Ficoll-Isopaque gradient (12 parts Ficoll, Pharmacia, Uppsala, Sweden, and 5 parts metrizoate Isopaque, Nyegaard and Co., Oslo, Norway) [5]. The Ficoll-Isopaque mixture was filtered (0.45 μ m millipore filter) and 4-ml aliquots were brought into sterile glass tubes. Next, 5-ml aliquots of the bone marrow suspension were carefully layered on the Ficoll-Isopaque and the tubes were spun at 420g for 20 min at room temperature. The clear upper layer was discarded to within 3 mm of the next cloudy layer; and this interphase layer was suspended in Medium 199, spun at 110g for 10 min, and washed three times. The cells were then resuspended in culture medium consisting of alpha modified Eagle's medium (Flow Laboratories, Irvine, Scotland) containing 15% (v/v) fetal calf serum (Flow Laboratories) and 35% (v/v) horse serum (Flow Laboratories). Both sera had been inactivated by incubation at 56°C for 30 min. Cell suspensions with a concentration of approximately 1×10^6 /ml were brought into Teflon culture bags prepared as described in detail elsewhere [20]; the bags were closed by a diathermic seal and incubated in a water-saturated atmosphere with 10% CO_2 in air at 37°C.

After incubation for a various number of days, the cells were harvested from the TCBs as described elsewhere [20], counted in a haemocytometer, and tested for viability by trypan-blue exclusion (2%) or sedimented on glass coverslips by centrifugation at 110g for 10 min and rapidly air-dried prior to fixation and staining.

Giemsa and May Grünwald Giemsa Staining

The cells on the coverslips were usually stained with May Grünwald Giemsa (Merck, Darmstadt, W. Germany), but for some experiments the coverslips were fixed for 20 min in absolute methanol and stained with a 1:30 dilution of Giemsa stain (Merck) in distilled water for 7 min.

In Vitro Labeling with ^3H -thymidine

For in vitro labeling, 0.1 $\mu\text{Ci/ml}$ ^3H -thymidine (specific activity 6.7 Ci/mmol; New England Nuclear Corp., Boston MA) was injected into the TCB just before the last 7.5 hr of culture. At the end of the culture period, cytocentrifuge preparations were made as described above. These preparations were carefully washed, fixed, dried, and prepared for autoradiography as described elsewhere [8]. Since the background radioactivity did not exceed five grains per cell area, all cells with more than five grains overlying the nucleus were considered positive.

Peroxidatic Activity

At the light-microscopic level, peroxidatic activity was studied according to Kaplow [17] in air-dried preparations fixed in 37% formaldehyde in absolute ethanol as described elsewhere [12]. Peroxidatic activity was also studied at the ultrastructural level (see Ultrastructural Studies below).

Esterase Activity

Esterase activity was studied with two substrates: that of mononuclear phagocytes (esterase-1) with an α -naphthyl butyrate (Sigma Chemical Co., St. Louis, MO) according to Ornstein et al [23], and the granulocyte esterase (esterase-2) with N-acetyl-DL-alanyl α -naphthyl ester (Fox Chemical, Los Angeles, CA) [23] as already described [12]. For these studies the cells were fixed in formaldehyde vapor.

Phagocytosis

To assess phagocytic activity, cells harvested from a TCB were counted and re-incubated together with *Staphylococcus aureus* 42D (bacteria-to-cell ratio 100:1) for 1 hr in culture medium containing 10% fresh human AB serum in siliconized glass tubes under rotation (4 rev/min) at 37°C, after which lysostaphin (Sigma Chemical Co., St. Louis, MO) was added to lyse the extracellular bacteria [9].

Fc Receptors

The presence of Fc receptors was detected with IgG-coated sheep red blood cells. The red blood cells were washed, and a 5% (v/v) suspension in buffered saline was made; 0.2 ml of this suspension was added to 0.05 ml heat-inactivated rabbit anti-sheep red cell serum (haemagglutination titre 1:1500) and the volume made up to 1 ml with medium. This suspension was incubated for 30 min at 37°C, after which the red cells were washed twice in buffered saline and resuspended in medium to a final concentration of 0.2% (v/v, 8×10^7 cells/ml). One ml of the suspension of opsonized red cells was then added to cells on coverslips, and incubated for 1 hr at 37°C to allow attachment and phagocytosis. The cells were then fixed in methanol and stained with Giemsa stain.

For the preparations used to establish the phagocytic index, the extracellular red cells were lysed osmotically with hypotonic ammonium chloride prior to fixation.

Ultrastructural Studies

After recovery from the TCB, the cells were fixed for 1 hr in 1.5% glutaraldehyde at 4°C and postfixed in OsO₄ for 30 min at 4°C as described elsewhere [3]. After complete dehydration in a graded series of alcohol, the cells were rinsed with propylene oxide, transferred to an Eppendorf tube, and concentrated to small pellets which were embedded in araldite. Ultrathin sections were cut on a Reichert Ultramicrotome (Vienna, Austria), stained with lead citrate and uranyl acetate on a grid, and examined with a Philips EM 301 at 40 KV.

For the ultrastructural studies on peroxidatic activity, the cells were fixed for 10 min in 1.5% glutaraldehyde at 4°C, washed three times in 0.1 M Na-cacodylate buffer (pH 7.4), preincubated in diaminobenzidine at pH 6.5, and next incubated in diaminobenzidine with 0.01% H₂O₂ for 1 hr at 20°C, as described in detail elsewhere [3]. The cells were then washed, postfixed in OsO₄, and further processed for electron microscopy as described above except that staining was omitted.

RESULTS

Culture Characteristics

Cells of liquid cultures in a teflon bag usually grow in suspension and do not adhere to the teflon surface; no colonies are observed under the inverted microscope. After recovery of the cell suspension from the TCB, only a few cells remain in the bag.

The numbers of nucleated cells in these liquid cultures of seven samples of human bone marrow are given in Figure 1, where each point represents the mean value for at least two TCBs of the same bone marrow sample counted in duplicate. Although there

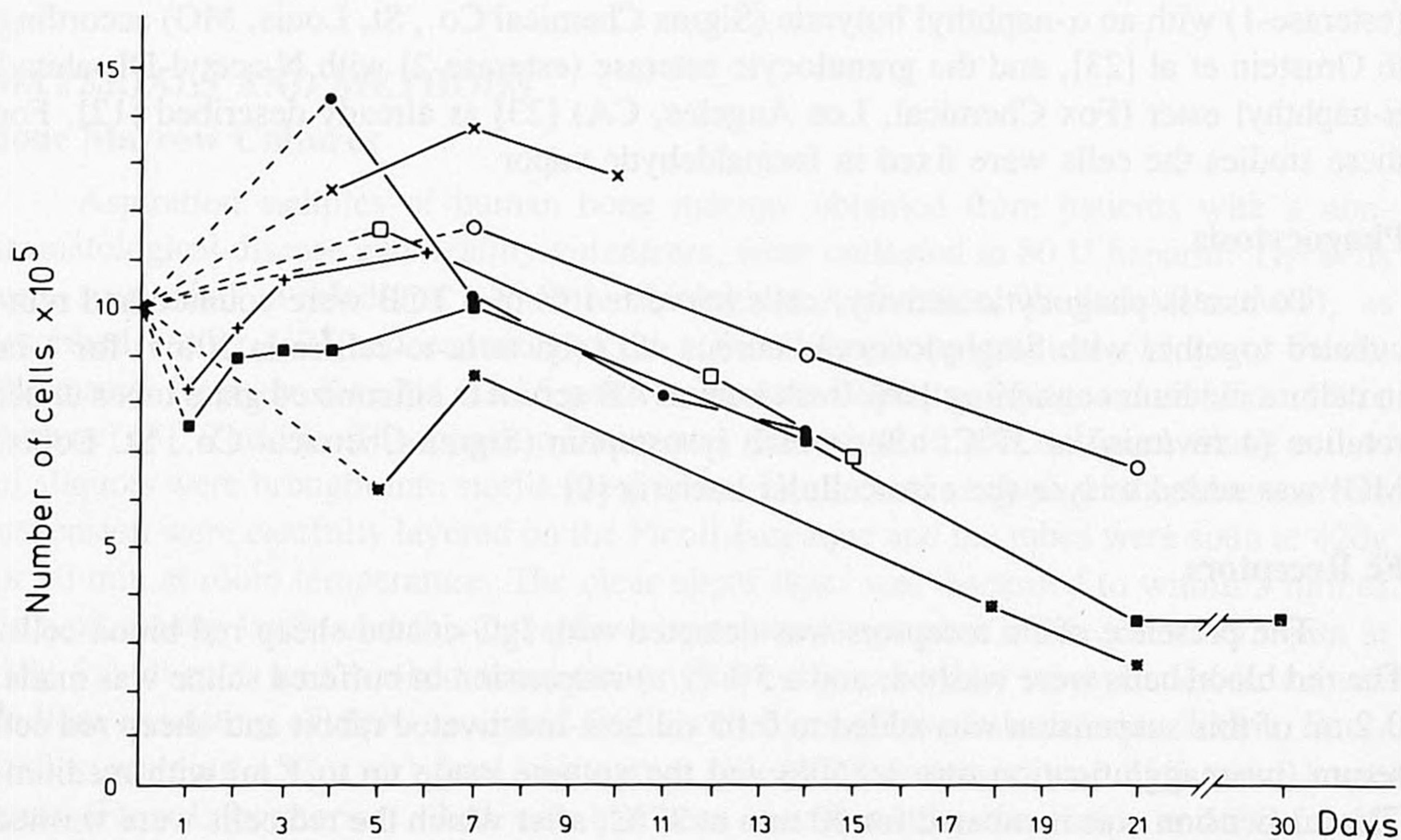


Fig. 1. Numbers of viable nucleated cells in liquid cultures of human bone marrow. Each curve represents the course of a bone marrow sample cultured at least in duplicate. The curves are standardized for an initial number of 10⁶ nucleated bone marrow cells.

is considerable variability between experiments, on average the total number of viable cells remains approximately constant over the first week of culture. On average, 14.6% of the cells are no longer viable at the various time points, as judged by trypan-blue exclusion. In the second and third weeks of culture the number of viable cells declines. Living cells have been maintained for more than 30 days.

Differentiation of the Cultured Cells

With the exception of megakaryopoiesis, all elements of normal haematopoiesis can be identified in May Grünwald Giemsa-stained preparations of these cultures. Although the relative numbers of the various cell types vary considerably between experiments, the sequential pattern is roughly the same; this is exemplified by the experiment shown in Figure 2. During the first week granulocytes and their precursors predominate in the cultures. Although the numbers of promyelocytes, myelocytes, metamyelocytes, band forms, and mature granulocytes decrease after the first week of culture, these cell types remain present up to day 30. The morphology of these cells is shown in Figure 3.

A relatively large number of "blast cells" is seen in these cultures (Fig. 3). With light microscopy we were unable to determine to which lineage these precursor cells belong. After about 4 days of culture their number decreases, but these cells are seen throughout the culture period. Less than 1% of all cells in culture are in mitosis, and mitoses are seen throughout the culture period.

Precursors of the mononuclear phagocyte series (monoblasts and promonocytes) cannot be recognized light-microscopically with certainty; a small number of cells (1–4%) with the morphology of monocytes are always present in the cultures (Fig. 3). After the third day of culture the first macrophages appear (Fig. 3); their number increases gradually over the next few days, and after about 14 days of culture the macrophage becomes the predominant cell type. Giant cells are only observed occasionally in these long-term cultures.

Eosinophils are also present in the cultures; although their number varies, the proportion does not exceed 2%. Lymphocytes also remain present in the cultures. During the first two weeks of culture their relative number is of the order of 10%; after that it drops below 3%. In the later days of culture, typical plasma cells are seen occasionally. Erythroid elements (erythroblasts and normoblasts) are encountered as a minor population (less than 2%) in the early days of culture, and are not seen after the first week of culture.

³H-thymidine Labeling

The variation in the numbers of cells of the various types between experiments is reflected by the ³H-thymidine labeling indices. Table 1 gives the labeling indices of the precursor cells (unidentified precursor cells, promyelocytes, myelocytes, and immature mononuclear phagocytes) of the experiment shown in Figure 2. Band forms, mature granulocytes, monocytes, and macrophages as well as lymphocytes are not labeled.

Cytochemical Characteristics of the Cells in Culture

The peroxidatic activity at the light-microscopic level of a bone marrow culture maintained for up to six days is given in Table 2. For comparison, the percentages of cells of the granulocytic series and of the monocytes, which could be expected to be peroxidase positive, as recognized in May Grünwald Giemsa preparations are also given. A large majority of peroxidase-positive cells are identifiable as granulocytes and their precursors. Some cells can be identified as peroxidase-positive monocytes. However, the nature of all peroxidase-positive cells cannot be determined. Some of the blast cells are

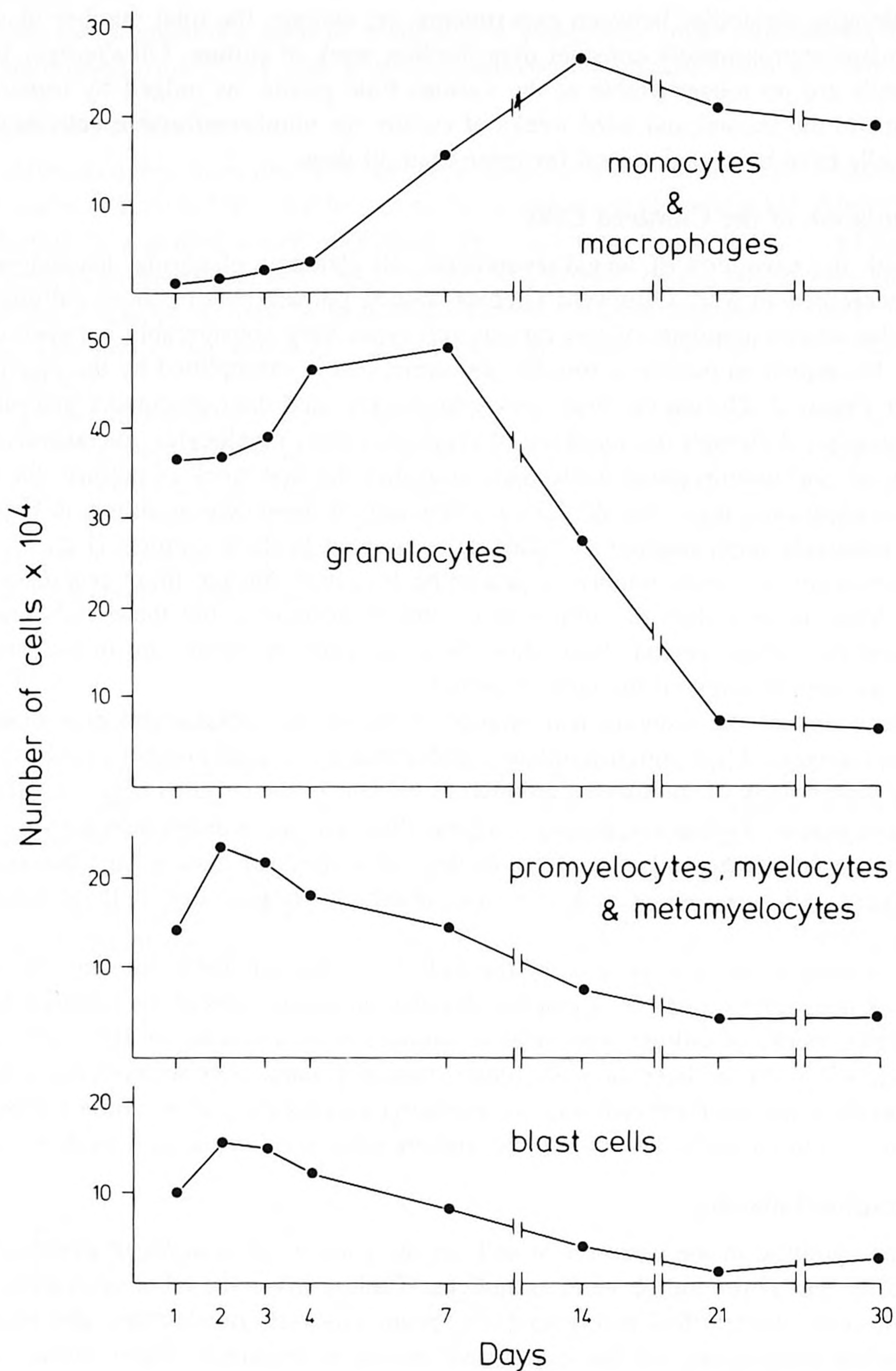


Fig. 2. Number of cells of the various types determined by lightmicroscopy of human bone marrow. The curves are standardized for an initial number of 10^6 nucleated bone marrow cells.

probably peroxidase-positive too, and among them promonocytes and monoblasts are presumably present (see Ultrastructure, below). Macrophages are peroxidase-negative. The percentage of peroxidase-positive cells agrees well with the percentage of cells of the granulocytic series and monocytes, which are expected to be peroxidase-positive.

The percentages of cells with activity for either esterase-1 or esterase-2 are given in Table 3. Most cells were so intensely stained that the stage of development could not

TABLE 1. ³H-Thymidine Labeling Index of Precursor Cells*

| Day of culture | Labeling index % |
|----------------|------------------|
| 1 | 39.9 |
| 2 | 46.4 |
| 3 | 51.4 |
| 4 | 49.9 |
| 7 | 35.2 |
| 14 | 28.3 |
| 21 | 39.5 |

*Reincubated for 7.5 hr in medium containing 0.1 μ Ci/ml ³H-thymidine. Precursor cells include cells of the granulocyte series (myeloblasts, promyelocytes, and myelocytes) and immature mononuclear phagocytes (monoblasts and promonocytes) of the experiment shown in Figure 2.

TABLE 2. Peroxidase Activity* and May Grünwald Giemsa-Staining Patterns in Cultured Human Bone Marrow Cells

| Day | Peroxidase-positive cells (%) | May Grünwald Giemsa staining ^a | |
|-----|-------------------------------|---|---------------|
| | | promyelocytes, myelocytes, metamyelocytes, granulocytes (%) | monocytes (%) |
| 1 | 72.0 | 73.8 | 2.4 |
| 2 | 80.0 | 77.5 | 3.0 |
| 3 | 90.3 | 79.9 | 3.7 |
| 6 | 88.4 | 80.0 | 2.4 |

*At the light-microscopic level, stained according to Kaplow [17].

^aExpressed as percentage of all cells present in the culture.

TABLE 3. Esterase Activity and May Grünwald Giemsa-Staining Patterns in Cultural Human Bone Marrow Cells

| Day | All cells positive for esterase-2 ^a (%) | Promyelocytes, myelocytes, metamyelocytes, and granulocytes ^b (%) | All cells positive for esterase-1 ^c (%) | mononuclear phagocytes ^b (%) |
|-----|--|--|--|---|
| 1 | 69.3 | 66.7 | 5.0 | 0.8 |
| 2 | 74.4 | 65.2 | 7.9 | 1.6 |
| 3 | 79.3 | 65.5 | 6.9 | 2.5 |
| 4 | 79.9 | 70.7 | 8.9 | 3.4 |
| 7 | 53.3 | 61.3 | 23.0 | 15.4 |
| 14 | 44.8 | 45.9 | 32.7 | 35.2 |
| 21 | 48.1 | 30.8 | 54.1 | 58.1 |

^aSubstrate: N-acetyl-DL-alanyl α -naphthylester. Ornstein et al [23].

^bIdentified in May Grünwald Giemsa-stained preparations.

^cSubstrate: α -naphthyl butyrate. Ornstein et al [23].

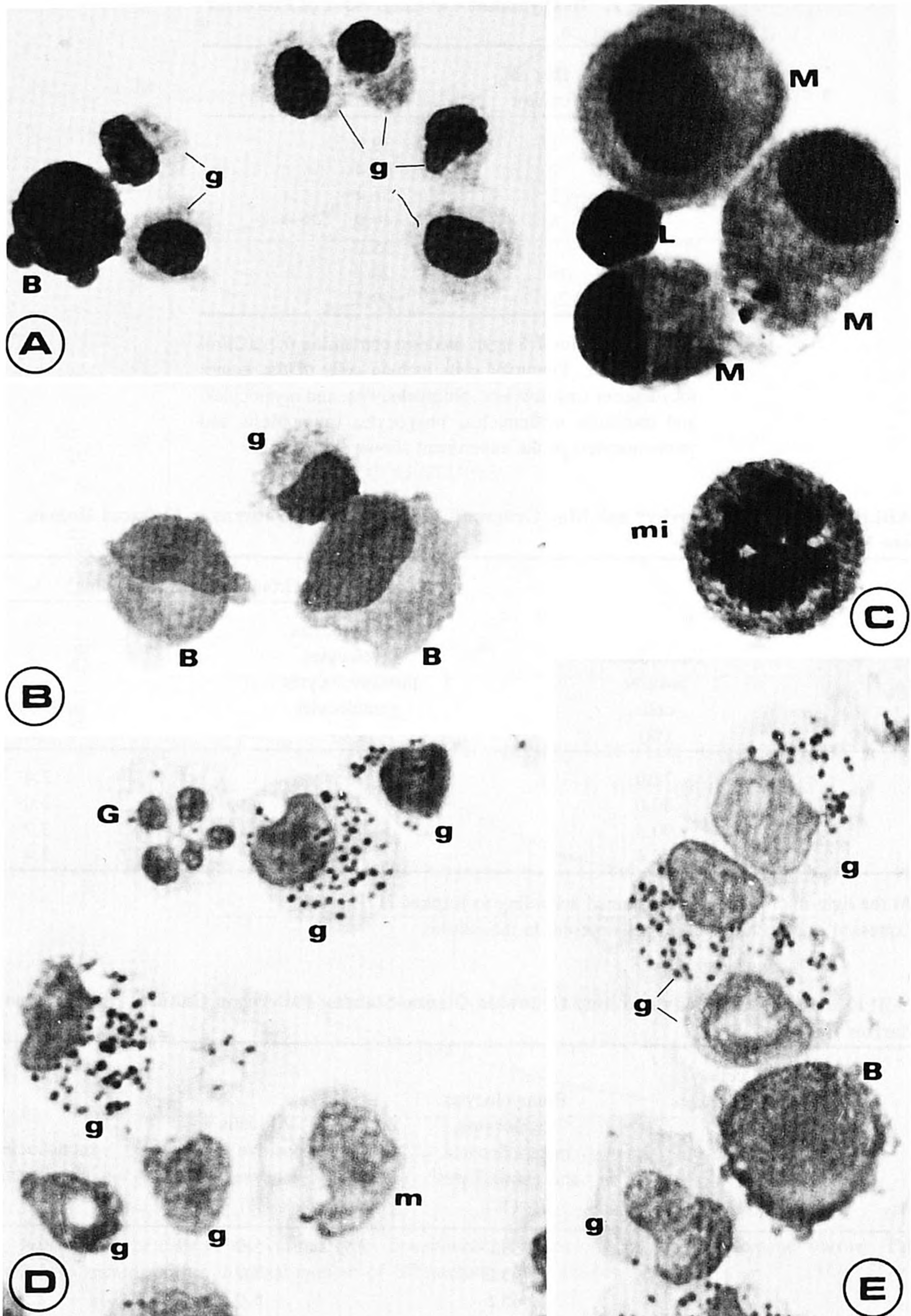


Fig. 3. The morphology of cultured human bone marrow cells. The cells were sedimented on glass coverslips and were stained with May Grünwald Giemsa. A) A blast cell (B) and a number of immature granulocytes (metamyelocytes/band forms (g) on day 3 of culture. $\times 950$. B) Two blast cells (B) and a band form (g) on day 6. $\times 1100$. C) Three macrophages (M), a lymphocyte (L), and a cell in mitosis (mi) on day 4. $\times 1400$. D,E) Phagocytosis of opsonized staphylococci on day 14. A mature granulocyte (G), some immature granulocytes (metamyelocytes/band forms (g), and a monocyte (m) have ingested bacteria. The blast cell (B) has not ingested bacteria. $\times 400$.

be determined. The intensity of the esterase staining (especially esterase-1) increased strongly during culture. The relative number of granulocytes with their precursors and of mononuclear phagocytes as identified with May Grünwald Giemsa staining are also given in Table 3. In the early days of culture the percentage of esterase-1-positive cells is higher than the percentage of cells identified as mononuclear phagocytes in Giemsa-stained preparations.

Since the number of esterase-1-negative mature macrophages is negligible in the early days of culture, a very rough estimate of the proportion of esterase-1-positive precursor cells (percentage of esterase-1-positive cells minus percentage of mononuclear phagocytes) can be obtained. This percentage averages 5.1 (range 4.2–6.3%) during the first four days of culture. For the granulocytic series a similar calculation can be made; on average, the percentage of esterase-2-positive precursor cells (percentage of esterase-2-positive cells minus cells of granulocytic series) amounts to 9.0 (range 2.6–14.8%). During the later days of culture, this calculation cannot be made, because esterase-1-negative mature macrophages and esterase-2-negative granulocytes are present by then.

Fc Receptors and Phagocytosis

When tested on day 8 of culture, 93.5% of the macrophages and 87% of the granulocytic cells proved to bear Fc receptors. Eighty-four percent of the macrophages with receptors and 97% of the granulocytes with receptors were found to ingest erythrocytes.

Phagocytosis of *Staphylococcus aureus* is shown in Figure 3, D,E. A high percentage of the band forms (on average 80.4%), mature granulocytes (on average 84.9%), monocytes and macrophages (on average 96.2%) phagocytosed bacteria throughout the culture period indicating that the cells are in good functional state. Sporadically blast cells that had ingested a few bacteria were seen. Whether these were mononuclear phagocyte precursors could not be established.

Ultrastructural Studies

Since the precursor cells of the mononuclear phagocyte series cannot be identified by light-microscopic techniques, electron microscopy—including cytochemical staining for peroxidatic activity—was applied. Monoblasts and promonocytes were recognized in cultures up to day 30 (Figs. 4A, 5A). Their morphology and peroxidatic-activity patterns were identical to those in the mouse. These cells could easily be distinguished from myeloblasts (Fig. 4B) and promyelocytes (Fig. 5B) [2,4,6], and a description of the characteristics of the cells is given in the legends to the figures. More mature macrophages were encountered resembling either monocytes (Fig. 6) or peroxidase-negative mature macrophages (Fig. 7). No transitional macrophages (macrophages with peroxidatic activity in both granules and rough endoplasmic reticulum and nuclear envelope) or resident macrophages (with peroxidatic activity only in rough endoplasmic reticulum and nuclear envelope) were observed.

DISCUSSION

The present paper describes a method for the culture of human bone marrow cells in liquid medium. The cells are incubated in culture medium containing high concentrations of heterologous sera, and no source of colony stimulating factor is added. Compared with bone marrow culture techniques using semisolid media, where only colonies can be counted, our method has the advantage that reliable quantitative data on the numbers of cells can be obtained easily and cytochemical and functional characteristics can be studied.

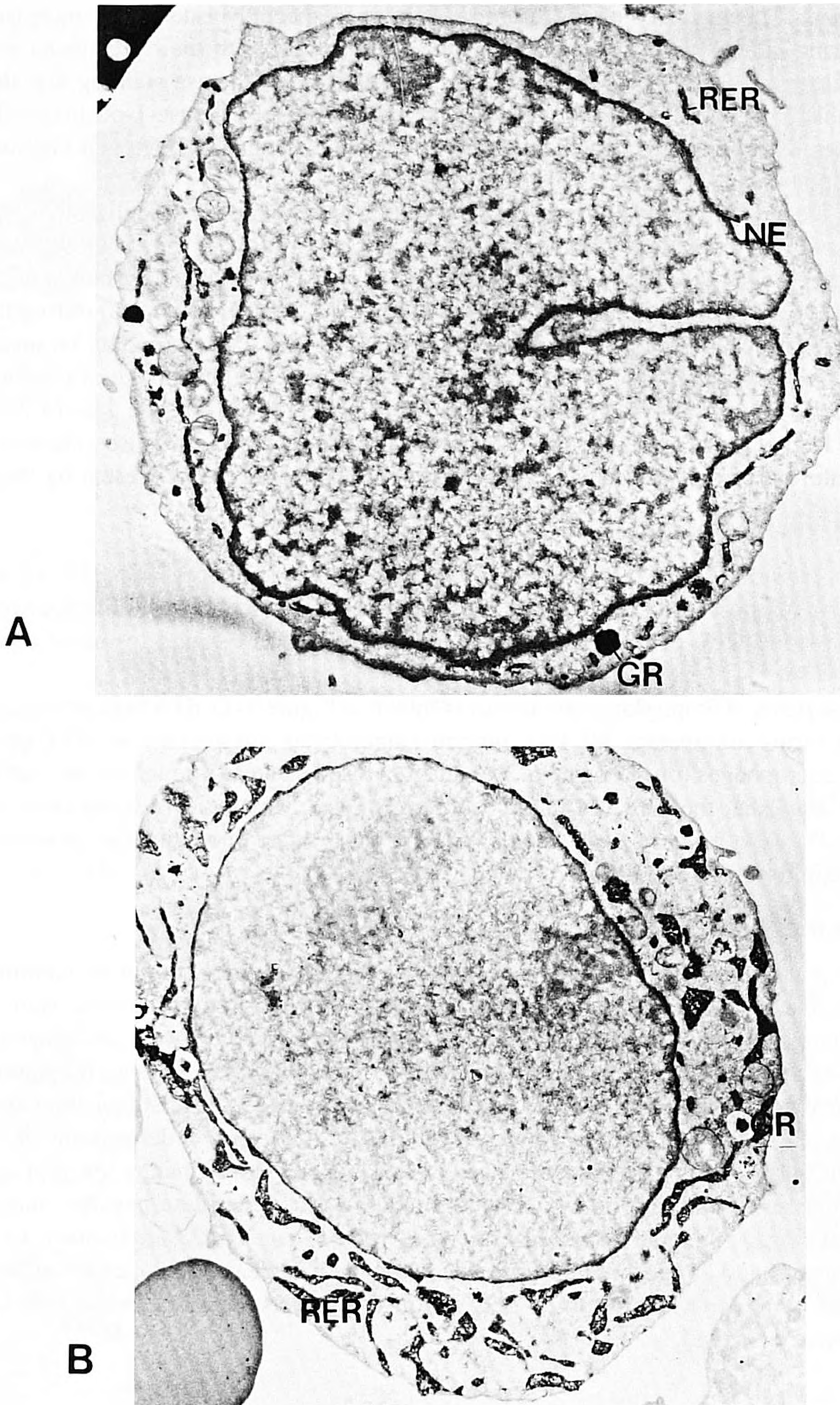


Fig. 4. A) Monoblast in a 1-day bone marrow culture. This cell shows the characteristic features of a monoblast: It is round with a few surface microextensions. The nucleus shows a sharp indentation; the nucleus-to-cytoplasm ratio is > 1 ; there are a few granules (GR); and the nuclear envelope (NE), the rough endoplasmic reticulum (RER), and the granules show peroxidatic activity. $\times 15,000$. B) Myeloblast in a 3-day bone marrow culture. This cell differs from the monoblast by the more rounded nucleus and the abundant, slightly dilated rough endoplasmic reticulum. The few granules present are less condensed than those of the monoblast. $\times 12,780$.

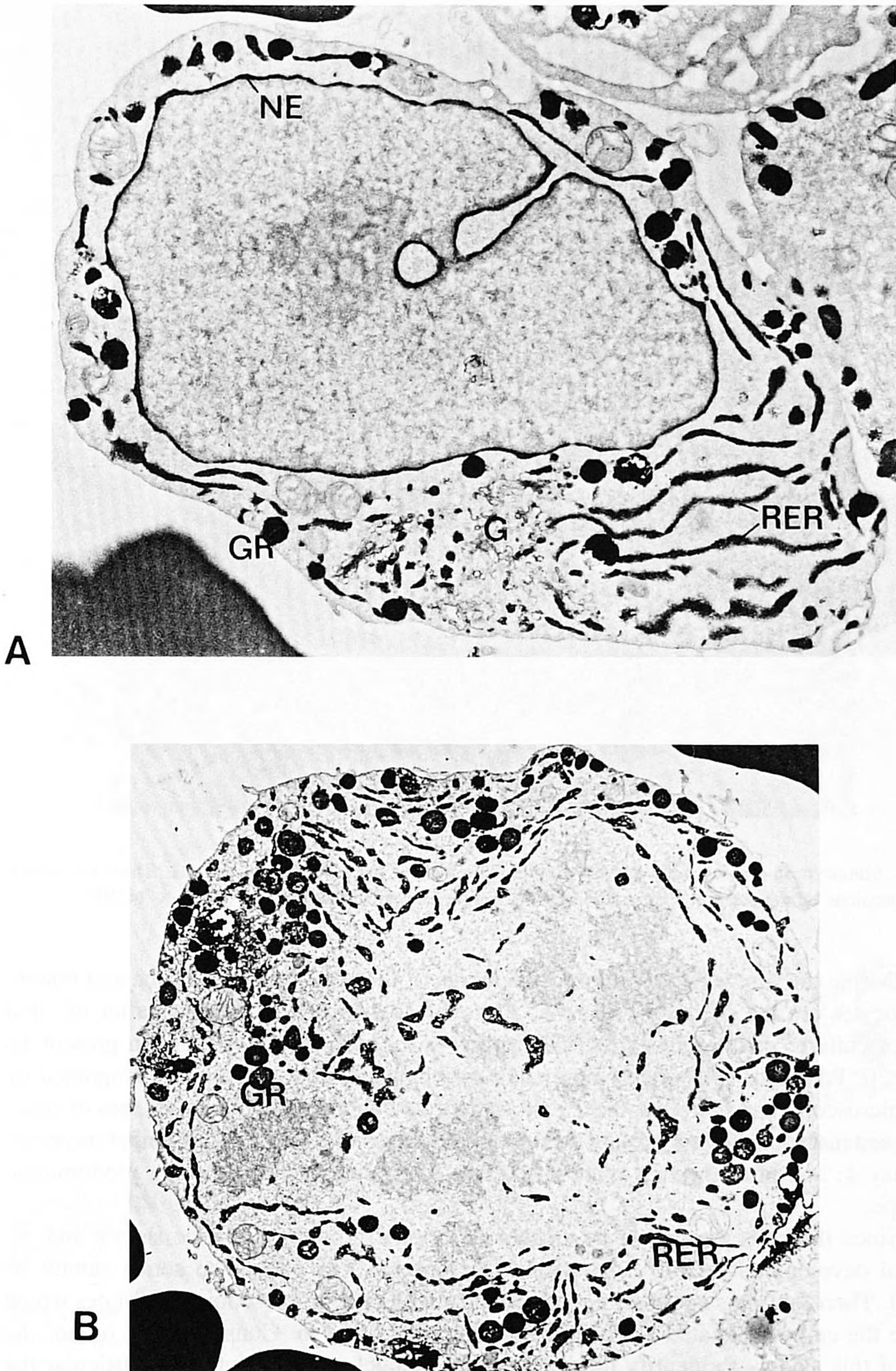


Fig. 5. A) Promonocyte in a 2-day bone marrow culture. The cell is somewhat elongated, the nucleus-to-cytoplasm ratio is about 1, and there are a few microextensions. The Golgi system (G) is well developed and shows peroxidatic activity. There is also peroxidatic activity in the nuclear envelope (NE), the rough endoplasmic reticulum (RER), and the numerous granules. $\times 15,000$. B) Promyelocyte (in mitosis) in a 1-day bone marrow culture. This cell type differs from the promonocyte by a less indented nucleus, the dilated rough endoplasmic reticulum, and the greater number of larger and less condensed granules. $\times 10,260$.

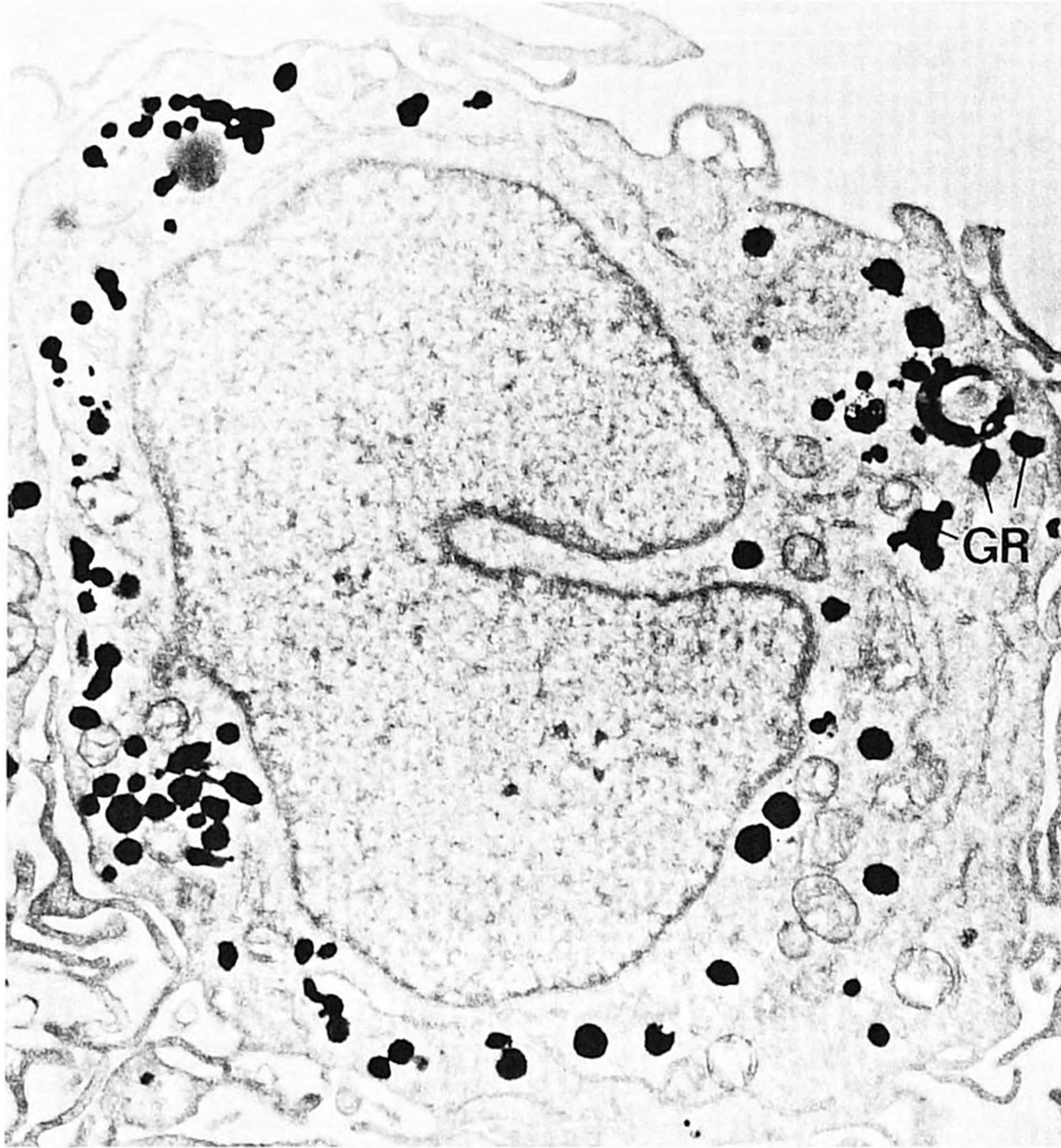


Fig. 6. Monocyte in a 2-day bone marrow culture. The nucleus-to-cytoplasm ratio is <1 . There are several microextensions on the surface. Peroxidatic activity is only present in the granules (GR). $\times 14,500$.

During the first week of culture, precursors of the granulocytic cell line and mature granulocytes are the predominant cells. Although their numbers decrease after the first week of culture, cells representing the various developmental stages remain present up to day 30. Precursor cells of the mononuclear phagocyte series cannot be recognized by light microscopy and therefore these cells cannot be quantitated. Small numbers of monocytes and macrophages are present during the early days of culture; their number increases after day 4, and after the first week of culture they gradually become the predominant cell type.

Since the cells are grown in suspension, clonal growth cannot be shown and sequential development of the cells within the mononuclear phagocyte series cannot be studied. Therefore, it is not possible to determine with light-microscopic techniques which lineage the cells designated as "blast cells" are committed to. Consequently, one of the goals of this study—to identify the immature mononuclear phagocytes, in particular the monoblast in human bone marrow—could not be achieved without the help of the electron microscope. The light-microscopic and ultrastructural characteristics of human promonocytes have been described in direct preparations and in short-term cultures of bone marrow [12,22]. In the present study of cultured bone marrow, use of the peroxidatic activity marker at the ultrastructural level made it possible to recognize cells identical to

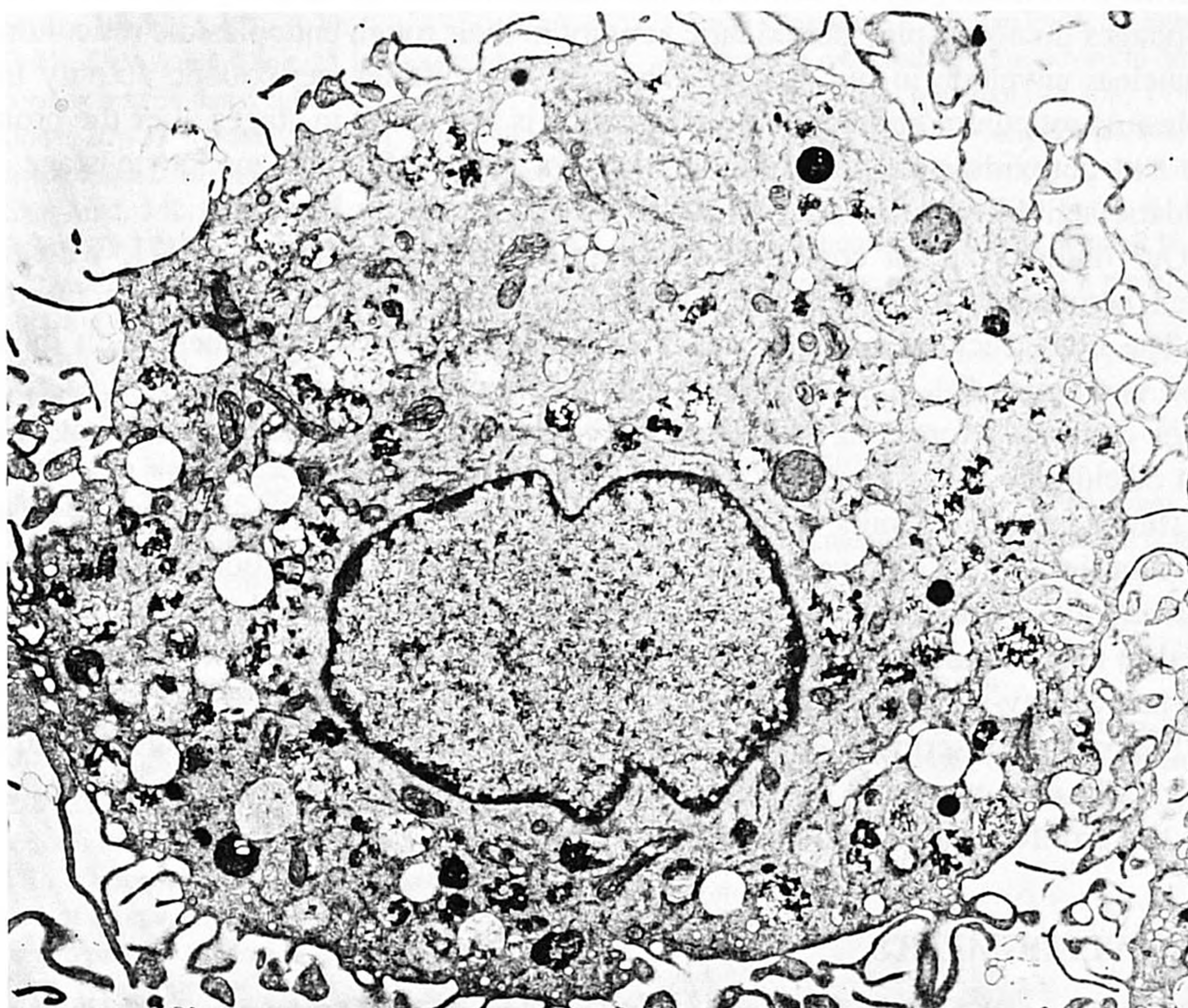
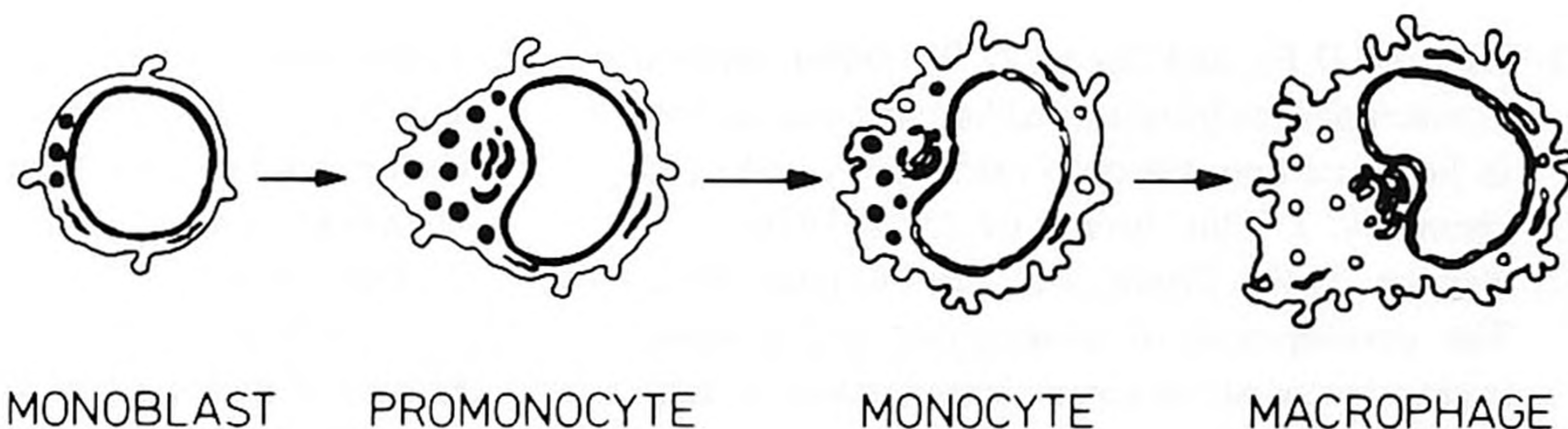


Fig. 7. Mature macrophage in an 8-day bone marrow culture. No peroxidatic activity present. $\times 8,500$.



LOCALIZATION OF PEROXIDATIC ACTIVITY

| | | | | |
|-----------------------|---|---|---|---|
| Endoplasmic reticulum | + | + | - | - |
| Nuclear envelope | + | + | - | - |
| Golgi apparatus | + | + | - | - |
| Lysosomal granules | + | + | + | - |

Fig. 8. Schematic representation of the peroxidatic activity patterns of human mononuclear phagocytes in culture.

the murine monoblast [10,18] and to the murine and human promonocyte [11,22]. Mature macrophages do not display peroxidatic activity in their rough endoplasmic reticulum and their nuclear envelope in our system. Thus, in our cultures peroxidatic activity in the endoplasmic reticulum and the nuclear envelope is not found in stages after the promonocyte, and peroxidase-positive granules are not found after the monocyte stage. The peroxidatic activity patterns are represented schematically in Figure 8.

Our findings are in contrast with those of Breton Gorius et al [7], who found transient peroxidatic activity in the nuclear envelope of human monocytes cultured in vitro. The ultrastructural findings in our study are largely in agreement with those of Bainton and Golde [1], although these authors did not report monoblasts in Marbrook-chamber cultures. Moreover, unlike us, they found many multinucleated giant cells at day 14 of culture.

The quantitative results obtained in our study are in some respects similar to those reported earlier [13,25]. However, the counts of viable cells in relation to time in culture reported by these authors were considerably lower; their initial cell number of 3×10^6 dropped to approximately 1×10^6 cells at day 4 and after that did not exceed 2×10^6 , even when colony-stimulating factor was added.

The method described in this paper offers a simple technique to culture human bone marrow cells in suspension. It can probably serve to study factors that stimulate and inhibit the proliferation of human marrow cells.

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