Characteristics of Long-Term Cultures of Proliferating, Mononuclear Phagocytes From Bone Marrow

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The characteristics of murine bone marrow mononuclear phagocytes in long-term cultures with embryonic fibroblast-conditioned medium were studied to determine the stage of development and state of activation of these cells. Two liquid culture systems were used: for studies on the morphology, cytochemistry, and functional characteristics at the cellular level, the cells were cultured adherent to a glass surface; and for experiments where the cells were needed in suspension (replating experiments, and studies on locomotion, intracellular killing, and cytotoxicity) use was made of Teflon culture systems.

Three developmental stages of mononuclear phagocytes could be recognized easily in these cultures: monoblasts, promonocytes, and macrophages. In cultures on a glass surface, these cells grow in colonies separate from granulocytic colonies. When incubation is prolonged beyond 7–9 days, the granulocytes die, leaving pure mononuclear phagocyte cultures. Primary cultures, in which monoblasts, promonocytes, and some macrophages proliferate, can be maintained for 3–4 weeks. Calculation showed that one monoblast present on day 0 gives rise to a progeny of more than 7×10^3 mononuclear phagocytes by day 14; after that, the rate of proliferation declines despite the addition of fresh media. Regular replating of the cells cultured on Teflon made it possible to maintain proliferation over a period of almost 200 days.

The cells in culture have the typical characteristics of mononuclear phagocytes, as judged by light microscopy, α-naphthyl butyrate esterase activity, lysozyme activity, presence of receptors for Fc and C3, and endocytic, microbicidal, and cytotoxic activity. The 5′nucleotidase activity, ingestion of erythrocytes via C3-receptor, locomotion, and antibody-dependent cytotoxicity indicate that the cultured bone marrow mononuclear phagocytes are more active than resident macrophages, and as active as or even more active than thioglycollate-induced macrophages. In conclusion, the population of mononuclear phagocytes in the liquid cultures of bone marrow is heterogenous with respect to developmental stage and state of activation.

Key words: proliferating mononuclear phagocytes, bone marrow, monocyte, hematopoietic stem cell, monoblast, promonocyte, macrophage

INTRODUCTION

Mononoclear phagocytes form a cell line originating in the bone marrow [43]. The most immature cell of this cell line is the monoblast, which has been identified and charcterized in liquid cultures of murine bone marrow [13,14]. In short-term cultures the monoblast has the morphologic, cytochemical, and functional characteristics of a mononuclear phagocyte [14,37,45]. This cell is probably not the direct descendant of the pluripotent hematopoietic stem cell, since there is evidence for the existence of other nonadherent precursors between this cell and the monoblast [2,23].

In the liquid culture system two distinct kinds of colony have been observed, that is, mononuclear phagocyte colonies and granulocytic colonies. These colonies differ in location, and mixed colonies were not observed in 4-day cultures with the media and sera used [14].

In mononuclear phagocyte colonies, monoblasts divide and form promonocytes [44,46], which in turn divide and form monocytes. In in vitro cultures, monocytes transform directly into macrophages [13,14].

The aim of the present study was to characterize the population of mononuclear phagocytes developing in long-term bone marrow cultures with respect to their morphologic, cytochemical, functional, and proliferative characteristics and to compare these characteristics with those of resident and elicited peritoneal macrophages.

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. For the study of Ia-antigens, use was made of inbred C3H mice (Erasmus University, Rotterdam, The Netherlands).

Culture of Bone Marrow Cells

The technique used for harvesting bone marrow cells from the mouse femur has been described elsewhere [14]. The culture medium consisted of Dulbecco's modified Eagle's medium (Grand Island Biologicals, Grand Island, NY) containing 20% horse serum (Flow Laboratories, Irvine, Scotland) and 20% conditioned medium (see below). Liquid cultures were prepared in plastic petri dishes (diameter 35 mm, Falcon Plastics, Cockeysville, Maryland) provided with a glass coverslip (21 mm × 26 mm) by incubating about 5×10^3 nucleated bone marrow cells in 2 ml culture medium. Suspension cultures were prepared by incubating 10^5 nucleated bone marrow cells in 5 ml of the same medium on disposable nontoxic Teflon FEP (fluorinated ethylene propylene resin) film, (gauge 25 μm, Jansens M&L, St. Niklaas, Belgium), either mounted in the aluminium Teflon film dish (TFD) [38] or diathermically sealed to form a Teflon culture bag [40]. All cultures were held in 37°C in a water-saturated atmosphere of 10% CO_2 in air.

Peritoneal Macrophages

Resident peritoneal macrophages were harvested by lavage of the peritoneal cavity of untreated mice; thioglycollate-induced peritoneal macrophages were obtained by lavage 4 days after an intraperitoneal injection of 1.5 ml of Brewer's thioglycollate (Difco Laboratores, Detroit, MI), prepared according to the manufacturer's directions and stored for at least 4 weeks. The peritoneal macrophages were used either immediately after harvesting or after culture in Medium 199 (Microbio-

logical Associates, Bethesda, MD) containing 20% heat-inactivated newborn calf serum (Grand Island Biologicals) in a TCB or TFD.

Conditioned Medium

An embryonic fibroblast conditioned medium was used as the source of colony stimulating factor 1 (CSF-1), the CSF with activity restricted to the mononuclear phagocyte series [33]. The preparation of this conditioned medium has been described elsewhere [12,39]; in short, 13- to 15-day-old murine embryos, after dissection of limbs, skull, liver, and spleen, were incubated for 20 min at room temperature in a solution composed of 0.25% trypsin (Difco Laboratories) in phosphate-buffered saline (hemagglutination buffer, Difco Laboratories).

After centrifugation (125 g) of the cell suspension, the precipitate was suspended in Waymouth medium (MB 752.1; Gibco) containing 5% newborn calf serum (Gibco). This suspension was divided into 10-ml aliquots, each containing about 3×10^6 embryonic cells, which were incubated in petri dishes (100 mm; Falcon Plastics) and held at 37°C in a humidified atmosphere under a flow of 10% CO_2 in air. The conditioned medium was collected at regular intervals, usually on the eighth day of incubation, after which new culture medium was added to the dishes. Subcultures were made by trypsinization and replating of the embryonic mouse cells; these subcultures were treated in the same way as the primary cultures. After collection, the conditioned medium was filtered (pore size 0.45 μm; Millipore S.A. Buc, France) and stored in 5-ml vials at -70°C. The activity of the various batches of conditioned medium was determined by counting the number of colonies grown from bone marrow cells in viscous methyl cellulose [55] after 7 days of incubation. Batches of about the same strength (at least 50 colonies per 5×10^4 nucleated bone marrow cells plated) were used in all subsequent experiments [12]. In three different batches of the conditioned medium used in this study, the CSF-1 concentrations were 1,730, 1,240, and 1,620 U/ml, respectively, as detected with a radioimmunoassay [33] (kindly performed by Dr. E.R. Stanley, Albert Einstein School of Medicine, NY). Unless stated otherwise, 0.1 ml of fresh conditioned medium was added every 3–4 days.

Light Microscopy

During incubation, cultures were examined at intervals in an inverted phase contrast microscope (Carl Zeiss, Oberkochen, West Germany) and photographed with a Polaroid camera. When culture was terminated, the coverslip was rapidly dried in flowing air, fixed in absolute methanol for 10 min, and stained with Giemsa's stain.

Counting of Colonies and Total Number of Cells per Culture

Colonies were counted on coverslips after fixation and staining. Groups of at least four cells were considered to be colonies [14].

To determine the total number of mononuclear phagocytes in cultures up to day 4, these cells were counted on coverslips after fixation; at later time-points, the cells were carefully removed from the surface by placing the petri dish on crushed ice (about 4°C) and scraping with a rubber policeman, and were counted in a hemocytometer. Cells adhering to the glass were fixed and stained. For calculation of the total number of cells in culture, a correction was made for the area of the petri dish surface not covered by the coverslip. The cells cultured on a Teflon surface were recovered by rinsing the surface with culture medium after aspiration of the suspension with a pasteur pipette (TFD cultures) or with a needle and syringe (TCB cultures). The cells in this suspension were counted in a hemocytometer, and cytocentrifuge preparations

[5] were made for distinction between mononuclear phagocytes and granulocytes (see below). Viability of cells on coverslips and Teflon was estimated by 0.1% trypan blue exclusion.

To compare the results of various experiments, all growth curves were standardized to 1×10^3 nucleated bone marrow cells; this number was chosen because an earlier study had shown that approximately one monoblast was present among 1×10^3 nucleated bone marrow cells [13].

Identification of Cells and Colonies in Culture

On coverslips, at least 200 cells of each cell type were examined per time-point in preparations from at least three experiments. In colonies of mononuclear phagocytes, cells were distinguished according to morphologic criteria (cell size, shape, nuclear-to-cytoplasmic ratio, basophilia of the cytoplasm, pinocytic vesicles), as previously described [14,37,45]. The following types of mononuclear phagocytes were distinguished: *monoblasts,* that is, round cells (diameter 10 μm $\times$ 12 μm) with a small rim of strongly basophilic cytoplasm; *promonocytes,* that is, slightly stretched cells (diameter 13 μm $\times$ 34 μm) with one pseudopod, basophilic cytoplasm, and a nucleus-to-cytoplasm ratio of about 1; and *macrophages,* that is, large elongated cells (diameter 17 μm $\times$ 69 μm) with two or more pseudopods and a grayish-blue cytoplasm and a nucleus-to-cytoplasm ratio lower than 1. Granulocytes were distinguished according to the criteria of Goud et al [13].

Replating of Cultures

After various times of incubation, cells cultured on a Teflon surface were recovered as described above. The recovered cells were counted in a hemocytometer, tested for viability, centrifuged (10 min at 100 g), resuspended in fresh medium of unchanged composition, and incubated in TFD or TCB as described above. Fresh conditioned medium was not added to these cultures after replating. At various time-points, cytocentrifuge preparations were made [5] in which the characteristics of these cells were studied.

[^{3}H]Thymidine Labeling Studies

For the study of DNA synthesis, medium containing 0.1 μCi/ml [^{3}H]thymidine (specific activity 6.7 Ci/mmol, New England Nuclear, Boston, MA) was added to the culture. Autoradiography was performed with Ilford Nuclear Research Emulsion K5 in gel form (Ilford, Essex, England). The exposure time was 10 days. Cells containing more than three grains over the nucleus were considered positive.

Cytochemistry

Esterase activity was investigated according to Ornstein et al [27] with a α-naphthyl butyrate (Sigma, St. Louis, MO) as the substrate at pH 6.1, and incubation for 25 min at room temperature.

Peroxidatic activity was investigated according to Kaplow as modified by Goud et al [14], with benzidine dihydrochloride (Fluka, Buchs, Switzerland) as substrate at pH 6.0 and hydrogen peroxide at a concentration of 0.002% (v/v).

Leucine–aminopeptidase activity was investigated with a modification of the method of Nachlas et al [25]. The reagent was prepared by mixing 4 mg 1-leucyl-4-methoxynaphthylamide (Koch Light Laboratories Ltd., Colinsbrooks, Bucks., England) dissolved in three drops of methanol and 5 ml 0.1 M tris-HCl buffer (pH 6.5) with 5 mg Fastblue B (Sigma Chemical Co., St. Louis, MO) in 4.5 ml buffered

saline. Cells on coverslips were fixed in formaldehyde vapor for 60 sec, washed in water, and dried; and after the coverslips had been placed horizontally, were incubated with the reagent in a water-saturated atmosphere at 37°C for 1.5 hr. The coverslips were then rinsed with tapwater, counterstained with hematoxylin (Merck, Darmstadt, West Germany), and mounted with Aquamount (Hopkin and William Chadwell Heath, Great Britain).

5'Nucleotidase was investigated with a modification of Wachstein and Meisel's method [52]. The incubation mixture consisted of 200 mg adenosine monophosphate (Boehringer, Mannheim, West Germany), 5 ml 0.05 M $MnCl_2$, 3 ml 2% lead nitrite, and 20 ml 0.2 M tris-maleate buffer (pH 7.2) in 22 ml distilled water. Coverslips fixed in formaldehyde vapor for 60 sec, rinsed, dried, and placed horizontally, were incubated in this mixture at 37°C for 2 hr, and then rinsed, incubated in a 1% ammonium sulfide solution, rinsed again, and counterstained with Kernechtrot. After dehydration in absolute ethanol and xylol, the coverslips were mounted with Malinol (Brunswick Chemie, Amsterdam, The Netherlands).

Lysozyme activity at the cellular level was demonstrated with an indirect immunoperoxidase technique as described elsewhere [6].

Lysozyme activity in the culture supernatants was assessed according to Parry et al [28] and Gordon et al [11] by measuring the rate of lysis of heat-killed *Micrococcus leisodeikticus* with a spectrophotometer (C. Zeiss, Oberkochen, West Germany). Eggwhite lysozyme (Worthington, Freehold, NJ) served as standard.

β-Glucuronidase activity in the culture supernatants was assayed fluorimetrically, with methylumbelliferyl glucuronide as a substrate (performed by Dr. J. Schnyder, Research Institute, Wander, Berne, Switzerland). Details of the assay have been given elsewhere [30].

Ia Antigens

The expression of Ia antigens on cultured bone marrow mononuclear phagocytes of inbred C_3H mice was studied with an indirect immunoperoxidase technique using a monoclonal anti-Ia antibody provided by Dr. W. van Ewijk (Erasmus University, Rotterdam). The methods used to prepare and test this antibody have been published elsewhere [41].

After fixation of the cells in acetone for 1 min, the cells were incubated with 25% normal rabbit serum for 10 min at room temperature. After being washed for 30 min with a buffer composed of Dulbecco's phosphate-buffered saline (pH 7.4), 1% (v/v) fetal calf serum, and 0.05% (v/v) Tween-20 [42], the cells were incubated with anti-Ia antibody for 30 min at room temperture. The preparations were then washed again and incubated with peroxidase-labeled rabbit anti-rat Immunoglobulin G (IgG) (dilution 1:50) [42] for 30 min at room temperature. After one wash the preparations were stained with diaminobenzidine [15]. The controls were frozen sections of thymus deriving from C_3H mice, prepared as described elsewhere [42], and bone marrow cells incubated without the monoclonal antibody.

Receptors at the Cell Surface

In cells recovered from the TFD and cells attached to a glass surface the presence of IgG- and C3-receptors was determined as described elsewhere [5].

Phagocytosis and Intracellular Killing of Bacteria

To study phagocytosis of bacteria at the cellular level, the supernatant of the culture was replaced by 1 ml of a suspension of 1×10^6 *Staphylococcus epidermidis*

in Dulbecco's modified Eagle's medium containing 10% newborn calf serum. After 1 hr at 37°C, the coverslip was washed thoroughly with medium, dried, fixed, and stained.

The rate of phagocytosis was determined as described in detail elsewhere [48]. For these experiments the cells were cultured in culture medium without antibiotics in TFD. After recovery of the cells from the TFD, about 10^7 viable cells were incubated with an equal number of preopsonized *Staphylococcus epidermidis* [48] suspended in gelatin–Hanks' salt solution containing 10% newborn calf serum under continuous rotation (4 rev/min) in a siliconized glass tube. At several time-points the number of viable bacteria in the supernatant was determined by a microbiologic method.

Intracellular killing of *Staphylococcus epidermidis* was determined independent of the rate of phagocytosis [48]. Phagocytosis of preopsonized staphylococci was allowed to continue for 15 min, after which the extracellular bacteria were removed by differential centrifugation at 110 g and thorough washing, and the cells containing ingested bacteria were reincubated in 10% serum at 37°C. At various time-points the number of viable intracellular bacteria was determined microbiologically.

Pinocytosis

Pinocytosis was studied during incubation of the cells for 24 hr with dextran sulphate (MW 500,000; Pharmacia, Uppsala, Sweden) in a concentration of 100 µg/ml culture medium. The cells on the coverslips were washed with medium, fixed in absolute methanol, and stained with 2.5% (v/v) Giemsa stain in water.

Cytotoxicity

Cytotoxic activity of mononuclear phagocytes was studied according to Fleer et al [9]. The capacity of the cells to lyse ^{51}Cr-labeled anti-D-coated human red cells was determined at ^{51}Cr release and expressed as the number of erythrocytes specifically lysed (difference between lysis of anti-D-coated and noncoated red cells). Aliquots of 100 µl containing 5×10^4, 2.5×10^4, 1.25×10^4, 0.6×10^4, and 0.3×10^4 mononuclear phagocytes were incubated with 1.6×10^5 anti-D-coated or noncoated red cells for 18 hr at 37°C in microtiter plates. The plates were then centrifuged and the supernatants analysed for ^{51}Cr release. (These studies were performed in the Central Laboratory of the Blood Transfusion Service of the Netherlands Red Cross, Amsterdam, The Netherlands.)

Oxygen Consumption

Oxygen consumption was studied with the K-ICC oxygraph (Gilson Medical Electronics, Middleton, WI) equipped with the YSI 5331 oxygen probe (Yellow Springs Instruments Co., Yellow Springs, OH). For these measurements, either heatkilled *Staphylococcus aureus* (10^9/ml) opsonized with 25% newborn calf serum or 0.1 µg phorbolmyristate acetate (PMA; Sigma) was added to 1×10^6/ml cells suspended in 0.14 ml Hanks' balanced salt solution.

Locomotion

Locomotion of the cells after culture in TCBs was assessed in a millipore chamber according to Wilkinson [54]. At least 10^6 cells were suspended in Gey's solution (Difco, Detroit, MI) and placed on the upper surface of a cellulose nitrate filter (pore size: 8 µm) (Sartorius, Göttingen, West Germany). The attractant casein (Merck, Darmstadt, West Germany) in concentrations of 1 and 2 mg/ml was added below the filter; for the controls, Gey's solution was applied instead of casein. The chambers were incubated at 37°C for 2 hr, after which the filters were detached,

dehydrated, stained and mounted. Locomotion was measured according to the leading-front method [56].

RESULTS

Morphology

The appearance of colonies of mononuclear phagocytes adhering to glass in liquid cultures in the period from day 4 to day 21 is essentially the same as that described by Goud et al [14] for mononuclear phagocyte colonies in the early days of culture. In the middle of the colony, round cells are crowded together, whereas toward the periphery there are dispersed elongated cells (Fig. 1a). During incubation, the area occupied by one colony expands due to the increase in the number of cells. After 2 or 3 weeks of culture the cell density in the centre of the colony decreases (Fig. 1b). Under the inverted microscope, clumps of round cells in the center of the colony are often seen to become detached from the glass and later to become attached to the glass in the vicinity of large colonies.

Cultures can be maintained for up to 3–4 weeks without replating, but after that the cells round up, the nucleus becomes pycnotic, and the cells are no longer attached to the glass.

Granulocyte colonies present in the early days of the liquid culture [14] are no longer seen after 7–9 days of incubation. Occasionally, fibroblasts are seen; overgrowth of these cells was never observed, and their number was so low that the total cell counts were not influenced.

The Number of Mononuclear Phagocyte Colonies

The number of colonies of mononuclear phagocytes on fixed slides per 10^3 plated nucleated bone marrow cells increased from 1.1 (SD $\pm$ 0.5) on day 4 to 2.6 (SD $\pm$ 1.2) on day 7 and to 5.2 (SD $\pm$ 3.4) on day 10. After day 10 the number of colonies cannot be evaluated accurately because of appreciable confluence of the large colonies.

The Number and Types of Mononuclear Phagocytes in Primary Cultures

The growth curves of bone marrow mononuclear phagocytes on a glass surface and on Teflon are very similar (Fig. 2). The number of cells augments very rapidly in the first two weeks and then levels off. This curve is normalized to the number of mononuclear phagocytes produced from 1×10^3 nucleated bone marrow cells on day 0.

Up to day 21 the viability of the glass-adherent cells in the cultures was over 99%; in the cultures on Teflon, viability was lower but still more than 80% (range 81–95%). This divergence is probably due to the fact that nonviable cells in the petri dishes are not counted because they detach from the glass surface, whereas in the cultures on Teflon both viable and nonviable cells are present in the suspension.

The proportion of monoblasts, promonocytes, and macrophages during 21 days of culture is shown in Fig. 3. The absolute number of monoblasts remained rather constant, whereas the number of promonocytes increased more than 60-fold between day 4 and day 14. The number of macrophages increased more than 70-fold during this culture period.

[^{3}H]thymidine Labeling of Cells in Mononuclear Phagocyte Colonies

The absolute numbers of monoblasts, promonocytes, and macrophages labeled after 1 hr of incubation with [^{3}H]thymidine are given in Figure 4 (black bars). For
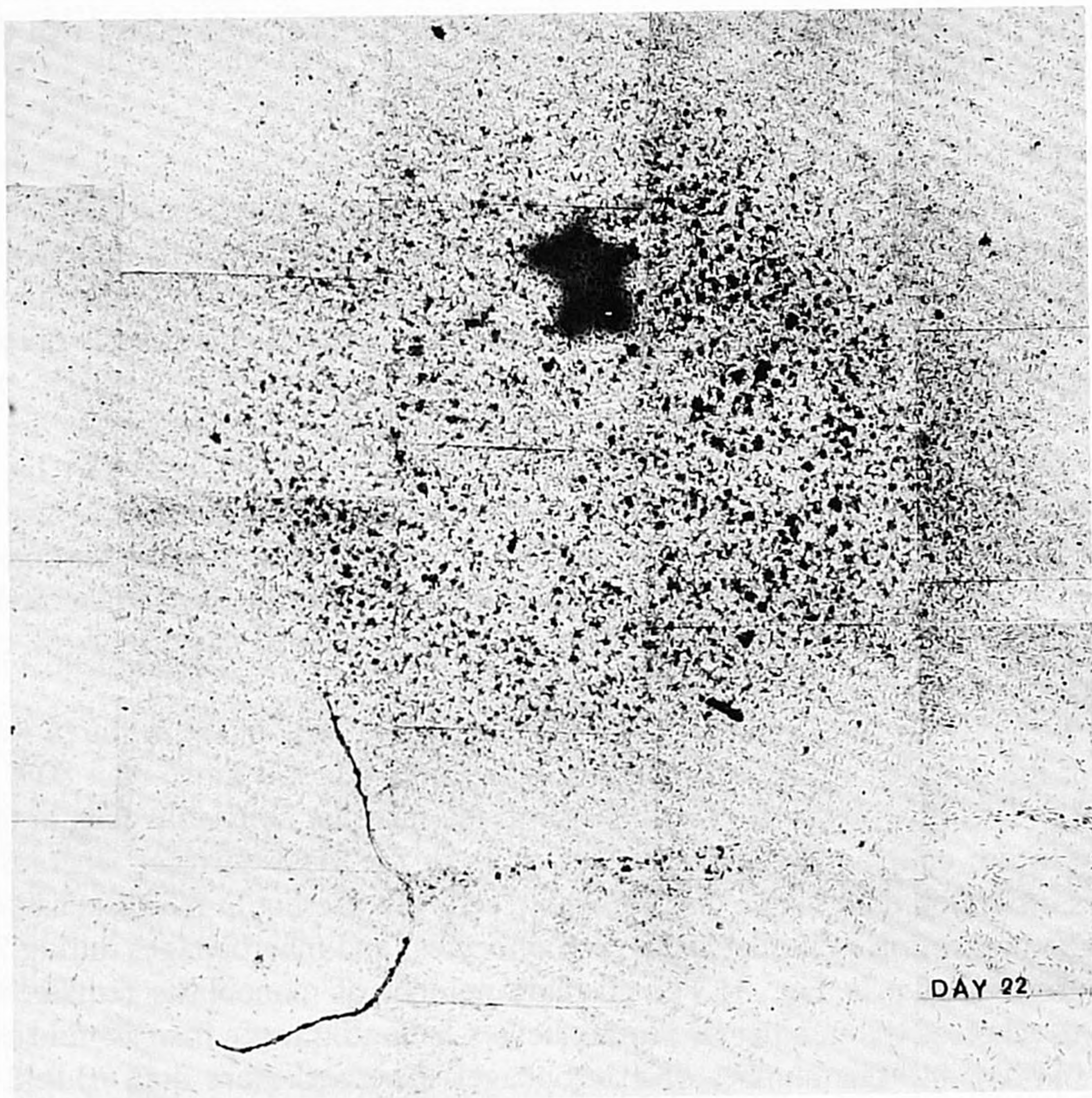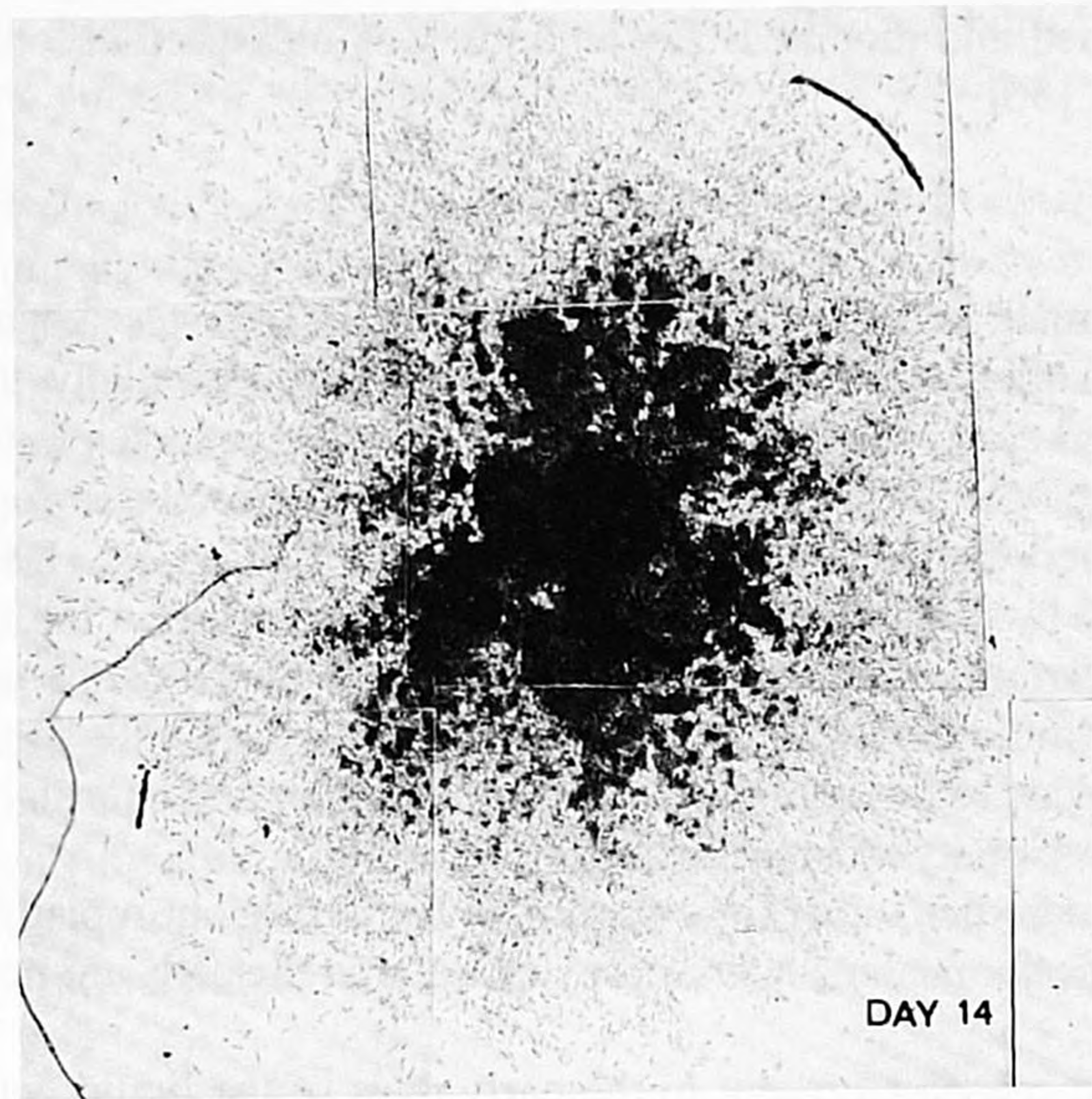
Fig. 1. (a) Mononuclear phagocyte colony on day 14; (b) the same colony on day 22, photographed under the inverted microscope. ×20 magnification for both.
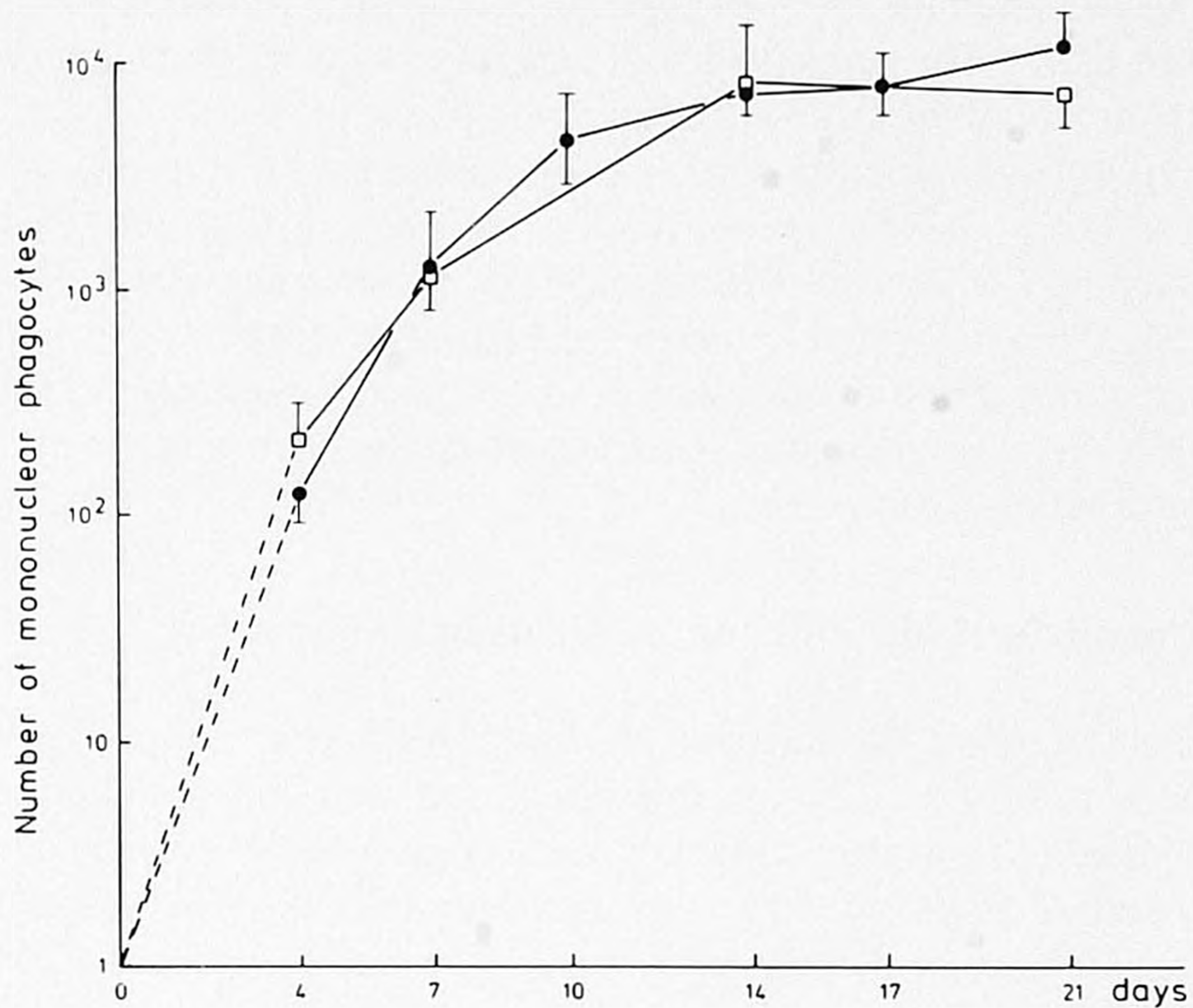
Fig. 2. Proliferation of mononuclear phagocytes from bone marrow on a glass surface (●) and on a Teflon surface (□) in the presence of conditioned medium. The curves are normalized to an initial number of 1×10^3 nucleated bone marrow cells. The bars indicate the SD.
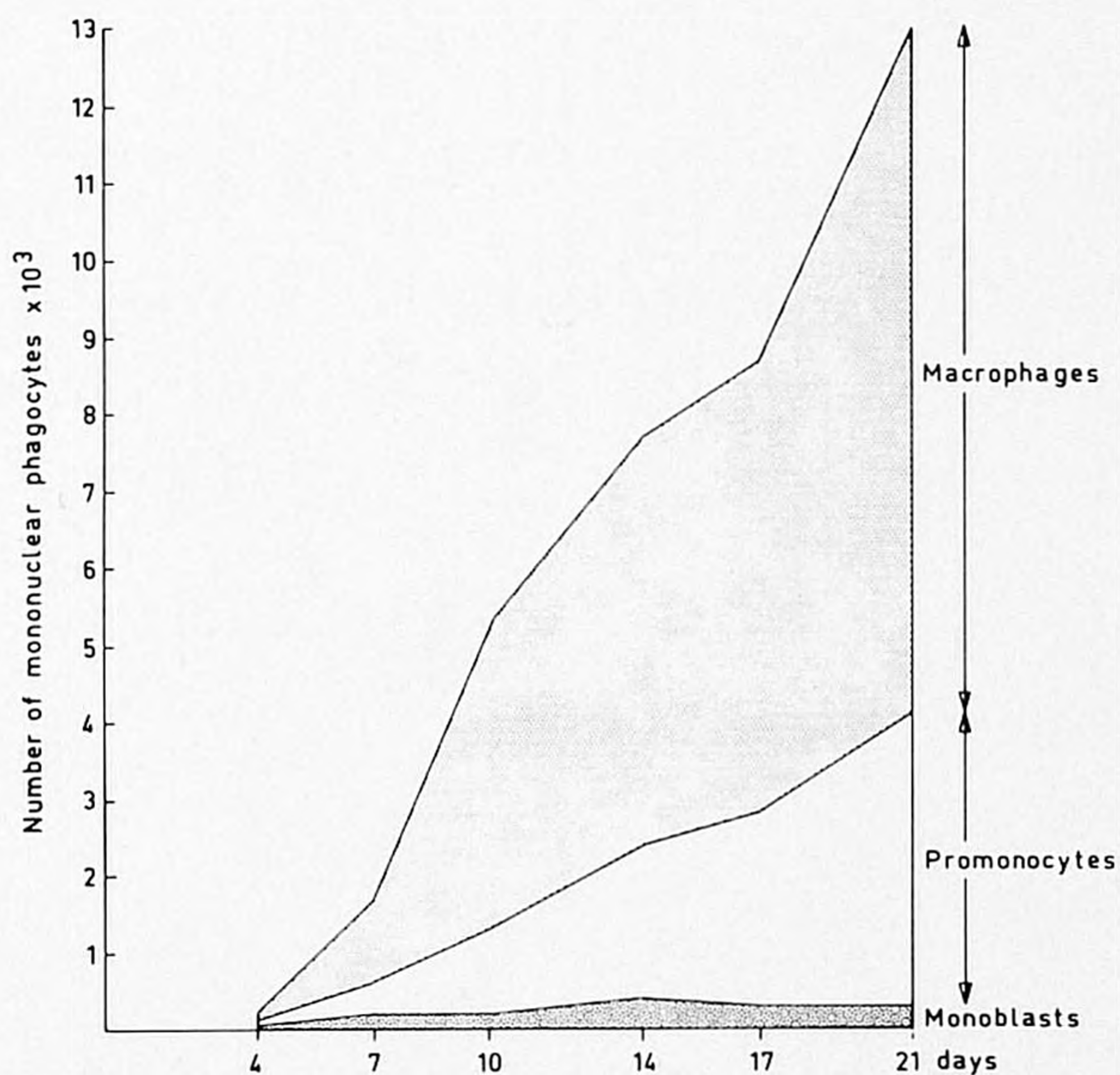

Fig. 3. Distribution of monoblasts, promonocytes, and macrophages in adherent colonies grown on a glass surface. The graph is normalized to an initial number of 1×10^3 bone marrow cells.

comparison the total number of each type of cell at each time-point is also given in Figure 4 (white bars). The percentages of labeling index of all categories of mononuclear phagocyte are given in an accompanying paper [47].

The highest labeling indices of the monoblasts (39% at day 4) and of the promonocytes (36% at day 4) indicate that the majority of these cells divide.

The percentage of labeled macrophages is lower (maximally 16% with 1 hr labeling). At day 7 the majority of labeled cells have the morphology of macrophages. This means that a proportion of cells with morphologic characteristics of macrophages synthesize DNA. In a mathematical analysis [47] we have tentatively called these macrophages "dividing macrophages."

The Role of Conditioned Medium and Nutrients in Proliferation

To find out whether the decrease of the proliferation rate and of the labeling index was due to loss of activity of conditioned medium during culture, the half-life of conditioned medium was determined by incubating 5×10^3 nucleated bone marrow cells in liquid culture as described above and removing the supernatant on successive days of culture.

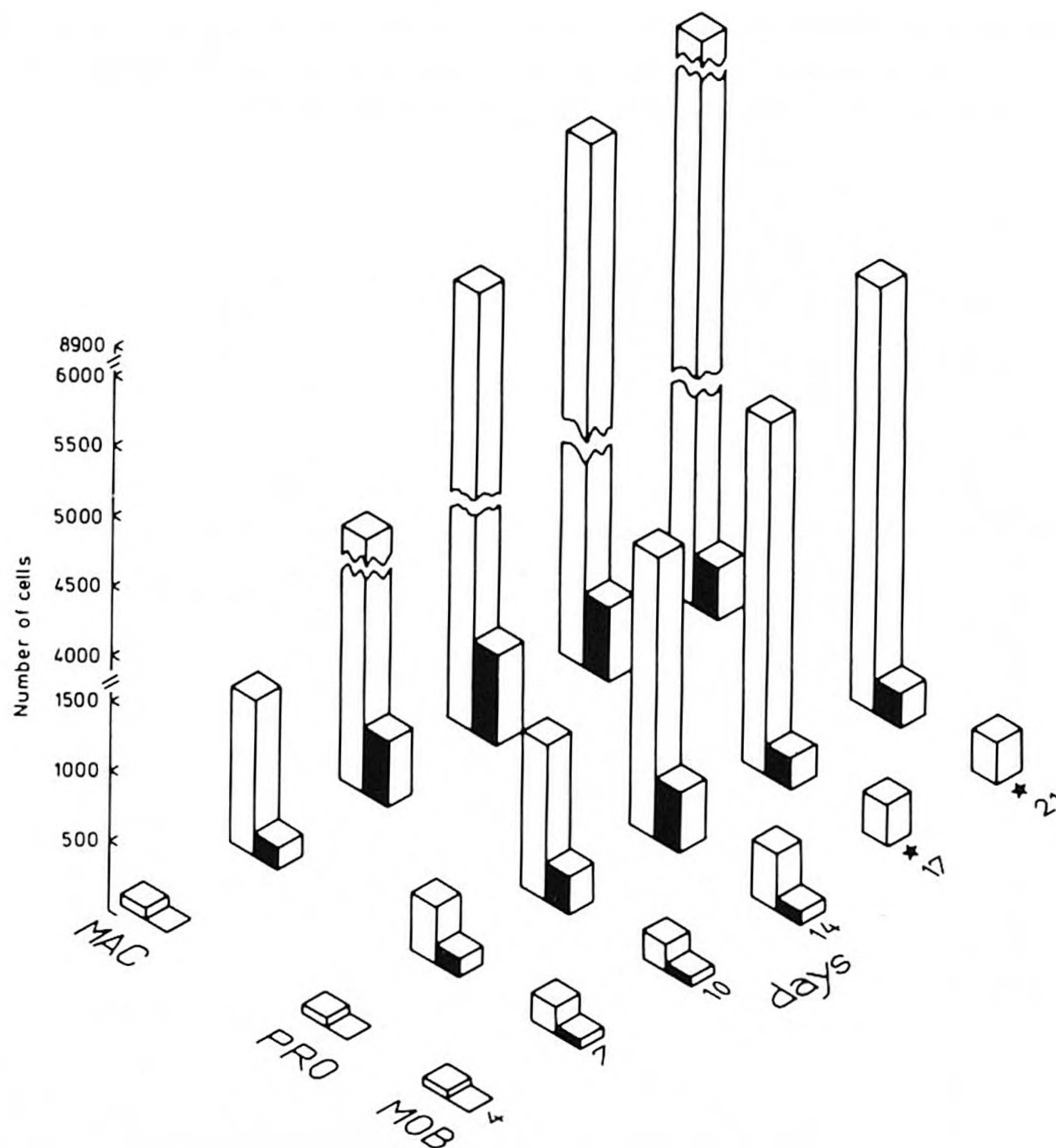

Fig. 4. [³H]thymidine labeling of monoblasts (MOB), promonocytes (PRO), and macrophages (MAC) in cultures on a glass surface. The black bars indicate the numbers of labeled cells after 1 hr incubation in [³H]thymidine. The white bars indicate the total numbers of cells. ★The total number of labeled monoblasts could not be calculated, because these cells could not be identified with certainty.

The supernatant, which was made cellfree by filtration through a millipore filter (pore size: 0.45 μm) was added to culture medium to be used for new bone marrow cultures in methyl cellulose. In these cultures the number of colonies was determined after 7 days of incubation. Computation with the data from these experiments gave a half-life of 4.8 days for the conditioned medium. During a 3-week culture period there was no decrease in the amount of amino acids, glucose, magnesium, or phosphate in the supernatant. Based on these findings, fresh conditioned medium was added to the cultures routinely twice a week.

Replating of Cells

Since we wanted to find out whether proliferation could be maintained over a long period, cultures in TFD and TCB were replated several times at weekly or 2-weekly intervals. In this way we were able to maintain cultures for 27 weeks (Fig. 5). Infection was the main reason for termination of these cultures. The rate of proliferation varied considerably between long-term cultures. The mean 1-hr [^{3}H]thymidine labeling index in the experiment with the highest rate of proliferation was 6.8% (SD $\pm$ 4.7) from day 21 onward; in the culture maintained for more than 190 days, the mean 1-hr labeling index from day 21 onward was 3.7% (SD $\pm$ 2.5).

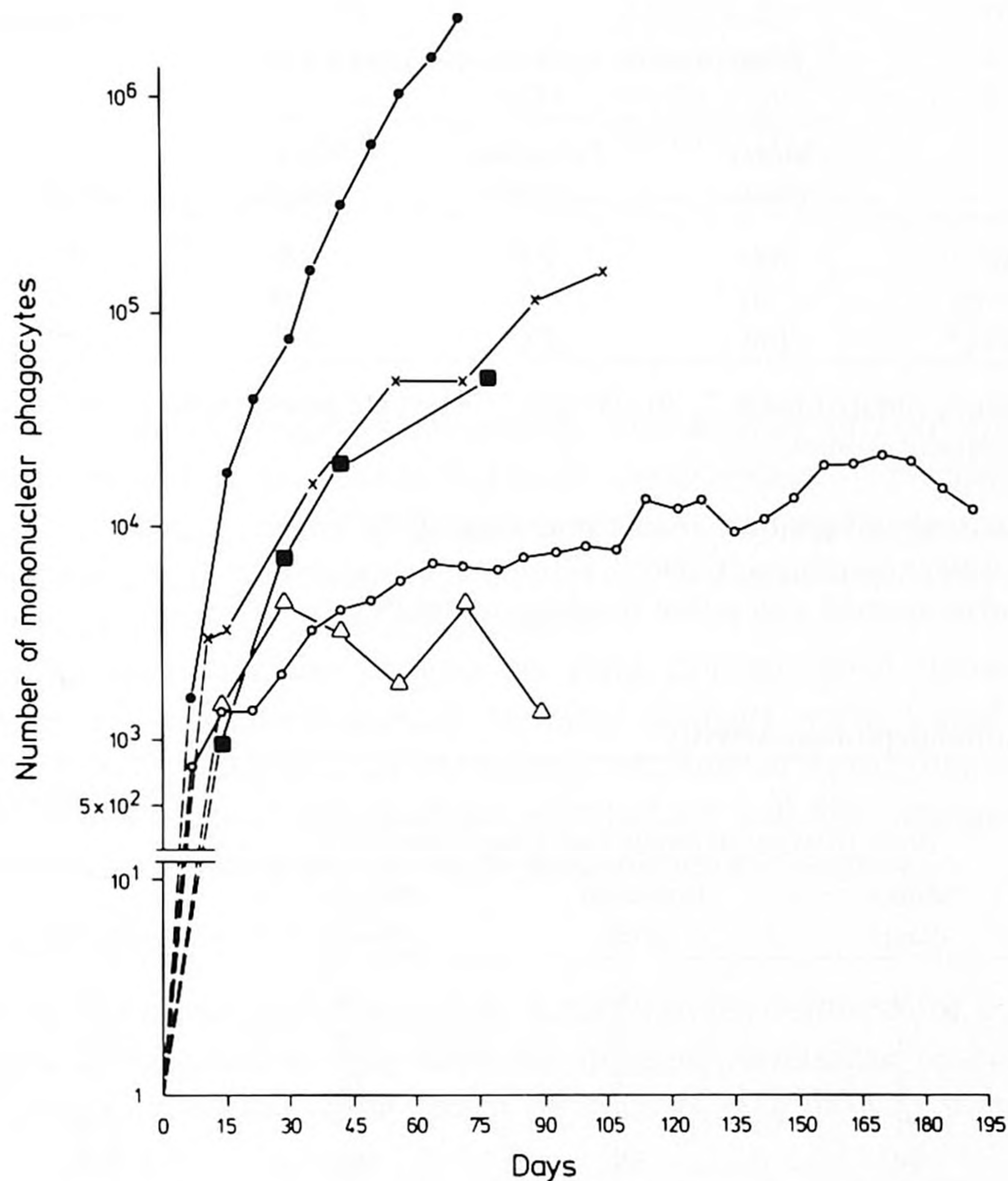Fig. 5. Proliferation of bone marrow mononuclear phagocytes maintained by replating. At varying intervals, indicated by a symbol, the cells were harvested and reincubated in fresh medium. The original cell density at day 0 varied between 5×10^3 and 5×10^4; the curves are normalized to 1×10^3 nucleated bone marrow cells at day 0. Different experiments are indicated by different symbols.

Cytochemical Characteristics

At all durations of culture studied, all of the mononuclear phagocytes were strongly positive for α-naphthyl butyrate esterase (Table 1). The intensity of the staining, as judged by light microscopy, did not differ between monoblasts, promonocytes, and macrophages, but increased with increasing culture time.

Peroxidatic activity could not be detected light microscopically in any of the cultures (Table 1).

The leucine aminopeptidase activity of bone marrow mononuclear phagocytes and resident- and thioglycollate-induced peritoneal macrophages, is given in Table 2. During the first 4 days of culture the enzyme activity of the bone marrow mononuclear phagocytes was low, similar to that of the resident peritoneal macrophages. After the first week of culture, there was an increase in both the intensity of staining and the number of positive cells.

The number of bone marrow mononuclear phagocytes positive for 5'nucleotidase, like the number of thioglycollate-induced peritoneal macrophages, was low compared with resident peritoneal macrophages (Table 3). The number of positive cells increased slightly with longer culture periods, and within one culture

TABLE 1.　Activity of Esterase, Peroxidase, and Lysozyme in Mononuclear Phagocytes

	Bone marrow mononuclear phagocytes (%)[a]			Peritoneal macrophages (%)[b]	
	Mono-blasts	Promono-cytes	Macro-phages	Resident	Thiogly-collate in-duced
Esterase activity[c]	100	100	100	100	100
Peroxidase activity[d]	0	0	0	0	0
Lysozyme activity[e]	100	100	100	100	100

[a]Studied in colonies cultured for 4, 7, 10, 14, and 21 days; the percentages of positive cells did not differ for the various days of culture.
[b]Freshly harvested.
[c]Cytoplasmic staining, α-naphthyl butyrate as substrate [27].
[d]Staining of granules according to Kaplow [17].
[e]Immunoperoxidase method with antirat lysozyme serum [6].

TABLE 2.　Aminopeptidase Activity

Day of culture	Bone marrow mononuclear phagocytes (%)			Peritoneal macrophages (%)	
	Mono-blasts	Promono-cytes	Macro-phages	Resident	Thiogly-collate induced
1	0	0	0	10	95
2	1	5	9	12	99
3	1	7	15	13	100
4	1	8	16	14	100
7	19	38	48	ND	ND
10	30	97	97	ND	ND
14		99	100	ND	ND
21		100	100	ND	ND

Abbreviations: ND, not done.

TABLE 3. 5'Nucleotidase Activity

Day of culture	Bone marrow mononuclear phagocytes (%)			Peritoneal macrophages (%)	
	Mono-blasts	Promono-cytes	Macro-phages	Resident (%)	Thiogly-collate induced
1	0	0	0	72	2
2	0	0	0	73	1
3	0	0	0	72	1
4	0	0	0	69	0
7	0	0	3	ND	ND
10	0	7	10	ND	ND
14		10	11	ND	ND
21		10	32	ND	ND

Abbreviations: ND, not done.

TABLE 4. Lysosomal Enzymes in Supernatants of Liquid Cultures

	Duration of culture (days)	Lysozyme[a] (μg/10^5 cells)	β-Glucuronidase (mU/10^5 cells)
Bone marrow cultures	4	1.0 ($\pm$ 0.5)[b]	0.27 ($\pm$ 0.01)
	7	1.6 ($\pm$ 0.4)	0.28 ($\pm$ 0.01)
	10	4.0 ($\pm$ 2.1)	0.61 ($\pm$ 0.03)
	14	4.8 ($\pm$ 1.0)	0.77 ($\pm$ 0.05)
	21	8.0 ($\pm$ 3.2)	0.77 ($\pm$ 0.10)
Resident peritoneal macrophages	4	0.9 ($\pm$ 0.1)	0.58 ($\pm$ 0.01)

[a]Standard: lysozyme egg white.
[b]Mean and SD.

some colonies were more positive than others. The activity of both aminopeptidase and nucleotidase varied considerably between experiments. Nevertheless, general activity patterns of bone marrow cells and peritoneal macrophages could be distinguished. Monoblasts and promonocytes cannot be distinguished from more mature cells on the basis of these cytochemical markers.

All cultured mononuclear phagocytes (and granulocytes) were positive for lysozyme (Table 1). No differences in staining intensity were found between the various stages of development. An increasing amount of lysozyme was produced during culture; the amount of this enzyme secreted per cell also increased (Table 4). A similar phenomenon was observed for β-glucuronidase (Table 4).

Ia-antigens

None of the mononuclear phagocytes from colonies cultured for 2, 4, 7, 10, 14, or 21 days showed Ia-positive cells with the immunoperoxidase technique. Frozen sections of the thymus of the same strain of mice (C_3H) showed many Ia-positive cells.

Receptors at the Cell Surface

The percentage of monoblasts, promonocytes, and macrophages with receptors for the Fc part of immunoglobulin G (Fcγ) and for complement (C3) on their

TABLE 5. Receptors at the Surface on Mononuclear Phagocytes

| | Bone marrow mononuclear phagocytes (%)[a] | | | Peritoneal macrophages (%)[b] | |
	Mono-blasts	Promono-cytes	Macro-phages	Resident (%)	Thiogly-collate induced
Fcγ-receptor rosettes	100	100	100	100	100
Phagocytosis of EIgG	95	99	98	91	98
C3-receptor rosettes	60	67	92	100	96
Phagocytosis of EIgMC	48	58	81	13	64

[a]Determined after 14 days of culture on a glass surface.
[b]Determined after 24 hr of culture on a glass surface.

TABLE 6. Phagocytosis by Mononuclear Phagocytes[a]

| Duration of culture on glass surface (days) | Bone marrow cultures (%) | | | Peritoneal macrophages (%) | |
	Mono-blasts	Promono-cytes	Macro-phages	Resident (%)	Thiogly-collate induced
1				98	99
4	30	60	93		
7	28	87	100		
10	30	88	100		
14	27	82	97		
17		81	99		
21		84	88		

[a]Incubated with Staphylococcus epidermidis (1 × 10⁶ bacteria/ml) in the presence of newborn calf serum.

membrane are shown in Table 5. More cells bearing complement receptors were found on the 14th day of culture than in the first 4 days [14]. For both types of receptor, the number of erythrocytes bound per cell increased in the sequence monoblast—promonocyte—macrophage. IgG-coated erythrocytes (EIgG) were ingested by more than 90% of the mononuclear phagocytes in the bone marrow cultures; more than 80% of the cells with C3b receptors ingested the IgMC-coated erythrocytes (EIgMC).

Phagocytosis and Intracellular Killing

The percentage of monoblasts, promonocytes, and macrophages phagocytosing *Staphylococcus epidermidis* remained roughly constant during 21 days of culture (Table 6). The phagocytic capacity increased in the sequence monoblasts—promonocytes—macrophages. Phagocytic capacity was retained in the cultures maintained by replating; for instance, on day 87 of culture 95% of the mononuclear phagocytes phagocytosed *S. epidermidis*.
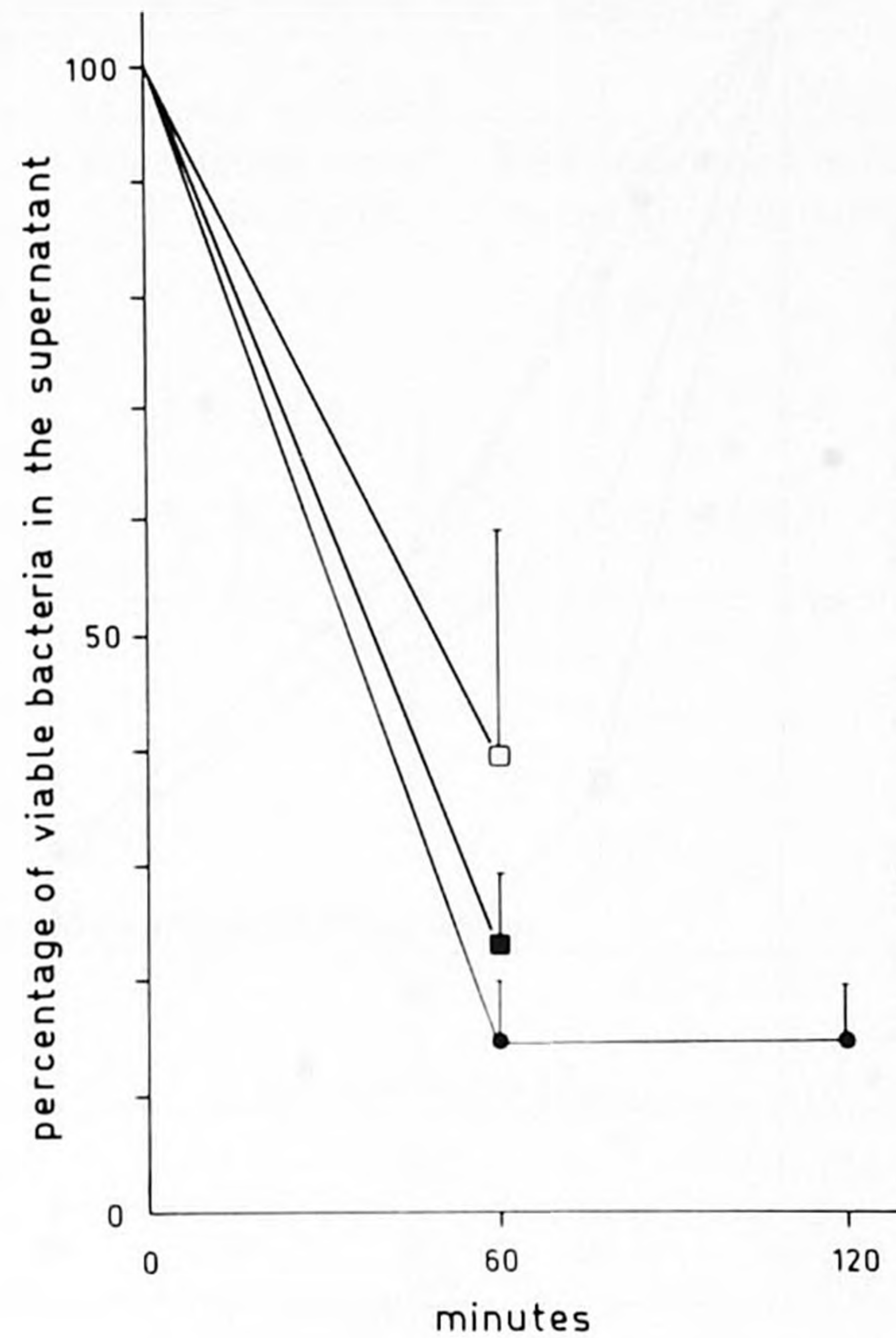
Fig. 6. Kinetics of phagocytosis of *Staphylococcus epidermidis* by bone marrow mononuclear phago-cytes and peritoneal macrophages. Bone marrow mononuclear phagocytes cultured for 14 days (●); resident peritoneal macrophages freshly harvested (■); resident peritoneal macrophages cultured for 24 hr in TFDs (□). The bars indicate the SD of three experiments.

The rate of phagocytosis measured in bone marrow mononuclear phagocytes cultured on Teflon for 14 days (Fig. 6) was similar to that of resident peritoneal macrophages collected directly from the peritoneal cavity or after 24 hr of culture on Teflon. It should be noted that in 14-day bone marrow cultures granulocytes were no longer present.

The rate of intracellular killing did not differ between bone marrow cultures incubated in TBC for 7 and 14 days and was similar to the value found for resident peritoneal macrophages cultured for 24 hr on Teflon and slightly lower than that for freshly harvested peritoneal macrophages (Fig. 7).

Thioglycollate-induced peritoneal macrophages do not provide a suitable control for killing assays, because they proved to be unable to kill staphylococci.

Pinocytosis

The percentage of cells that pinocytosed dextran sulphate is given in Table 7. Monoblasts are less active in pinocytosis than promonocytes and macrophages. The number of cells performing pinocytosis at the time-points studied was rather constant.

Cytotoxicity

The mean cytotoxic activity of 7-day-old cultures of bone marrow cells was 3.4 erythrocytes lysed per effector cell (range 2.2–2.5). Mononuclear phagocytes in 14-
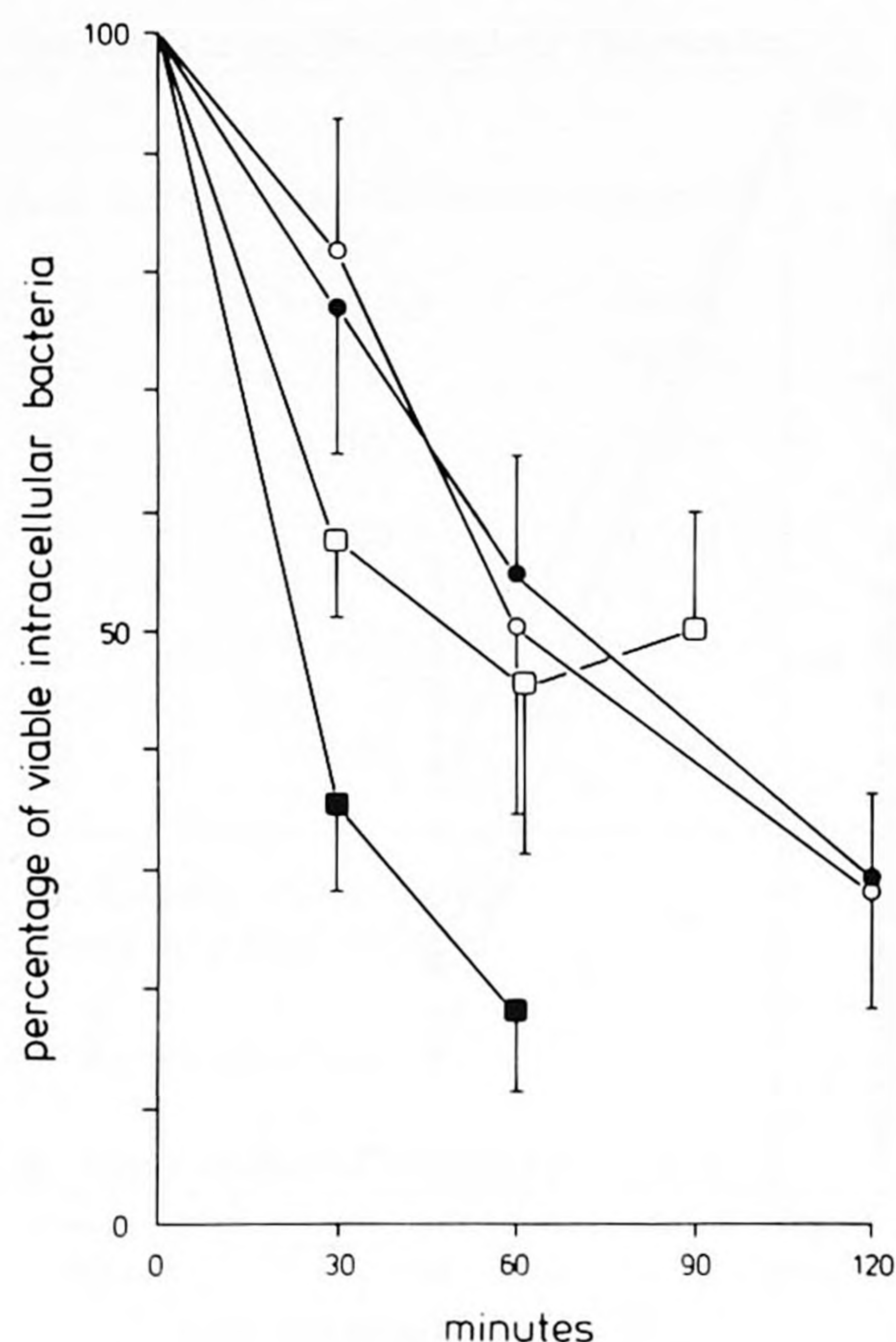
Fig. 7. Kinetics of intracellular killing of opsonized *Staphylococcus epidermidis* by bone marrow mononuclear phagocytes and by peritoneal macrophages after 15 min of phagocytosis. Bone marrow mononuclear phagocytes cultured for 7 days on Teflon surface (○); bone marrow mononuclear phagocytes cultured for 14 days on Teflon surface (●); resident peritoneal macrophages, freshly harvested (■); resident peritoneal macrophages, cultured on Teflon for 24 hr (□). The bars indicate the SD of three experiments.

TABLE 7. Pinocytosis by Mononuclear Phagocytes in Colonies[a]

Duration of incubation (days)	Monoblasts (%)	Promonocytes (%)	Macrophages (%)
7	25	96	99
10	28	99	100
14	27	95	99
17		100	98
21		100	100

[a]Incubation with 100 μg/ml dextran sulfate for 24 hr.

day-old bone marrow cultures lysed 4.5 erythrocytes per effector cell (range 3.3–6.5). The activity found for resident peritoneal macrophages and thioglycollate-induced macrophages was low (0.1 and 1.6 erythrocytes, respectively). Human peripheral blood monocytes lysed 2.0 (SD ± 0.1) red cells per cell in 18 hr in this system.

TABLE 8. Oxygen Consumption by Mononuclear Phagocytes

| | In Hanks' balanced salt solution (nmol/ 10⁶ cells/5 min) | Stimulation with | |
		Killed opsonized bacteria (nmol/10⁶ cells/5 min)	PMA[a] (nmol/10⁶ cells/5 min)
Bone marrow mononuclear phagocytes[b]	5.6 ± 4.1^c	11.8 ± 4.2	7.1 ± 3.4
Resident peritoneal macrophages	4.6 ± 3.4	5.0 ± 4.3	6.3 ± 4.1
Thioglycollate-induced macrophages	8.2 ± 5.2	10.1 ± 8.0	9.6 ± 3.4

[a]Phorbol myristate acetate.
[b]Cultured for 14 days.
[c]Mean $\pm$ SD.

TABLE 9. Locomotion of Mononuclear Phagocytes

| | Gey's solution (%) | Casein (%) | |
		1 mg/ml	5 mg/ml
Bone marrow mononuclear phagocytes[a]	100	124 ± 7.9	167 ± 25.5
Peritoneal macrophages[b]	100	101 ± 4.1	118 ± 15.5

[a]Cultured for 14 days on Teflon in the presence of conditioned medium.
[b]Freshly harvested, noninduced.

Oxygen Consumption

The oxygen consumption of bone marrow mononuclear phagocytes cultured for 14 days was of the same order as that of resident and thioglycollate-induced macrophages (Table 8). After stimulation with killed opsonized bacteria, increment of oxygen consumption by the bone marrow mononuclear phagocytes was observed. Because of their somewhat higher resting oxygen consumption, the increment was less pronounced for thioglycollate-induced cells; for resident peritoneal macrophages virtually no increment in oxygen consumption was measured. Stimulation with PMA gave little increment of oxygen consumption.

Locomotion

The results of the studies on locomotion are given in Table 9. To compare the distance of migration between three different experiments, the migration in the absence of a chemoattractant is arbitrarily put at 100%. The bone marrow mononuclear phagocytes cultured in vitro migrated more actively than freshly harvested peritoneal macrophages did.

DISCUSSION

The results of the present study, in which the development of mononuclear phagocytes from mouse bone marrow in long-term cultures were studied and the

morphologic, cytochemical, and functional characteristics of monoblasts, promono-cytes, and macrophages were compared with those of resident macrophages, provide a basis for an attempt to define the characteristics under study in terms of maturation and activation.

A number of these characteristics seem to be intrinsic properties of all mono-nuclear phagocytes, which means that they must be relatively independent of maturity and of state of activation. The activity of esterase and lysozyme and the presence of Fcγ-receptors are examples of this. Other characteristics change appreciably during maturation, such as peroxidatic activity [37], the presence of C3-receptors, and pinocytic and phagocytic activity. A third category is formed by characteristics that seem to be dependent on the state of activation and may change during culture (more or less) independent of the stage of maturation (i.e., whether a cell is a monoblast, a promonocyte, or a macrophage). According to the literature [8,50,51] and our findings, aminopeptidase activity and 5'-nucleotidase activity are probably examples of the latter category. Finally, adherence, spreading of cells, and ingestion of particles via the C3-receptor seem to be dependent not only on activation [1,29] but also on maturation. It should, however, be kept in mind that this classification is not absolute, since a number of the intrinsic characteristics can be modulated: the intensity of esterase activity increased during culture, and, as we have shown elsewhere, Fc-receptor expression is enhanced by CSF [39]. Moreover, the notion that CSF-1 is not only required for survival of bone marrow mononuclear phagocytes and for prolifer-ation [36], but also stimulates Fc-receptor expression and the synthesis and secretion of plasminogen activator [16,20], interleukin-1 [24], and prostaglandins [19], implies that the state of activation and the stage of development cannot always be assessed separately in in vitro culture systems where CSF is present.

For a number of functions (intracellular killing, oxygen consumption, locomo-tion, and cytotoxicity) the relative contribution of monoblasts, promonocytes, and macrophages could not be assessed yet, but the functional state of the whole mono-nuclear phagocyte population in bone marrow cultures could be compared with peritoneal macrophages. The ability of the mononuclear phagocytes in such cultures to phagocytose and kill bacteria appears to be similar to that of resident peritoneal macrophages, and their oxygen consumption after stimulation by bacteria is compar-able to that of thioglycollate-induced macrophages; however, their motility and antibody-dependent cytotoxicity is considerably better than that of peritoneal macro-phages. Thus, with respect to the last of these functions, the bone marrow mononu-clear phagocytes should be considered more activated than resident or even elicited macrophages. This is supported by other authors' finding that cells of these bone marrow cultures produce plasminogen activator [20,26] and may be rendered tumor-icidal [22].

Using the liquid culture system for bone marrow cells, Lohmann-Matthes et al showed that on day 5 of culture some cells display natural killer activity [21]; the effector cell in these studies was thought to be a nonphagocytic, nonadherent mono-nuclear phagocyte [7]. The claim of these authors that this effector cell is a promon-ocyte can easily be rejected in view of the results obtained in the present study and previous studies done in our laboratory, which established the adherent and phagocytic properties of the promonocyte [14,44,46].

Unlike others [53], we were unable to detect Ia-antigen on the membrane of the cultured bone marrow mononuclear phagocytes. The percentage of mononuclear phagocytes positive for Ia in bone marrow cultures has been reported to range from zero up to 80% [4,31,53]. However, it is not clear which types of mononuclear phagocyte in the cultures are positive. The strain of mice, the type of antiserum used, and methodological differences may account for the differences in Ia-antigen expression encountered. With the technique used in the present study, Ia-positive cells are readily detected in frozen sections of spleen, lymph nodes, and thymus [41] which means that if Ia-antigens occur at all on cultured bone marrow mononuclear phagocytes, they are not present in high density. Others have shown that Ia-antigen expression on bone marrow mononuclear phagocytes is inducible with lymphokines [4], and the disappearance of Ia-antigens from the surface of macrophages in culture has also been described [4,34].

The proliferative characteristics of the cells in our culture systems reflect that of mononuclear phagocytes in vivo: monoblasts and promonocytes proliferate actively, and some macrophages seem to proliferate too. In primary bone marrow cultures, on day 4, approximately one mononuclear phagocyte colony is found per 10^3 nucleated bone marrow cells plated; on the basis of this figure, the progeny of one monoblast would be put at approximately 1.7×10^3 mononuclear phagocytes on day 7, and at more than 7.7×10^3 mononuclear phagocytes on day 14. If this estimate is correct, there must have been approximately 11 divisions in the primary cultures by day 7, and 13 divisions by day 14. Such calculations are based on the assumption that the observed increase in colony number from day 4 to day 10 is due solely to the formation of satellite colonies and not to proliferative activity of colony-forming cells with a longer lag time [35]. There is a decrease in the proliferative activity and in the [^{3}H]thymidine labeling index after the first week of culture; this trend becomes even more pronounced after the second week. Our results do not indicate that a shortage of nutrients is responsible for this decrease. This decrease in the labeling index occurred in spite of addition of fresh conditioned medium twice weekly. The amount of CSF-1 added to these cultures can be estimated to be in the order of 150 U of CSF-1. In view of the recent data of Tushinski et al [36], this concentration may have been suboptimal at the high number of cells present in the second week of culture.

Production of inhibitory substances could also explain the decreased proliferation. Substances inhibiting cell division, such as prostaglandins [19], monocytopoiesis inhibitor [49], lactoferrin [3], interferon [10,26], thymidine [32], and arginase [18], might be produced in bone marrow cultures. Since no decrease of the arginine concentration was found in our cultures, arginase can be ruled out as the cause of the decline in proliferation. Spontaneous interferon production was not found in our cultures either (van der Meer, Heremans, and Billiau, unpublished observations). No search has been made yet to find any of the other inhibitors of proliferation.

Replating makes it possible to maintain the cultures for long periods. Calculations similar to those described above indicated that in the experiments with the highest proliferation rate more than 20 divisions took place during a period of 10 weeks. The divergence in proliferation rate in different experiments (which were carried out over a number of years) may be due to the use of different batches of conditioned medium and serum. Nevertheless, all our experiments point to at least

limited self-renewal in the long-term cultures. Self-renewal properties of the mono-blast in vitro have already been suggested by Goud et al [14]. Moreover, our findings that in primary cultures of bone marrow the number of monoblasts is rather constant after day 4 also suggests that the monoblasts have stem cell property.

From the present study it is clear that these liquid culture techniques for the culture of bone marrow cells yield pure cultures of mononuclear phagocytes. A pure population of mononuclear phagocytes of good quality, grown in vitro has value not only for the study of the biological characteristics of these cells but also for cell transfer studies and immunologic investigations. With respect to functional quality and proliferative capacity these cells are more physiologic than the macrophage tumor cell lines. However, when cultured bone marrow mononuclear phagocytes are to be used for further experiments, it must be kept in mind that the population is not homogeneous with respect to developmental stage, and that the cells are more active than resident macrophages in many respects.

The characteristics of murine bone marrow mononuclear phagocytes in long-term cultures with embryonic fibroblast-conditioned medium were studied to deter-mine the stage of development and state of activation of these cells. Two liquid culture systems were used: for studies on the morphology, cytochemistry, and functional characteristics at the cellular level, the cells were cultured adherent to a glass surface; and for experiments where the cells were needed in suspension (replating experi-ments, and studies on locomotion, intracellular killing, and cytotoxicity) use was made of Teflon culture systems.

Three developmental stages of mononuclear phagocytes could be recognized easily in these cultures: monoblasts, promonocytes, and macrophages. In cultures on a glass surface, these cells grow in colonies separate from granulocytic colonies. When incubation is prolonged beyond 7–9 days, the granulocytes die, leaving pure mononuclear phagocyte cultures. Primary cultures, in which monoblasts, promono-cytes, and some macrophages proliferate, can be maintained for 3–4 weeks. Calcula-tion showed that one monoblast present on day 0 gives rise to a progeny of more than 7×10^3 mononuclear phagocytes by day 14; after that, the rate of proliferation declines despite the addition of fresh media. Regular replating of the cells cultured on Teflon made it possible to maintain proliferation over a period of almost 200 days.

The cells in culture have the typical characteristics of mononuclear phagocytes, as judged by light microscopy, α-naphthyl butyrate esterase activity, lysozyme activ-ity, presence of receptors for Fc and C3, and endocytic, microbicidal, and cytotoxic activity. The 5'nucleotidase activity, ingestion of erythrocytes via C3-receptor, loco-motion, and antibody-dependent cytotoxicity indicate that the cultured bone marrow mononuclear phagocytes are more active than resident macrophages, and as active as or even more active than thioglycollate-induced macrophages. In conclusion, the population of mononuclear phagocytes in the liquid cultures of bone marrow is heterogenous with respect to developmental stage and state of activation.

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