# PROLIFERATIVE CHARACTERISTICS OF MONOBLASTS GROWN IN VITRO*

By THEO J. L. M. GOUD and RALPH VAN FURTH

*(From the Department of Infectious Diseases, University Hospital, Leiden, The Netherlands)*

A liquid culture system, in which mononuclear phagocyte colonies are grown on a glass cover slip, was developed to identify and characterize the promonocyte precursor (1), which was not possible with the techniques then available (2, 3). The mononuclear phagocyte colonies developing in these cultures are composed of three types of cells, i.e. monoblasts, promonocytes, and macrophages, each having distinct morphological, cytochemical, and functional characteristics (1). In these colonies the monoblasts and promonocytes are dividing cells, whereas the macrophages do not proliferate.

The characteristics of promonocytes and macrophages developing in colonies are similar to those shown by the same types of cells isolated from animals (1, 2, 4, 5). The monoblast had not been previously characterized. In earlier studies the proliferative characteristics of the promonocyte—such as the DNA synthesis and cell-cycle times—were determined during the normal steady state, an acute inflammatory response (3, 6), and treatment with anti-inflammatory drugs (7, 8). The multiplication of immature mononuclear phagocytes obtained in liquid cultures has now made it possible to study the proliferative behavior of the monoblast.

The present study was performed to determine the duration of the various phases of the cell cycle of the monoblast and the promonocyte grown in mononuclear phagocyte colonies. In addition, the number and size of these colonies were estimated and the development of individual mononuclear phagocyte colonies was studied with respect to the change in cellular composition and growth rate.

## Materials and Methods

*Animals.* In all studies specific pathogen-free Swiss male mice weighing 25–30 g (Central Institute for the Breeding of Laboratory Animals, TNO, Bilthoven, The Netherlands) were used.

*Bone Marrow Cell Cultures.* The techniques for harvesting and culturing bone marrow cells have been described in detail elsewhere (1). Unless otherwise stated, about $5 \times 10^4$ nucleated bone marrow cells were suspended in 2 ml Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N. Y.) containing 20% horse serum (Flow Laboratories, Inc., Ayrshire, Scotland) and 20% conditioned medium, obtained by incubation of embryonic mouse fibroblasts.[1] The cell suspension was incubated in a Leighton tube with a flying cover slip (10 × 35 mm) at 37°C in a humidified atmosphere with a constant flow of 10% $CO_2$ in air.

*Colony Mapping.* The bone marrow cell suspension was cultured in a stoppered Leighton tube gassed with 10% $CO_2$ in air. The Leighton tube was then placed in a strictly horizontal position on an inverted phase-contrast microscope (Carl Zeiss, Oberkochen, West Germany). The inner

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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.

atmosphere of the tubes was kept at 37°C with an air-curtain incubator (Sage Instruments, White Plains, N. Y.) guided by a thermistor in another Leighton tube placed parallel to the first with respect to the air-curtain incubator. The room was kept dark during the culture period. All colonies in a strictly defined area of the cover slip were studied; the growth of the colonies during incubation was followed by frequent observations from 24 h onward and at each observation the colonies were photographed with a Polaroid camera (magnification of 250).

[$^3$H]*Thymidine Labeling.* The techniques for cell labeling with 0.1 μCi/ml [$^3$H]thymidine (sp act 6.7 Ci/mmole, New England Nuclear, Boston, Mass.) and autoradiography have been described in detail elsewhere (9). The exposure time was 10–11 days. Cells containing less than three grains over the nucleus were considered negative.

*Light Microscopy.* After gentle removal from the Leighton tube the cover slips were rapidly air dried, fixed in absolute methanol for 10 min, and stained with Giemsa stain for 15 min.

*Colony and Cell Counts.* Each group of four or more cells was considered a colony. Counts were done in the Giemsa-stained preparations or in the Polaroid micrographs.

*Identification of the Cells.* Mononuclear phagocytes grown in in vitro colonies were defined according to the criteria described elsewhere (1). The following types of cells can be distinguished.

MONOBLASTS. Round cells (diameters 10 × 12 μm) with a small rim of strongly basophilic cytoplasm.

PROMONOCYTES. Slightly stretched cells (diameters 13 × 34 μm) with one pseudopod and a basophilic cytoplasm; nuclear-to-cytoplasmic ratio about 1.

MACROPHAGES. Large, elongated cells (diameters 17 × 69 μm) with two or more pseudopods and a grayish-blue cytoplasm; nuclear-to-cytoplasmic ratio lower than 1.

## Results

*Number of Mononuclear Phagocyte Colonies.* The number of immature bone marrow cells that can initiate a mononuclear phagocyte colony was determined in Giemsa-stained preparations by counting the number of colonies developing from $2.5 \times 10^4$ nucleated bone marrow cells. The results show that the number of colonies increases with time (Table I), which indicated that the colony-forming cells start to divide at different intervals after the initiation of the culture. From 96 h of incubation on no new colonies develop; this shows that all colony-forming cells have divided and given rise to at least four cells. The mean number of mononuclear phagocyte colonies at 96 h, calculated for four experiments (two from Table I and two additional experiments only counted on day 4) amounts to $25/2.5 \times 10^4$ nucleated bone marrow cells.

*Size of Mononuclear Phagocyte Colonies.* If colony-forming cells do not start to proliferate at the same time after plating, the number of cells per colony can be expected to be variable at given time points of incubation. Information about the distribution of colony size after various periods of incubation was obtained

TABLE I

*Mean Number of Mononuclear Phagocyte Colonies Per Cover Slip**

| | Incubation time in hours | | | | | |
|---|---|---|---|---|---|---|
| | 24 | 48 | 72 | 96 | 120 | 168 |
| Exp. 1 | 2.0 | | 20.5 | 27.0 | | 27.0 |
| Exp. 2 | 0.8 | 14.8 | 21.8 | 24.2 | 24.2 | |

* $2.5 \times 10^4$ nucleated bone marrow cells plated per cover slip; in each experiment four cover slips per time point.

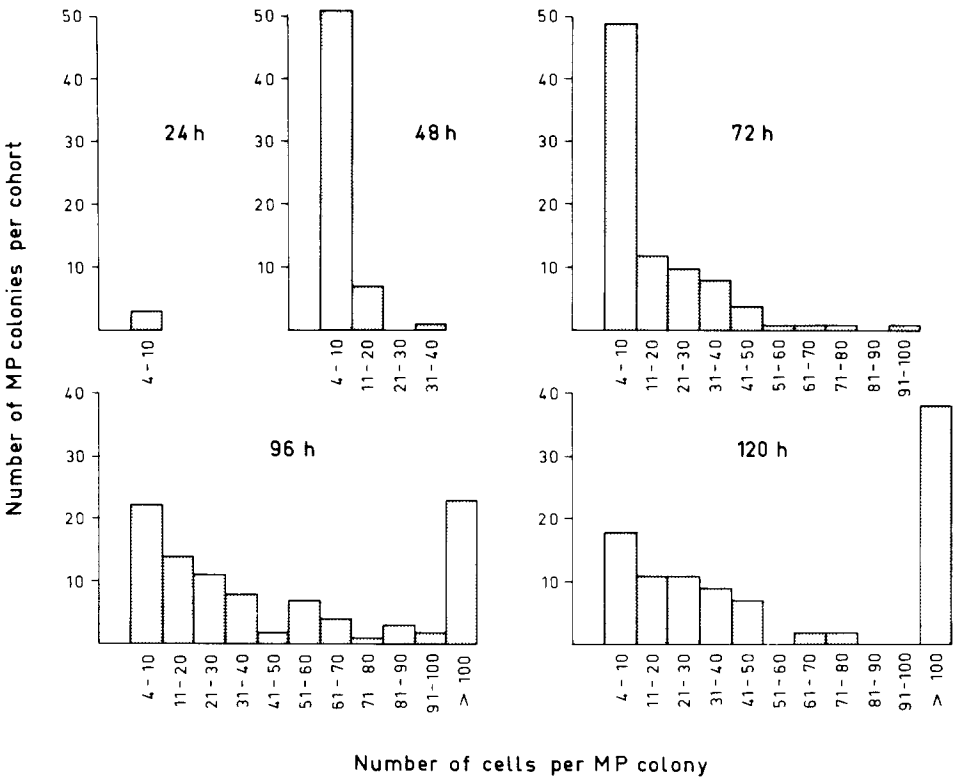


FIG. 1. Distribution of the size of mononuclear phagocyte (MP) colonies after various periods of incubation. $2.5 \times 10^4$ nucleated bone marrow cells were plated per cover slip and four cover slips counted per time point to determine the number of cells in each mononuclear phagocyte colony. The numbers of colonies of comparable size are expressed by bars representing cohorts of 4–10, 11–20, 21–30, etc., cells per colony.

by counting the number of cells in each colony (done for exp. 2 of Table I). Counts were not done in cultures after 120 h of incubation, because after that crowding of the cells in the center of the colonies leads to inaccuracy. The results given in Fig. 1, where the colonies are arranged according to size in cohorts of 4–10, 11–20, 21–30, etc. cells per colony, show a continuous increase in the number of cells per colony with increasing duration of incubation.

The progressive heterogeneity in the size of the various colonies is remarkable; for instance, at 120 h of incubation there are both very large colonies of more than 100 cells and also a considerable number of colonies still having only 4–10 cells. Calculation of the logarithm ($\log_2 N$) of the number of cells per colony, which gives the mean number of cell generations produced by the cell initiating that colony, shows, however, that the distribution of the numbers of cell generations, computed from the cell numbers per colony at 120 h of incubation, is normal ($\chi^2 = 5.42$, d.f. $= 8$, $0.70 < P < 0.80$) (Fig. 2).

*Growth Rate of Mononuclear Phagocytes Colonies.* To determine the growth rate of mononuclear phagocyte colonies, the regression line was computed for the increase of the geometric mean of the cell numbers per colony during

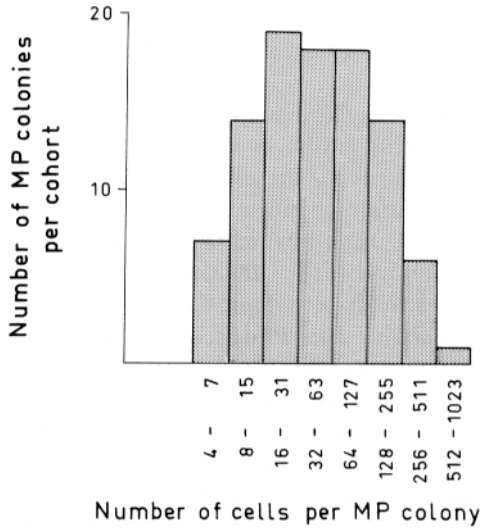


FIG. 2. Distribution of the size of mononuclear phagocyte (MP) colonies in cultures incubated for 120 h (see Fig. 1). The colonies are arranged on the basis of the logarithm of the number of cells per colony ($\log_2 N$) which gives the mean number of cell generations produced per colony. Colonies with a comparable number of cell generations are shown by bars representing cohorts of 4–7, 8–15, 16–32, etc., cells per colony.

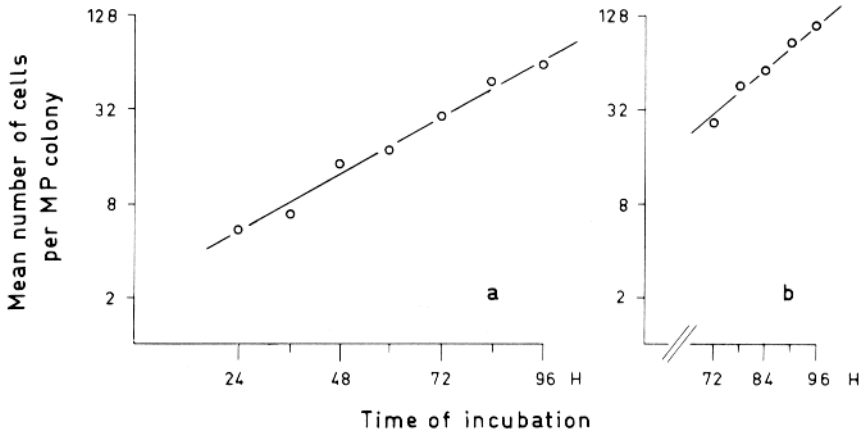


FIG. 3. Growth curves of mononuclear phagocyte (MP) colonies. Each point represents the geometric mean of the number of cells per colony ($\log_2 N$). For (*a*) four cover slips and for (*b*) eight cover slips were counted per time point.

incubation (Fig. 3 *a*). The mean colony-doubling time, i.e. the time needed to double the number of cells per colony, is calculated from the slopes of the regression lines and amounts to 19.5 h (mean of three experiments). But the mean colony-doubling time only gives a rough approximation of the growth rate of the proliferating cells. In the initial stage of the culture the slope of the regression line is influenced, because the calculated mean number of cells per colony is artificially increased by the absence of groups of cells which have not yet reached the four-cell stage (scoring criterion for a colony). In the later period

of incubation an increasing number of mononuclear phagocytes become mature, i.e. macrophages and stop proliferating, which also leads to a decrease of the slope of the regression line.

To reduce these influences, the growth rate was studied during a short period at a later stage of incubation (72–96 h) in the same number of colonies (the five largest colonies per cover slip), all of which are made up almost solely of proliferating cells (8-h labeling index with [$^3$H]thymidine, 86%). The mean colony-doubling time, calculated from the regression curve (Fig. 3 *b*), now amounts to 11.9 h, which indicates that the cell-cycle time of the proliferating mononuclear phagocytes in these colonies is rather short.

*Growth Pattern of Mononuclear Phagocyte Colonies.* The growth pattern of the mononuclear phagocyte colonies was studied in more detail by the method of colony mapping, i.e., the development of the individual colony was followed with the inverted phase-contrast microscope at 12-h intervals.

The serial micrographs of a mononuclear phagocyte colony show the cells to be discrete and single-layered during the initial days of the incubation; in the later stages some crowding is seen in the center (Fig. 4). The numerical course of this colony shows an initially rapid exponential growth which levels off at 72 h (Fig. 5).

When the cytological picture and the growth pattern of this colony are compared, it is striking that during the initial phase only round cells are present in the colony and that stretched cells appear when the rapid exponential growth starts to slow down. The round cells were identified in a previous study as monoblasts; the stretched cells belong to two distinct cell types, promonocytes and macrophages (1). 29 other colonies studied in this way showed similar growth curves and a similar distribution of the cell types.

Fig. 5 shows that the colony growth occurs mainly during the initial growth phase. This is confirmed by mathematical analysis of all of the computed growth curves (the slopes of both parts of the growth curve were computed separately from the cell counts in the micrographs; if less than 10% of the cells of a colony were stretched the observation was used to calculate the initial part, if 10% or more for the second part), which demonstrates a very high degree of correlation between the number of cells per colony at the end of incubation and the number of cells reached when the exponential growth begins to slow down ($r = 0.88$, $P < 0.001$) (the latter time point was determined by computing the intersection of the slopes of both parts of the growth curve). The mean colony-doubling time during the exponential growth phase, computed from the initial slopes of the 30 growth curves, amounts to 11.0 h (95% confidence limits, 10.4–11.5 h). Since the colony consists only of monoblasts during the initial growth phase and nearly all of these cells proliferate (labeling index after incubation with [$^3$H]thymidine for 8 h, 92–96%), their cell-cycle time must be roughly the same as the calculated colony-doubling time. The small differences between the colony-doubling times of the various colonies do not contribute to differences in colony size at the end of incubation ($r = 0.23$, $P > 0.1$).

There is considerable variation in the calculated time points of the onset of retardation of the growth (calculated range, 54–>96 h), which shows no correlation with the number of cells per colony at the end of incubation ($r = 0.04$). It

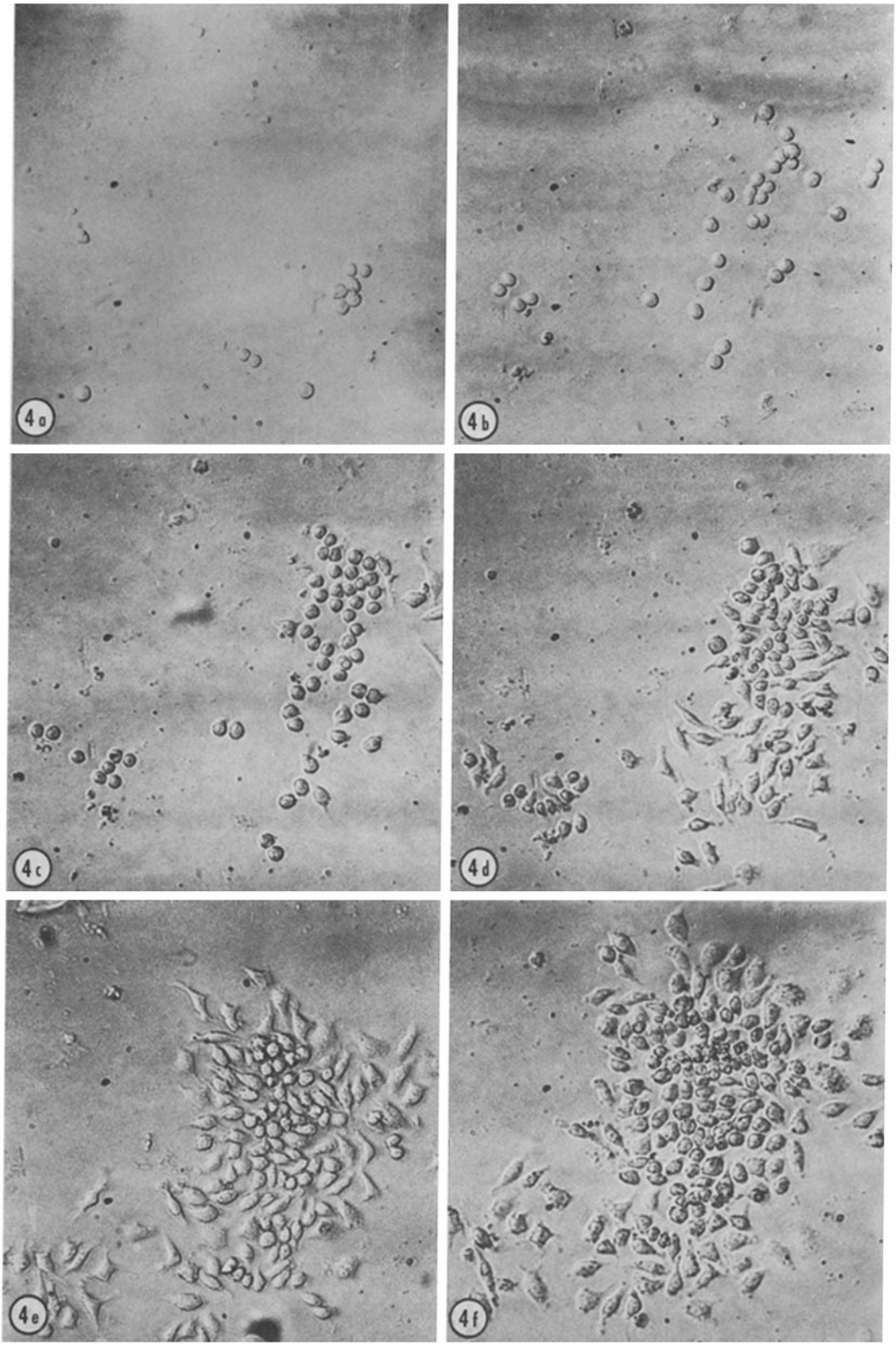


FIG. 4 *a–f*. Colony-mapping based on serial micrographs of a mononuclear phagocyte colony during the incubation period from 36 to 96 h. Initially, only round cells (monoblasts) are present; from 72 h onward, stretched cells (i.e., promonocytes and macrophages) appear. Note the loose structure of the colony. Phase contrast, × 211.

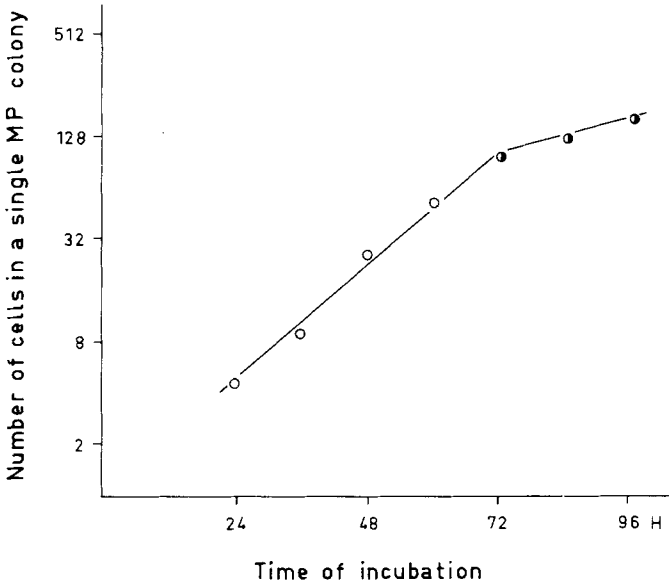


FIG. 5. Growth curve of the mononuclear phagocyte (MP) colony shown in Fig. 4. Initially there is rapid exponential growth, levelling off occurring after 72 h. Open circles, only round cells (monoblasts) present; half-closed circles, round cells and stretched cells (i.e., promonocytes and macrophages) present.

should be noted that at 96 h, 7 of the 30 colonies are still in the stage of rapid proliferation; for the remainder, the mean time point of the onset of retardation is calculated to be 65.6 h.

Extrapolation of the initial part of the growth curves to the time axis gives the mean lag time, i.e. the interval between the start of the culture and the time at which the colony-forming cell divides for the first time, which amounts to 23.2 h (calculated range, −6.4–54.2 h). Statistical analysis of the lag times shows that they are normally distributed. ($\chi^2 = 1.01$, d.f. = 5, $0.95 < P < 0.98$) (Fig. 6). The correlation between the lag time and the colony size at the end of incubation is significant ($r = -0.55$, $P < 0.01$), suggesting that the single main factor in the variation of the colony size is the time point at which the colony-forming cell starts to divide.

When the development of individual cells is followed at frequent intervals (every 15 min) with the phase-contrast microscope, the cell-cycle time of individual cells can be measured by recording the time points at which a particular cell originates from a mitosis and subsequently divides again (Fig. 7). For two monoblasts the measured cell-cycle times amount to 11.1 and 11.6 h; and for three promonocytes to 12.0, 12.7, and 13.7 h.

*Determination of Cell-Cycle Time and DNA-Synthesis Time of the Monoblasts and Promonocytes by [$^3$H]Thymidine labeling.* The cell-cycle time of the monoblasts and promonocytes in colonies was also studied by incubating the cells of 96-h cultures with [$^3$H]thymidine for 45 or 90 min and then assessing the increase in the labeling index. To eliminate the influence of the unequal distribution of the cells over the various cell-cycle phases, occurring in expand-

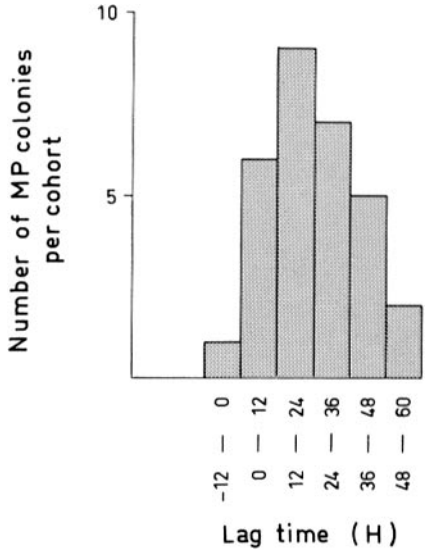


FIG. 6. Distribution of lag times after which mononuclear phagocyte (MP) colony-forming cells start to divide. The lag times are computed by extrapolation of the initial part of the growth curve to the time axis. The numbers of colony-forming cells with comparable lag times are shown by bars representing cohorts with lag times of −12–0, 0–12, 12–24 h, etc.

ing populations, the 45-min incubation was started 22.5 min after the beginning and terminated 22.5 min before the end of the 90-min incubation period.

From the labeling indices of the monoblasts and promonocytes the cell-cycle times are calculated according to the equation:

$$t_c = (100/\Delta i) \times \Delta t$$

in which $t_c$ is the cell-cycle time, $\Delta i$ the increment of the labeling index during $\Delta t$, and $\Delta t$ the difference between the respective incubation times. This equation may only be applied if proliferation occurs in all monoblasts and promonocytes, which may be assumed because 8 h of cell labeling with [$^3$H]thymidine gives labeling indices of 92.1 and 88.2% for the monoblasts and promonocytes, respectively (1). The cell-cycle time obtained in this way amounts to 11.9 h for the monoblast and 11.4 for the promonocyte (Table II).

From these labeling indices the DNA-synthesis time can be computed according to the equation:

$$t_s = (i_t/\Delta i) \times \Delta t - t$$

in which $t_s$ is the DNA-synthesis time, $i_t$ the labeling index after incubation during time $t$, and $t$ the incubation time. The calculated DNA-synthesis times are 5.7 h for monoblasts and 5.5 h for promonocytes (Table II).

*Determination of the G2, Mitosis, and G1 Times of the Proliferating Cells of Mononuclear Phagocyte Colonies.* The mitosis time was measured by incubation of the cells with [$^3$H]thymidine for various periods to determine the course of the labeling index of the cells in mitosis. Since the cells round up during mitosis, differentiation between monoblasts and promonocytes is not possible in this phase.

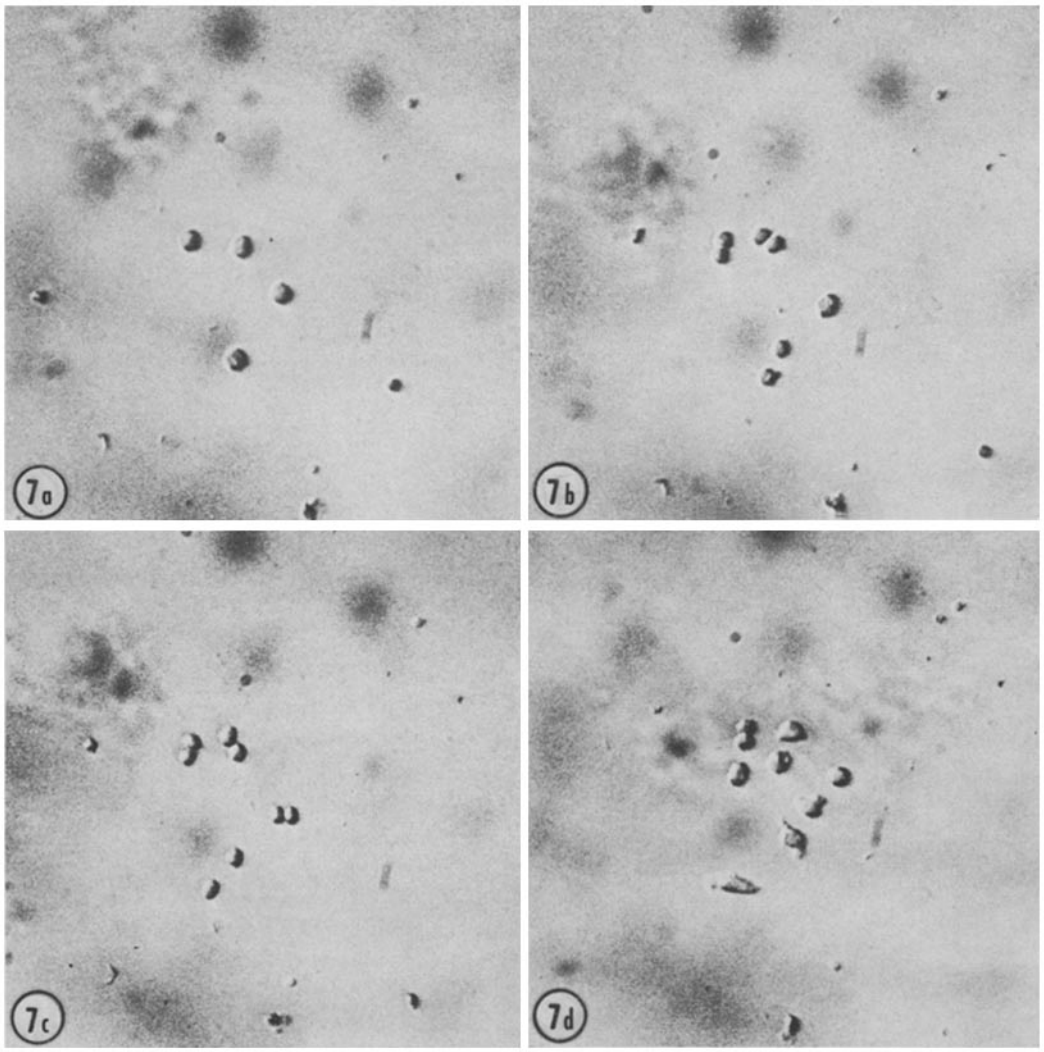


FIG. 7. *a–h*. Serial micrographs of mononuclear phagocyte colony on the 2nd and 3rd days of incubation. In (*b*), three cells have divided and one cell is about to divide. In (*d*) the cell at the upper left shows another division, and two cells have begun to stretch while retaining their ability to divide, as shown in (*e*) and (*f*). Phase contrast, × 250.

The results of this study in 96-h cultures are shown in Fig. 8. After an initial period without labeled cells in mitosis, the labeling index gradually increases until finally all mitotic figures are labeled. The mean mitosis time, calculated from the slopes of the curves obtained in two experiments, is 1.8 h. The G2 time, i.e. the interval between the end of DNA synthesis and the beginning of mitosis, can be derived from the duration of incubation before the first labeled mitosis occurs and amounts to 0.6 h. The duration of the G1 phase can be estimated by subtraction of the values calculated for the DNA synthesis, G2 phase, and mitosis times from the cell-cycle time, and amounts to approximately 3.5–3.8 h.

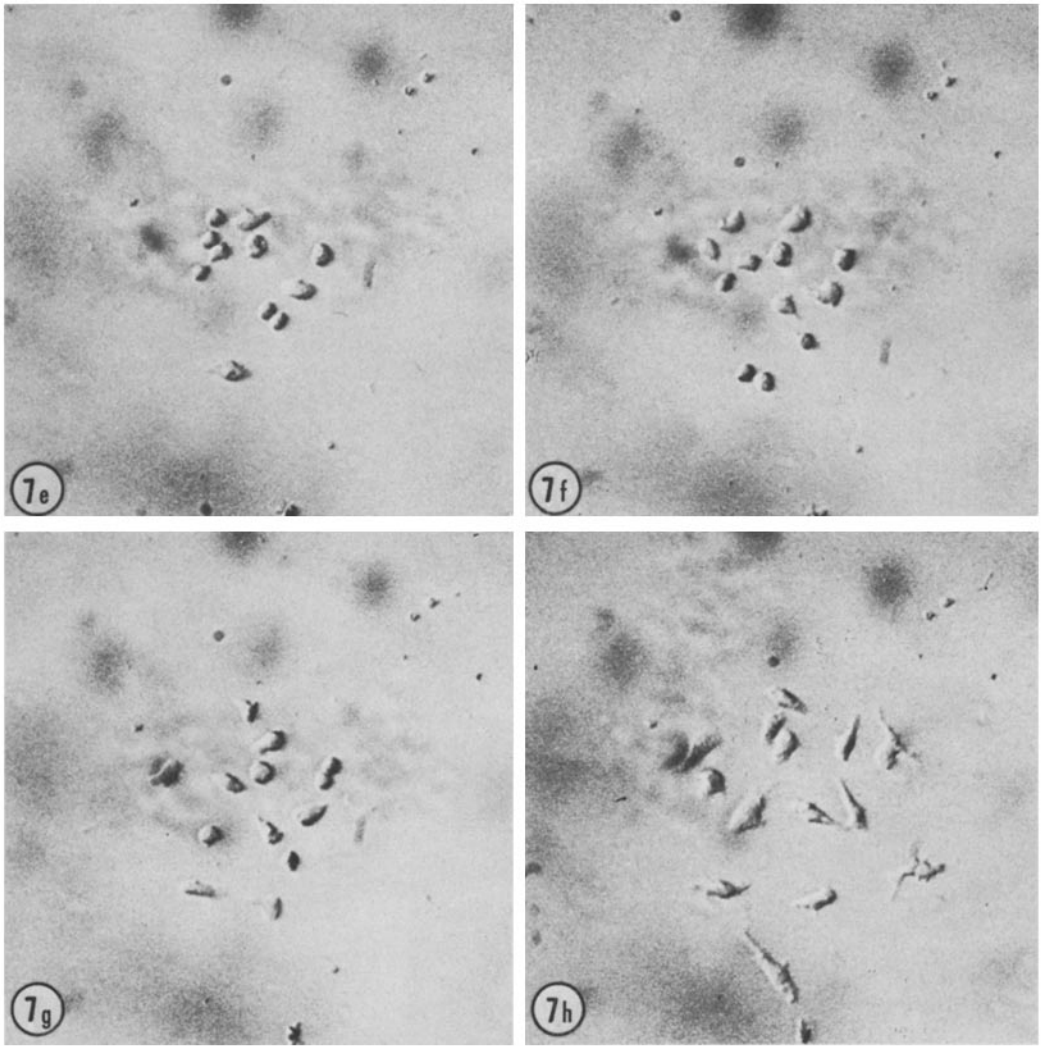


FIG. 7E–7H.

## Discussion

This study performed with the liquid culture technique concerned the growth characteristics of the mononuclear phagocytes growing in colonies. From the growth curves determined for individual colonies, observed with the inverted phase-contrast microscope, it was computed that after a lag period, which is different for each colony (mean lag time, 23.2 h), the colony-forming cell starts to divide and all of the cells thus produced proliferate rapidly for a number of generations. In this initial proliferation phase only monoblasts are present in the colony. After a certain time the growth rate of the colony levels off and this retardation invariably coincides with the appearance of promonocytes and macrophages in the colony. These findings lead to the conclusion that in the development of mononuclear phagocyte colonies the monoblast is the precursor

TABLE II

*Cell-Cycle Time ($t_c$) and DNA-Synthesis Time ($t_s$) of Monoblasts and Promonocytes in In Vitro Colonies**

| | Monoblasts | Promonocytes |
|---|---|---|
| $i_t$ | 54.5% | 55.0% |
| $\Delta i$ | 6.3% | 6.6% |
| $t_c$ | 11.9 h | 11.4 h |
| $t_s$ | 5.7 h | 5.5 h |

$i_t$, labeling index after incubation during time $t$ (in the table, 45 min). $\Delta i$, increment of the labeling index during $\Delta t$. $\Delta t$, difference between the respective incubation times.

* Incubation in medium containing 0.1 μCi/ml [$^3$H]thymidine for 45 min or 90 min; studies performed on the 4th day of incubation.

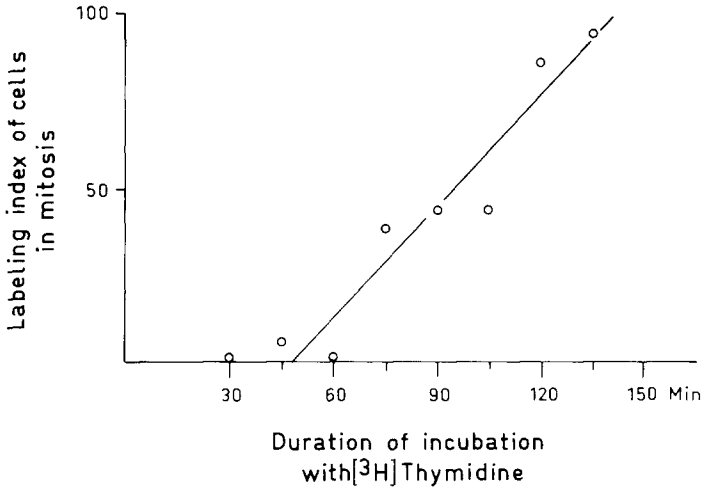


FIG. 8. The effect of incubation for various periods in medium containing 0.1 μCi/ml [$^3$H]thymidine on the labeling index of the cells in mitosis in mononuclear phagocyte colonies of 96-h cultures.

of the promonocyte, which confirms the cell sequence previously established on the basis of morphological, cytochemical, and functional criteria (1).

The cell-cycle time of the monoblast was determined by three methods. Colony-mapping gave a cell-cycle time of 11.0 h; the cell-cycle time determined by [$^3$H]thymidine labeling amounted to 11.9 h; and when individual cells were followed from mitosis to mitosis with the inverted phase-contrast microscope a cell-cycle time of 11.4 h was found. For the promonocyte, the cell-cycle time determined by the [$^3$H]thymidine labeling method amounted to 11.4 h and in the individual cell studies to 12.8 h.

The independently obtained results for the monoblasts and promonocytes show an excellent agreement, and are confirmed by the colony-doubling time (11.9 h) calculated for the largest colonies from cell counts in Giemsa-stained preparations. The cell-cycle time found for the promonocyte differs from that

determined in vivo for this cell type (16.2 h) (6), probably due to the lack of regulatory mechanisms in the in vitro situation. It is noteworthy that in the acute inflammatory reaction the promonocyte cell-cycle time reaches the same magnitude (10.8 h) (6) as found in this study.

The DNA-synthesis time determined by [$^3$H]thymidine labeling amounted for the monoblast to 5.7 h and for the promonocyte to 5.5 h. These values are in agreement with those obtained by Testa and Lord (10), who calculated a DNA-synthesis time of 5.8 h for the whole group of proliferating granulocytes and mononuclear phagocytes in colonies. It seems that the short cell-cycle time of the monoblasts and promonocytes in vitro is determined by the short DNA-synthesis time. For the promonocytes in vivo it has already been demonstrated that reduction or prolongation of the cell-cycle time, e.g. during an inflammatory reaction or after administration of azathioprine (6, 8), is governed mainly by the duration of DNA synthesis.

The variation in colony size found in the Giemsa-stained preparations and which has also been observed by others (11–13), raises the question of whether the cell population from which the colonies arise is heterogeneous. Since mathematical analysis shows that the numbers of cell generations produced by the colony-forming cells are normally distributed, the variation in colony size must be based on a normal biological variability of the colony-forming cells. This is confirmed by analysis of the lag times, the factor mainly responsible for the variation in colony size, which too proved to be normally distributed. All this indicates that the variation in colony size is not likely to be dependent on heterogeneity of the initiating cells, and this is also supported by the fact that the size of the colony can be greatly influenced by changes in the culture conditions, e.g. the amount of colony-stimulating factor added to the culture (references 11, 12, 14–17 and footnote 1).

Insight into the proliferation pattern of mononuclear phagocytes in vitro can be obtained by analysis of the increase of each cell type during incubation (Fig. 9). The curves show that the monoblast is initially predominant in a colony; the increase of the promonocytes occurs later, and still later macrophages appear in increasing numbers. From this pattern it may be concluded that promonocytes are derived from monoblasts and that the macrophages originate from promonocytes.

A mathematical analysis of the numerical course of the different cell types, described in detail in the Appendix, shows that some of the dividing monoblasts and promonocytes replicate themselves, and that after division others differentiate into the next cell stage. It is of interest that the proportion of dividing cells that are self-replicating diminishes with the duration of incubation. Initially (day 2), about 75% of the monoblasts are self-replicating and the remainder give rise to promonocytes, whereas on the 4th day of incubation about 90% of the progeny of the dividing monoblasts are promonocytes (Table III, see Appendix). For the promonocytes, calculation shows that until the 4th day of incubation about 70% of the cells are self-replicating and about 30% give rise to macrophages and on the 4th day the percentage of self-replicating promonocytes becomes smaller (Table III, see Appendix). This pattern of proliferation and differentiation of mononuclear phagocytes only pertains to the in vitro situation;

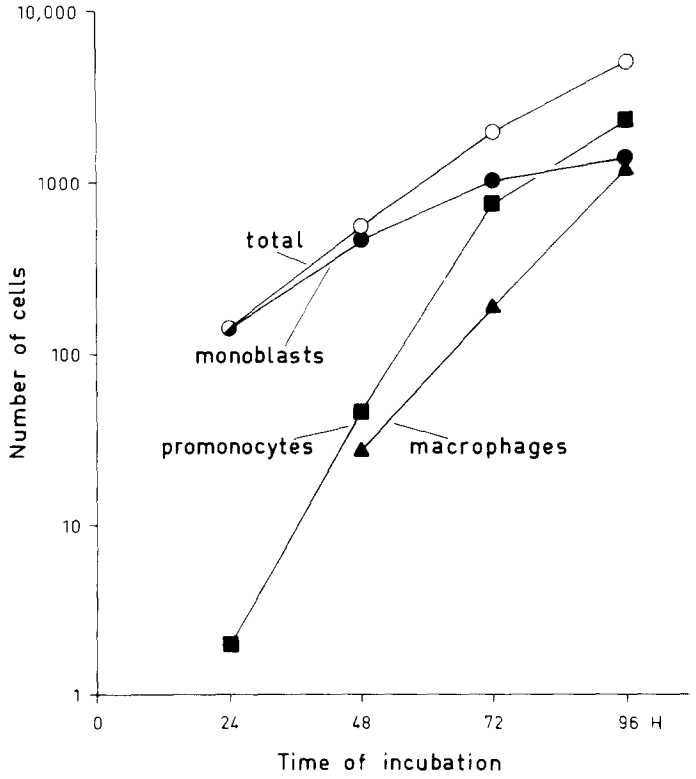


FIG. 9. Total number of monoblasts, promonocytes, and macrophages developing in a culture, that was initiated with $1 \times 10^5$ bone marrow cells (for details see Appendix).

in vivo the monoblasts and promonocytes, show a different pattern of division (3, 6).

Since each colony originates from a single cell the number of colonies per culture reflects the number of colony-forming cells present in the initial bone marrow cell suspension. On average, 25 colonies were found per $2.5 \times 10^4$ plated nucleated bone marrow cells, which means that a mouse has a total of $2.5 \times 10^5$ cells that can initiate a mononuclear phagocyte colony, since the bone marrow of a mouse contains $2.5 \times 10^8$ nucleated cells (6, 18). This computation is, however, only valid if each plated colony-forming cell is stimulated to proliferate in vitro. Since the conditioned medium used is maximally active in respect to colony stimulation, as shown by dose-response experiments,[1] it is highly probable that each colony-forming cell does in fact proliferate in these cultures.

It is remarkable that the number of cells initiating a mononuclear phagocyte colony ($2.5 \times 10^5$) is half the number of promonocytes per mouse ($5 \times 10^5$) (6). The implications of this ratio for the in vivo pattern of cell division all depend on the identity of the cell initiating the mononuclear phagocyte colony.

If the colony-forming cell is the monoblast, the ratio of one monoblast to two promonocytes implies that in vivo the monoblast must divide symmetrically, one monoblast giving rise to two promonocytes. This also means that the monoblast must be preceded by at least one other cell type with self-replicating properties.

On the other hand, if the colony-forming cell is the precursor of the monoblast, two division models in vivo are conceivable. These precursors could be self-replicating cells giving, per generation, $2.5 \times 10^5$ similar precursor cells and $2.5 \times 10^5$ monoblasts, the latter in their turn dividing symmetrically and producing $5 \times 10^5$ promonocytes or the precursor cells could divide symmetrically, giving $5 \times 10^5$ monoblasts. In the latter case the monoblast would have to be a self-replicating cell, since symmetrical division of the monoblast in vivo would give rise to a higher number of promonocytes per mouse than has been determined (6). Of the various possibilities, the monoblast seems to be the most likely colony-forming cell, since light- and electron-microscopic studies (reference 1 and footnote 2) indicate that the monoblast is the most immature cell of the mononuclear phagocyte colony and replating experiments have shown that monoblasts initiate new mononuclear phagocyte colonies (1).

It should be mentioned that in the preceding argumentation it is silently assumed that, as found for monoblasts and promonocytes in the colonies, the cycle times of promonocytes and their precursors in vivo are roughly similar. This assumption is probably justified, because in vivo the labeling index of the promonocytes, whose division gives rise to two monocytes, remains fairly constant during the first 24 h after the administration of [$^3$H]thymidine (3); this must be due to an influx of cells deriving from a precursor with a similar labeling index.

The proliferation pattern of monoblasts and promonocytes in vitro and in vivo is clearly different. Promonocytes do not renew themselves in vivo but after division give rise to two monocytes (3, 6), whereas in vitro some of them are self-replicating cells; the monoblasts are mainly self-replicating cells in vitro, although the self-replicating fraction decreases with time, probably due to culture conditions. Since all of the available evidence points to the monoblast as being the cell that initiates the mononuclear phagocyte colony, the most likely pattern for in vivo division of the proliferating mononuclear phagocytes is a self-replicating precursor cell giving rise to monoblasts, which divide only once into two promonocytes; these cells in turn divide once and form two nonproliferating monocytes.

## Summary

In a previous study also done with a liquid culture technique, the monoblast was identified and characterized as the most immature cell of the mononuclear phagocyte cell line recognized so far. The present study concerned the proliferative behavior of the monoblast and promonocyte in colonies.

The cell-cycle times of both cell types were determined on the basis of four independent methods. The resulting values all show excellent agreement: for the monoblast 11.0–11.9 h, and for the promonocyte 11.4–12.8 h. The DNA-synthesis time found for the two cell types amounted to 5.7 h for the monoblast and 5.5 h for the promonocyte. The duration of the other phases of the cell cycle of the proliferating mononuclear phagocytes proved to be: G2 phase, 0.6 h; mitosis phase, 1.8 h; and G1 phase, 3.5–3.8 h.

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

The individual colonies showed a biphasic pattern of colony growth, an initial phase of rapid proliferation being followed by a stage with a markedly decreased growth rate. In the initial stage only monoblasts are present in the colony; when the growth rate slows down promonocytes and macrophages appear. These observations support the earlier conclusion that the monoblast is without doubt the precursor of the promonocyte.

Colony size was found to vary widely. The main factor underlying this variation proved to be the lag time between the start of the culture and the time point at which the colony-forming cells begin to divide. Mathematical analysis showed that the variation in colony size probably does not arise from heterogeneity of the population of colony-forming cells.

A mathematical approach was used to determine the proportion of self-replicating and differentiating cells among the dividing monoblasts and promonocytes in the colony. The results indicate that initially in vitro the majority of the cells of both types are self-replicating cells, but later an increasing proportion of the dividing cells give rise to another, more mature type of cell.

On the basis of the conclusion that the monoblast initiates the mononuclear phagocyte colony, the number of monoblasts ($2.5 \times 10^5$) present in vivo was estimated to be half the number of the promonocytes. In view of this ratio the most likely pattern for the proliferation of mononuclear phagocytes in the bone marrow is that a monoblast divides once, giving rise to two promonocytes which in their turn divide once and form two nonproliferating monocytes.

## APPENDIX

### *Mathematical analysis of the proliferation and differentiation of mononuclear phagocytes in vitro (together with Dr. H. Mattie and Ir. P. C. J. Leijh)*

Using the values of the total number of mononuclear phagocytes on four cover slips after various periods of incubation (data taken from Fig. 1; since the total number of mononuclear phagocytes cannot be determined accurately at 24 h, this value was calculated by extrapolation) and the percentage of monoblasts, promonocytes, and macrophages (taken from Table III of reference 1) the total number of each type of cell has been calculated. The results are presented in Fig. 9.

If all proliferating monoblasts were self-replicating cells the doubling time of the population of monoblasts $t_d^B$ would be:

$$t_d^B = t_c^B/\bar{i},$$

in which $t_c^B$ is the cell-cycle time of the monoblast, (see Table II) and $\bar{i}$ the mean labeling index of the monoblasts (taken from Table VII of reference 1). The rate constant of monoblast doubling ($k_d^B$) would then be:

$$k_d^B = \ln 2/t_d^B$$

which gives $0.0564\ h^{-1}$.

However, the number of monoblasts do not increase exponentially as can be seen from the semilogarithmic plot. Mathematically, the number of monoblasts at time $t$ can be described by applying the equation:

$$\ln N_t^B = \ln N_{24}^B + k_i^B \cdot (t - 24) - \tfrac{1}{2}\alpha \cdot (t - 24)^2,$$

in which $N_t^B$ is the number of monoblasts at time $t$, $k_i^B$ the initial rate of monoblast

TABLE III

*Percentage of Dividing Cells Giving Rise to a Differing Cell Type*

| Duration of incubation | Monoblasts giving rise to promonocytes | Promonocytes giving rise to macrophages |
|---|---|---|
| *h* | % | % |
| 24 | 0 | |
| 48 | 26.1 | 41.9 |
| 72 | 58.7 | 41.2 |
| 96 | 91.2 | 68.2 |

formation, and $\alpha$ a constant which governs the decline in the rate of monoblast formation. Using the numbers of monoblasts in Fig. 9, calculation of the values of $\alpha$ and $k_i^B$ gives: $\alpha = 7{,}644 \times 10^{-4}\ h^{-2}$ and $k_i^B = 0.060\ h^{-1}$, which is close to the value of $k_d^B$. The monoblast curve calculated with these values using the least squares method, agrees excellently with the observations and has a mean standard coefficient of 0.4%.

The rate at which monoblasts are formed at each time point is then

$$dN_t^B/dt = [k_i^B - \alpha \cdot (t + 24)] \cdot N_t^B.$$

The difference between this real rate of monoblast formation during exponential growth and the rate constant of monoblast doubling ($k_d^B$) represents the rate of promonocyte formation at each time point which is determined by applying the equation:

$$dN_t^{B \to P}/dt = [k_d^B - k_i^B + \alpha \cdot (t - 24)] \cdot N_t^B,$$

in which $N_t^{B \to P}$ is the number of promonocytes formed from monoblasts at time $t$.

The percentage of monoblasts giving rise to promonocytes ($p_t^{B \to P}$) at each time point can be calculated with the equation:

$$p_t^{B \to P} = \frac{k_d^B - k_i^B + \alpha \cdot (t - 24)}{k_d^B} \times 100.$$

The results of this calculation are given in Table III.

The rate of production of macrophages can be regarded as a function of the number of promonocytes:

$$dN_t^M/\mathrm{dt} = q \cdot N_t^P,$$

in which $N_t^M$ is the number of macrophages at time $t$ and $N_t^P$ the number of promonocytes at time $t$. Integration of this equation gives:

$$N_{t_j}^M - N_{t_{j-1}}^M = q \cdot \int_{t_{j-1}}^{t_j} N_t^P \cdot dt.$$

During the interval between $t_{j-1}$ and $t_j$, $q$ is considered to be constant. Since $N_t^P$ increases more or less exponentially, the best approximation of

$$\int_{t_{j-1}}^{t_j} N_t^P \cdot dt$$

is given by the area under the exponential growth curve of $N_t^P$. A value of $q$ can then be calculated for each time interval.

The rate of change in the number of promonocytes can now be expressed as follows:

TABLE IV

*Rates of Monoblast, Promonocyte, and Macrophage Production Calculated for the Experiment Shown in Fig. 9*

| Duration of incubation | Rate of monoblast self-renewal | Rate of production of promonocytes from monoblasts | Net rate of promonocyte increase | Rate of promonocyte self-renewal | Rate of production of macrophages from promonocytes |
|---|---|---|---|---|---|
| *h* | *cells/h* | *cells/h* | *cells/h* | *cells/h* | *cells/h* |
| 48 | 10.2 | 3.6 | 5.4 | 1.8 | 1.3 |
| 72 | 24.0 | 34.1 | 63.5 | 29.4 | 20.6 |
| 96 | 7.7 | 79.8 | 112.7 | 32.9 | 70.5 |

$$dN_t^p/dt = [k_a^B - k_l^B + \alpha \cdot (t - 24)] \cdot N_t^B + r \cdot N_t^p,$$

in which $r\,N_t^p$ represents the rate at which promonocytes form new promonocytes. $dN^p/dt$ can then be estimated graphically by measuring the slope of the tangent of the promonocyte growth curve at time $t$, so that $r$ can be calculated. For each time interval the percentage of dividing promonocytes giving rise to macrophages will then be:

$$[q/(q + r)] \times 100.$$

The calculated values are given in Table III.

The results of the calculations of the various rates of cell formation for the population under study (Fig. 9) are given in Table IV.

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