PROLIFERATIVE CAPACITY OF MOUSE PERITONEAL MACROPHAGES IN VITRO*

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Intraperitoneal injection of thioglycolate medium or other phlogogenic stimuli into mice or rats will increase the number of mononuclear phagocytes several-fold within a few days (1-3). This is due to an invasion of newly formed cells originating from the bone marrow (1, 2, 4-6). Whereas normal "resident" macrophages rarely synthesize DNA and divide in vivo, "activated" macrophages will synthesize DNA and proliferate.

In the presence of L-cell-conditioned medium, activated macrophages can also be induced to proliferate in vitro (7) and, under appropriate culture conditions, will produce colonies of mononuclear phagocytes with a relatively high plating efficiency (3, 8). The component in the L-cell-conditioned medium that induces this response is a glycoprotein of about 60,000 daltons, referred to as macrophage growth factor (MGF; 9-11). In contrast, resident mononuclear phagocytes cannot be induced to proliferate in vitro (3, 8).

Activated macrophages also differ in many other respects from resident macrophages. Activated macrophages (a) have greater pinocytic activity (12); (b) show enhanced phagocytosis (13); (c) spread out and attach more firmly to wettable surfaces (14); (d) spontaneously synthesize and excrete neutral proteases like collagenase (15), elastase (16, 17), and plasminogen activator (18-20); and (e) have a lower membrane bound 5'-nucleotidase activity (21). These differences suggest that activated macrophages represent a cell population distinct from resident macrophages.

In this study, we sought to determine whether mononuclear phagocytes could be subcultured, how many times cells could be passaged and still maintain exponential proliferation, the average number of progeny a single precursor cell could produce, and the role of MGF in the regulation of their proliferation. In addition, we used 5'-nucleotidase activity as a marker for activation to determine whether senescent macrophages, exhausted in their proliferative capacity,

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Abbreviations used in this paper: CFC, colony-forming cells, clonogenic cells; MEM, Eagle's minimum essential medium; MGF, macrophage growth factor.

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behaved like resident nonactivated macrophages. These studies have both practical and theoretical importance. A single progenitor cell must be capable of producing a sufficient number of progeny if functional studies, such as those mentioned above, are to be carried out on individual colonies. Using these cells, we can directly address questions concerning the functional heterogeneity of mononuclear phagocytes. Knowledge of their proliferative capacity would also contribute to our understanding of this cell system's ability to expand in the host defense to infections and malignant growth.

Materials and Methods

Macrophages. Cells were obtained from 11- to 16-wk-old C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, Maine) as described before (3). Briefly, mice were injected intraperitoneally with 1.5 ml Brewer's thioglycolate medium (Difco Laboratories, Detroit, Mich.) 72 h before harvest. Cells were collected after intraperitoneal injection of 5 ml α-MEM (Eagle's minimum essential medium [22]; Flow Laboratories, Inc., Rockville, Md.) medium supplemented with 10% fetal calf serum and 5 U heparin/ml, and were washed once with the same medium without heparin. Growth medium consisted of α-MEM, supplemented with 10% L-cell-conditioned medium. For cell counts, the cells were lysed by cetrimide, and nuclei were counted with an electronic particle counter (23). Pronase digestion of dead cells was omitted because detachment is concomitant with cell death.

Conditioned Medium. L-cell-conditioned medium was used as the source of MGF for most experiments. We prepared large batches by plating 175 ml of L cells at 5 × 10^4 cells/ml in 1,300 cm^2 roller bottles. After 4 days the culture medium (α-MEM supplemented with 10% fetal calf serum) was collected.

To prepare fibroblast-conditioned medium, we obtained embryo fibroblasts from 14- to 19-day-old fetuses and established cultures at 5 × 10^5 viable cells per milliliter (10 ml) in replicate 100-mm tissue culture dishes. The fibroblast-conditioned medium was harvested 7 days later.

Culture Conditions. Cells were grown in a humidified incubator having a 5% CO₂ atmosphere. Unless otherwise noted, cells were plated in 35-mm plastic tissue culture dishes containing 3 ml growth medium. If necessary, parallel 100-mm dishes were plated with the same cell suspension and the same volume to surface ratio (25 ml per dish).

To determine the plating efficiency, we established cultures, derived from either the primary cells or the removed subcultured cells plating 1,000 cells in 3 ml growth medium on 35-mm dishes. After 14 days incubation, we stained these cultures and counted the number of colonies, defining "colony" as a cluster of 50 or more cells.

When unstimulated macrophages were used, the nonadherent cells (about 75%) were removed by rinsing the plates repeatedly with medium 2 h after the cells were plated.

Phagocytosis Assay. Phagocytosis of autoclaved bakers' yeast in the presence of guinea pig complement has been described (3). Briefly, 5 × 10^7 yeast particles and guinea pig complement (3% final concentration) were added to culture dishes. After 30 min incubation at 37°C, the dishes were rinsed with phosphate-buffered saline and stained with methylene blue.

Pulse Labeling with [3H]Thymidine and Autoradiography. Cells grown in dishes having an 18 x 18-mm sterile coverslip were supplemented with 5 μCi/ml [methyl-3H]thymidine (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y., 58 Ci/mmol). After 2 h, the coverslip was removed, washed once with phosphate-buffered saline, twice with 5% trichloroacetic acid, and once with ethanol/ether 1:1 by volume. The dry coverslips were mounted onto microscope slides, dipped into NTB-2 liquid photographic emulsion (Eastman Kodak Co., Rochester, N.Y.), and exposed for 3-4 wk before developing. After staining with Giemsa, we determined the fraction of labeled cells (labeling index).

Subculturing of Macrophages. Cells were incubated at 37°C for 30 min. in medium supplemented with 1/6 vol of 2% lidocaine-HCl (Astra Pharmaceutical Products, Inc., Framingham, Mass.), giving a final concentration of 12 mM (24, 25). We used 2 ml of medium for 35-mm dishes and 5 ml for 100-mm dishes. The cells rounded up during this incubation period and were removed by a jet of medium from a Pasteur pipette. The cells were diluted 10-fold with α-MEM.
supplemented with 10% fetal calf serum; they were then collected by centrifugation (200 g for 5 min), resuspended in growth medium, counted, appropriately diluted, and replated.

5'-Nucleotidase Assay. We adapted the method used by Edelson and Cohn (21) to assay the activity in a smaller number of cells. The cells in a 35-mm culture dish were rinsed twice with serum-free medium and then lysed with 1 ml buffer solution (54 mM Tris-Cl, pH 9.0, 12 mM MgCl₂, 0.05% Triton X-100). A volume of 0.1-0.5 ml of this lysate was incubated at 37°C in a final volume of 0.7 ml buffer solution containing 5 × 10⁻⁵ M unlabeled AMP and 8.4 × 10⁻¹° M (0.014 μCi/ml) [HS]AMP (adenosine-[2-SH]-5' monophosphate, ammonium salt, 17 Ci/mmol, obtained from Amersham/Searle Corp., Arlington Heights, Ill.). The reaction was terminated by adding 0.25 M solutions, 0.15 ml each of ZnSO₄ and Ba(OH)₂. Unhydrolyzed AMP and BaSO₄ coprecipitate under these conditions. We removed this precipitate by centrifugation (10,000 g for 10 min) and determined the amount of adenosine that had been formed by counting 0.5 ml of the supernate in 3 ml xylene-Triton X-117 scintillation fluid (26). The amount of adenosine formed after 1- and 2-h incubation periods was plotted, and the reaction rate was calculated from the slope of this curve.

Enzyme levels were expressed as nanomoles per minute per 10⁶ cells; this number of cells corresponds to 238.3 ± 43.0 μg protein determined by the Lowry method (27) with bovine serum albumin as a standard.

Results

Growth Kinetics of Primary Cultures of Thioglycolate-Stimulated Macrophages. Under our present culture conditions, when macrophages were plated at cell concentrations of approximately 3,000 cells/ml or less, the number of cells doubled every 40 h (Fig. 1 A, closed points), somewhat faster than previously reported (3). The duration of exponential growth, however, was dependent on the initial cell concentration, and cells entered plateau phase when the cell number reached 2-3 × 10⁶ cells per culture plate (35 mm). These cultures were not completely confluent, and if the cells were maintained for a longer time in culture many rounded up and detached from the plate. Within 4 days of growth, the fraction of cells in S-phase during a 2-h [³H]thymidine pulse labeling reached over 30% (Fig. 1 B, closed points). This labeling index remained relatively constant during the exponential growth period and then dropped abruptly as cells entered stationary phase.

Synthesis of MGF by Contaminating Fibroblasts. When peritoneal cells are obtained, they are invariably contaminated by a small number of fibroblasts; we usually find 1 colony-forming fibroblast per 1,000 exudate cells (0.1%) or about 1 colony-forming fibroblast per 50 colony-forming macrophages (2%). Because fibroblasts can produce MGF, they could, in time, produce enough endogenous factor to stimulate the macrophage precursors to proliferate, provided that the precursors had survived that long.

To determine the survival of the macrophage colony-forming cells (CFC), we plated peritoneal exudate cells into 35-mm culture dishes at 1,000 cells per dish in 3 ml α-MEM containing only 10% fetal calf serum and 5% horse serum. Fibroblast-conditioned medium was added to replicate dishes on days 0, 1, 4, 7, or 14 to a final concentration of 10%; the number of colonies was determined 14 days later. The results are shown in Table I. No significant difference was found in the number of colonies formed as a function of the time at which the conditioned medium was added. Thus, the CFC survive in culture as resting cells for at least 14 days and can be induced to proliferate when the conditioned
FIG. 1. Dependence of macrophages on the continuous presence of MGF for cell division and DNA synthesis. (A) Macrophages were grown in the continuous presence of MGF (●). On day 4, the medium of some plates was removed, the cells were washed and refed with medium lacking MGF (○). On day 8, the medium removed on day 4 was readded to the cells (▲). (B) Parallel plates were pulse labeled with [3H]thymidine; the fraction of the labeled cells was then determined after autoradiography. Phagocytosis on other parallel plates was done on days 4, 8, and 12. In all cases >99% of the adherent cells phagocytized yeast particles.

TABLE I

| Day FCM* added | Assay | Macrophage colonies per 1,000 cells |
|----------------|------|-----------------------------------|
|                |      | With FCM | Without FCM |
| day            |      |          |              |
| 0              | 14   | 42 ± 35  | 0             |
| 1              | 15   | 48 ± 35  | 0             |
| 4              | 18   | 54 ± 33  | 0             |
| 7              | 21   | 35 ± 10  | 0.7 ± 0.6     |
| 14             | 28   | 48 ± 6   | 6 ± 5         |

* Fibroblast-conditioned medium.
† The number of contaminating fibroblast colonies, all groups, was 0.97 ± 0.8 per 1,000 cells.
§ 1 SD.
medium is added. Table I also shows that the number of macrophage colonies in culture increases after 21 days in the absence of conditioned medium.

To test whether the contaminating fibroblasts produce enough MGF to stimulate some of the CFC to proliferate, we established cultures containing 10,000 embryo fibroblasts in 3 ml medium. After 2, 4, 7, 9, or 14 days of incubation, medium was removed from replicate cultures of fibroblasts. After all media were collected, they were tested individually for their ability to stimulate macrophage proliferation. The results are shown in Table II. By day 7 the fibroblasts had produced enough MGF to maximally stimulate all the macrophage CFC, suggesting that contaminating fibroblasts are likely to provide this kind of stimulation. As direct proof that a single fibroblast colony can produce enough MGF to stimulate CFC to proliferate, Fig. 2 shows that visible colonies of macrophages form near a fibroblast colony but not at a distance from it. Because the expected frequency of fibroblast contamination (1% 1,000 cells) is 63%, not all macrophage cultures are contaminated; when contamination does not occur, macrophage colonies never form; the latter possibility is also shown in Fig. 2, for comparison.

Control of Proliferation by MGF. To determine whether MGF is required continuously or acts merely as a trigger for cell division, the growth medium was removed on day 4 and replaced with culture medium containing only 10% fetal calf serum and 5% horse serum. The results, shown in Fig. 1, indicate that removal of MGF causes cells to enter a stationary phase; < 1% of these cells synthesized DNA 48 h later. In fact, by 12 h after removal of MGF, the percentage of labeled cells had fallen from 33 to 17% and by 24 h was only 0.5%. Cells surviving during stationary phase are able to phagocytize bakers' yeast but some cells become detached from the plates.

If growth medium is readded to the cultures 4 days later, the "resting" macrophages reenter the cell cycle as evidenced by an increase in the labeling index to 37% by day 10 (Fig. 1). This means that about the same fraction of cells is in cell cycle as just before removal of the MGF. It appears that not all the cells enter the cell cycle during the first 24 h because the maximum labeling index of 37% was not found until 48 h. Also by day 10, the number of cells increases again, at a rate equal to that found for the control cultures from which growth medium was not removed.

Results similar to those shown in Fig. 1 have been obtained with cells that had been deprived of MGF for longer intervals, up to 7 days. In each case, its
readdition resulted in reentry of macrophages into the growth cycle. However, when the interval of time without the factor is long, an increasing number of macrophages were found to enter the cell cycle spontaneously, even though exogenous growth factor was absent. This was due to contamination of these cultures by fibroblasts that produce endogenous factors, as was shown in Table II and Fig. 2. This stimulation did not occur in cultures which, upon microscope examination, lacked fibroblasts.

**Proliferative Capacity of Macrophages.** When macrophages are cultured in 35-mm culture dishes, the cell number never exceeds $5 \times 10^5$ and generally reaches a plateau at $2-3 \times 10^5$. This represents about a 20% confluent monolayer of cells on a 35-mm culture dish. The failure of these cells to establish a confluent monolayer could be due to the limited proliferative ability of precursor cells, to a depletion of essential nutrients, or to some contact inhibitory phenomena within the colony. When cultures were initially established at $10^3$, $5 \times 10^3$, or $10^4$ cells per plate in 3 ml growth medium, the final cell numbers achieved were very much the same (compare the curves for primary macrophages in Fig. 3A–C). Thus, it seems likely that depletion of essential nutrient(s) or contact inhibition is responsible for cell entrance into stationary phase. We have previously shown that peritoneal exudate cells completely deplete glucose in less than 48 h when cultured as a confluent monolayer (24). Thus, the cells must be subcultured at lower cell densities in order to evaluate their proliferative capacity.

Primary cultures of peritoneal exudate cells were established at either $10^4$, $5 \times 10^3$, or $10^3$ cells in 35-mm culture dishes. The cell number was determined frequently and when the level reached $1-3 \times 10^5$ cells per culture, the cells from a parallel 100-mm dish were removed with lidocaine, diluted, and replated onto 35-mm dishes for determining cell numbers and onto 100-mm dishes for eventual
subculturing. The results of this study are shown in Fig. 3. Subcultured cells continued to divide at the same rate as did the primary macrophages. The doubling time for the population was approximately 40 h. However, with each subculture, the final yield of cells decreases. A final cell density of < 20% of that achieved by the primary macrophages (5 × 10⁴ cells per 35-mm dish) was assumed to mean that the cells had nearly exhausted their proliferative capacity.

The data suggest that the precursor cells can undergo only a limited number of cell divisions; however, this limited number is considerably greater than that attained in the original culture. The total number of cells that would have accumulated from subculturing all of the cells was 6.0 × 10⁷ for primary cultures established at 10⁴ cells per culture, an overall increase of 6,000 times. For an initial cell number of 5 × 10⁴, the increase was 4,400-fold or 2.2 × 10⁷ cells; for 10³ cells, it was 4,600-fold or 4.6 × 10⁶ cells. The average increase for all concentrations was 5,000 times the original cell number, and the degree of increase did not depend greatly on the number of cells cultured or the number of subcultures.

The plating efficiency for the primary cultures and for each subculture thereafter was determined to find out whether the fraction of CFC (presumed stem-line) decreased with subculture. The plating efficiency of the primary cells was found to be 20.1%. With each successive subculture, the plating efficiency decreased to 6.2, 2.6, and 1%, respectively. An initial plating efficiency of 20.1% implies that starting with 10⁴ cells per culture, 2,010 CFC were able to produce 6 × 10⁷ cells by the end of the third subculture. If we assume that all of these cells are derived from the initial clonogenic fraction, the average CFC underwent 14.9 divisions and produced 3 × 10⁴ progeny. When the initial number was
10^3 cells per culture, the average CFC underwent 14.5 divisions and produced 2.3 × 10^4 progeny.

Every 4 days throughout these experiments we assayed parallel cultures for phagocytic activity to be sure that the proliferating cells were macrophages and not contaminating fibroblasts. In all cases, nearly every cell was phagocytic. Occasional fibroblasts were encountered on some plates but not on others; these fibroblasts were found in discrete localized colonies surrounded by macrophages and represented, at the most, 5% of the cells on the plate at the time stationary phase was achieved.

Lidocaine Removal of Macrophages. If not all of the cells survive the preparative procedure for subculturing, the proliferative ability of macrophages might actually be greater than that described above. To evaluate what fraction of the cells recovered with lidocaine is viable, primary cultures of macrophages were treated with lidocaine at different times after plating: 1, 4, and 23 h and 7 and 8 days. The removed cells were washed and then resuspended in their original volume of medium. Samples of 3 ml were replated, and either 1 or 24 h later we determined the number of cells that would reattach. To compare the plating efficiency of these cells to that of untreated cells, we established cultures of 10^3 cells in 3 ml growth medium. Colonies were counted 14 days later. For this experiment 10^3 cells were initially plated onto 35-mm dishes in 3 ml α-MEM containing only 10% fetal calf serum for the 1-, 4-, and 23-h determinations. For the 7- and 8-day assays 10^4 cells were plated onto 35-mm cultured dishes in complete growth medium. All plates were washed 1 h after plating to remove nonadherent cells.

As shown in Table III, 75-94% of the cells could be removed with lidocaine with an overall recovery, after centrifugation, of between 58 and 77%. Table III (column 5) shows the recovery of viable macrophages after they were allowed to reattach for either 1 or 24 h. The results were surprising. When primary cultures are treated with lidocaine during the first 24 h, the overall recovery of viable macrophages is very poor, between 5 and 11%. However, if these cells are cultured in growth medium 7-8 days and then treated with lidocaine, the recovery of viable cells improves to around 40%. This same pattern is reflected in the number of CFC (columns 6 and 7). We included the 24-h determination of attached cells in this study because cells treated with lidocaine might require a period of time to recover and, therefore, might not attach as quickly as untreated cells. Alternatively, the treated cells, although viable soon after treatment, may have accumulated lethal damage which requires time for expression. The latter effect appears to be significant for freshly obtained macrophages, because reattachment after 1 h incubation was greater than that after 24 h.

5'-Nucleotidase Activity of Macrophages. The activation state of proliferating macrophages in culture is not known. One possibility is that when macrophages reach proliferative senescence they might behave like normal, unstimulated, "resident" macrophages. Edelson and Cohn (21) have shown that the membrane-bound enzyme 5'-nucleotidase can be used as a probe for macrophage activation. "Activated" macrophages have a low enzyme activity, whereas "resident" macrophages have a rather high enzyme activity. Our
TABLE III
Viability of Primary and Cultured Macrophages Treated with Lidocaine

| Time of treatment* | % Recovered† | Assay time | Percentage attached viable cells§ | Plating efficiency after lidocaine treatment |
|-------------------|-------------|------------|-----------------------------------|---------------------------------------------|
|                   | Removed from plate | After centrifugation | h | 1,000 cells | Percentage of control |
| 1 h               | 85          | 70         | 1 | 18 | 1 ± 0.82 | 0.7 |
|                   | 24          | 5          | - | 24 | - |
| 4 h               | 75          | 58         | 1 | 17 | 3.62 ± 1.01 | 2.4 |
|                   | 24          | 6          | - | 24 | - |
| 23 h              | 94          | 77         | 1 | 12 | 9.3 ± 1.01 | 6.2 |
|                   | 24          | 11         | - | 24 | - |
| 8 days            | 88          | -          | - | - | 74 | 49 |
| 7 days            | 81          | 75         | 1 | 45 | 60 | 40 |
|                   | 24          | 58         | - | 24 | - |
| 7 days            | 81          | 66         | 1 | 39 | 52 | 35 |
|                   | 24          | 39         | - | 24 | - |

* This is the time after cells were placed in dishes.
† The fraction of macrophages removed from the original plate was determined by dividing the number of cells removed by the number of attached cells on a replicate untreated plate. This was verified by also counting the number of cells left behind on the lidocaine-treated plate.
§ The percentage reattachment was calculated from the ratio of the number of cells that reattach to a culture dish to the number of cells on the original culture dish before treatment with lidocaine. This figure represents the yield of viable macrophages.
|| The number of colonies on untreated control plates was 150 ± 10 per 1,000 cells.

results confirm their findings. We compared unstimulated and thioglycolate-stimulated macrophages that had been cultured for 24 h in α-MEM with 10% fetal calf serum. We used 35-mm dishes containing about 5 × 10⁴ cells in 3 ml medium. The unstimulated macrophages had a 5'-nucleotidase level of 14.35 ± 0.35 nmol/min per 10⁶ cells. In thioglycolate-stimulated cells we could not detect any enzymatic activity. However, when these same cells were allowed to proliferate in the presence of MGF, they acquired detectable enzyme levels. For instance: 10⁴ cells divided in 4 days to about 5 × 10⁴ cells. These logarithmically growing cultures had an enzymatic activity of 4.4 nmol/min per 10⁶ cells or 31% of the value found for resident macrophages. Table IV shows the 5'-nucleotidase levels of these macrophages and their subcultured progeny, determined every 4 days. At first sight the enzyme levels fluctuate randomly between 3 and 44% of the value found for resident macrophages. A more careful look at the growth kinetics of these cells (see Fig. 3) indicates that enzyme levels are high in logarithmically growing cells and lower when cultures reach the stationary phase. There is no indication that the cells acquire enzyme levels similar to those found in resident macrophages when they have nearly exhausted their proliferative capacity.

Discussion

This study shows that macrophage precursors obtained from the peritoneal
Table IV

| Days | 10⁴ Cells plated per dish | 10⁵ Cells plated per dish |
|------|---------------------------|---------------------------|
|      | 5'-Nucleotidase            | Subculture               | 5'-Nucleotidase            | Subculture               |
|      | nmol/min/10⁶ cells        | nmol/min/10⁶ cells        |
| 4    | 4.4                       | 6.3                      |
| 8    | 1.4                       | 0.9                      |
| 13   | 3.4                       | 1                        |
| 16   | 0.8                       | 1                        |
| 16   | 1.5                       | 2                        |
| 20   | 2.0                       | 2                        |
| 24   | 0.5                       | 0.5                      |
| 28   | 2.6                       | 1                        |

* The cultures were replicates of those used in the experiments reported in Fig. 3.
† After establishing the initial culture.

The results of our studies show that a positive stimulus (MGF) must continuously be present to maintain macrophages in a growth cycle. Removal of the stimulus causes these cells to return to a nonproliferative "resting" state; a state that can be entirely reversed by the readdition of the stimulus. Other hematopoietic cells seem to be similarly regulated: for example, the formation of granulocyte and macrophage colonies by bone marrow cells in the presence of colony stimulating factor (10), and the differentiation and division of erythroid cells to hemoglobin-producing cells by erythropoietin (28). Also the lymphocyte proliferative response requires the continuous presence of mitogens (29, 30).

The results shown in Table III indicate that even though lidocaine yields more viable cells when it is used to remove cultured macrophages than when it is used to remove freshly obtained cells, still more than half of the former cells do not survive the treatment. Cultured macrophages have been growing in culture and they have been bathed in medium containing 10% fetal calf serum, 5% horse serum and 10% L-cell-conditioned medium. Only further exploration will reveal whether either or both of these variables affect the viable cell recovery after lidocaine treatment. Even with the relatively poor recovery of viable macrophages, their proliferative ability is quite extensive and might...
have been even greater had they all been removed in a viable state for subculture.

The plating efficiency dropped from 20% for primary macrophages to 1% for quaternary cultures. As shown by our viability studies after lidocaine treatment, we can account for at least part of the decrease in plating efficiency by the failure to recover all of the cells in a viable state for subculture. However, this cannot explain the observation that the maximum density, achieved when cells enter stationary phase, decreases after each subculture. Because subculture results in further proliferation with a doubling time similar to that of the original cell population (Fig. 3), it might be expected that cells, as long as they have remaining proliferative capacity, will continue to proliferate up to the same culture density of $2-3 \times 10^5$ cells per plate. The fact that these cells do not achieve this level suggests that each colony must reach some maximum size at which macrophages cease to proliferate though potentially capable of more divisions. Not until the individual colony is disrupted and the cell density reduced by subculture are the cells again capable of proliferation, albeit a smaller proportion of them. One possible explanation for this phenomenon is contact inhibition within the colony. Although we have no direct evidence that this is the cause, such a hypothesis would explain the continual reduction in total cell yield. Alternatively, the failure of the macrophages to continue to proliferate may be an artifact introduced by the lidocaine treatment.

We have referred to the cells from a thioglycolate medium-elicited exudate as activated macrophages. When the most recent classification for mononuclear phagocytes was proposed (31), there was no need to provide for cells with self-replicating properties not localized in the bone marrow. Clearly, we and others (3, 7-9) have accumulated ample evidence to show that these peripheral cells can replicate. Therefore, the proposed classification should be changed to include these precursor cells which, though derived from the bone marrow, retain an extensive self-replicating potential when they infiltrate peripheral tissues.

Although current thought favors the concept that activated peritoneal macrophages induced by agents like thioglycolate medium are derived from the bone marrow via the blood, the source of the resident macrophage is not as clear; these resident macrophages might be the senescent progeny of activated macrophages. It has been our thought that once cultured activated macrophages have exhausted their proliferative capacity, they might behave like resident, nonactivated macrophages. Thus 5'-nucleotidase activity was chosen as a probe for the state of activation of cultured macrophages. The results of our studies on this point are somewhat equivocal. Even though exponentially growing macrophages showed higher 5'-nucleotidase activity than did their nonproliferating, newly derived counterparts, they never acquired the levels found for resident macrophages, nor did subculture produce any trend in that direction. Lidocaine treatment cannot be overlooked as having an influence on the results. Nevertheless, these macrophages are a mixture of proliferating and nonproliferating cells, and this population could be expected to have a lower activity than one predominantly composed of resident macrophages. It is clear that further work will be necessary before any firm conclusion can be derived.
Because activated macrophages can be cultured easily and can be made to proliferate or rest at will, they lend themselves to further study of the part of the cell cycle where the decision is made to continue to proliferate or not. It is unlikely that removal of the growth factor results in immediate cessation of all events involved in proliferation. Rather, cells that have progressed past the point of decision proceed through cell cycle. By using this system as an example, it may be possible to look more precisely at the events that occur when the decision is made. Their proliferative capacity can also be exploited to amplify single clones of macrophages into sufficient numbers for studies dealing with macrophage function. These clones would be absolutely uncontaminated by other cells because they would have been derived from a single isolated precursor cell.

Summary
Thioglycolate-stimulated mouse peritoneal macrophages cultured in the presence of macrophage growth factor (MGF) will continue to proliferate when they are removed from culture dishes with the local anesthetic lidocaine and subcultured. The number of times the cells can be subcultured and remain in a proliferative state is dependent on the number of previous cell divisions. One precursor cell (colony-forming cell) yields about $2.6 \times 10^4$ daughter cells.

When MGF is removed from actively proliferating macrophages, they leave the cell cycle and enter a "resting" condition. When MGF is readded, cells reenter the cell cycle and proliferate with the same doubling time as if MGF had not been removed.

Membrane 5'-nucleotidase activity was used as a probe to identify the state of macrophage activation. Proliferating macrophage populations had significantly higher enzyme levels than stimulated macrophages cultured without MGF. These enzyme levels were, however, lower than those found for resident (unstimulated) macrophages.

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