Bone Marrow Macrophage Precursors.  
I. Some Functional Characteristics of the Early Cells of the Mouse Macrophage Series

By Martin J. Cline and Margaret A. Sumner

Mouse bone marrow cells were cultured in vitro under conditions that allowed little granulopoiesis but permitted proliferation of mononuclear phagocytes. During 10 days' culture, the population gradually shifted from a preponderance of undifferentiated blast cells and promonocytes to macrophages. Cells up to the A (immature) macrophage stage were capable of DNA synthesis and mitosis. B (mature) macrophages were nondividing. Blast cells were not phagocytic and lacked glass adhesiveness and demonstrable surface receptors for IgG immunoglobulin. With progressive cellular maturation, peroxidase activity disappeared after the monocyte stage. Glass adhesiveness, phagocytic ability, and surface receptors for immunoglobulins appeared to increase with cell maturation from the promonocyte through the macrophage stage of development.

THE ORIGIN AND KINETICS of proliferation of monocytes and macrophages have been the subject of study for many years. The introduction of tritiated thymidine-labeling techniques established that the proliferating precursor of mononuclear phagocytes resides in the bone marrow. In the bone marrow and subsequently in the blood and tissues, cells of this series undergo a series of functional and morphologic maturation steps that culminate in the mature tissue macrophage. The recent introduction by Van Furth et al. of a glass-adhesiveness technique has provided a method of identifying early bone marrow precursors of the macrophage series. A liquid culture system introduced by us now makes possible identification of nonadhesive cellular precursors and assessment of their functional characteristics.

MATERIALS AND METHODS

Cell Isolation and Cultivation

Bone marrow was expressed from the fractured femoral shafts of 2- to 3-mo-old female (CBA × C57B1)F1 mice by the injection of culture medium. Viable nucleated cells, 2.5 × 10^6, in modified Eagle's medium containing 10% fetal calf serum, 5% horse serum, and one-tenth volume of a partially purified extract of 16- to 20-day embryos, pregnant
uterus, and membranes from B L6/c, C57B1, and (CBA × C57B1)F1 mice were distributed in 1-ml volumes and incubated undisturbed in an upright position in 10% CO₂ in air at 37°C for 1-21 days.

³H-Thymidine Labeling

Mice were injected i.v. with 50 µCi ³H-thymidine (23 Ci/mM) 1 hr before the preparation of marrow cell suspensions. Alternatively, a pulse of ³H-thymidine 0.25–0.5 µCi/ml was given to unlabeled bone marrow cells 3 days in culture. Unlabeled thymidine 10⁻⁵ M was added to all cultures containing labeled cells to prevent reutilization of radioactive thymidine.

Cell suspensions were removed at intervals, deposited on glass slides, or allowed to adhere to sterile cover slips at 37°C for 4–6 hr, fixed, coated with Kodak NTB emulsion, and exposed for 2–21 days before development. Exposure time was varied to produce a medium grain count of approximately 30–50 grains/cell at reference time intervals. Background (usually less than 0.2 grains/cell) was subtracted in all calculations.

Morphology

Cells were fixed in absolute methanol and stained with Giemsa. The method of Rytomaa was used for peroxidase staining.¹⁰

Phagocytosis

Candida albicans and Staphylococcus aureus were prepared as previously described¹¹,¹² and were suspended in medium containing 5% normal mouse serum. Organisms at a concentration of approximately 5 × 10⁷/ml were added to glass-adherent cells or cells in suspension and incubated for 30–60 min at 37°C. The cells were then fixed, stained, and examined for ingested organisms.

Surface Receptors for IgG Immunoglobulin

Tests for identification of surface receptors for IgG immunoglobulin were carried out as previously described using a rosette technique with sheep erythrocytes coated with mouse immunoglobulin.⁹,¹³

RESULTS

Morphology

The following morphologic categories of cells were identified in differential counts of cultured bone marrow.

Undifferentiated Blast Cells: Round cells 12–25 µ in diameter with centrally located, large, round nucleus with fine chromatin structure and containing one to three prominent nucleoli were observed. These cells are peroxidase positive.

Promonocytes: Cells 12–20 µ in diameter similar to those described in detail by Van Furth et al.⁴,⁵ were identified. These had a slightly elongated and eccentric nucleus with prominent nucleoli and fine chromatic structure, and sparse peroxidase-positive granules were clustered in the perinuclear area within a deep-blue cytoplasm (Fig. 1).

Monocytes: Round or slightly oblong cells 10–18 µ in diameter were identified. The nucleus was lobulated with a fine, lacy chromatin structure, and nucleoli were rarely visible. Sparse peroxidase-positive granules were observed scattered throughout the cytoplasm.

“A” Macrophages (Immature): Round or oval cells 15–40 µ in longest axis with a nuclear-to-cytoplasmic ratio much less than 1; an eccentric round
nucleus with either a fine or a slightly clumped chromatin structure and one or two nuclei; abundant basophilic cytoplasm containing numerous peroxidase-negative inclusions and occasional vacuoles were seen (Fig. 2).

“B” Macrophages (Mature): Oval cells 20–50 μ in longest axis, containing an eccentrically located nucleus with a dense and often clumped chromatin structure and rarely one or two dense nucleolar remnants; light acidophilic cytoplasm containing a variety of peroxidase-negative inclusions and prominent vacuoles were seen (Fig. 3).

Granulocytes: At various stages of maturation granulocytes were identified.
Cellular Proliferation

The variation with time of the numbers of cells in culture, of the distribution of cells according to morphologic type, and of the ability of cultured cells to incorporate $^3$H-thymidine is illustrated in Fig. 4. As noted previously for cultures of this type, in the absence of stimulating factor the cell count was near zero by day 8. In the presence of stimulating factor, total cell numbers declined for the first 2–3 days of culture as a result of loss of granulocytes and nucleated erythrocyte precursors; they then rose, reaching maximal values...
between days 6 and 8. During this period, there was a gradual shift from undifferentiated blast cells and promonocytes, which together constituted 75–80% of the mononuclear population at day 1, to an increasing proportion of A and then B macrophages.

Morphologically identifiable erythroblasts showed evidence of degeneration by 24 hr of culture. Morphologically identifiable granulocytes constituted less than 26% of the cells by day 4 and less than 10% of the cells by day 8.

During the first 5 days of culture, approximately 40% of the mononuclear cells were labeled by a 1-hr pulse of ³H-thymidine. By day 9, 1.0% or less of the cells were labeled (Fig. 4). Mitoses and nuclear labeling were observed in all mononuclear cells types except B macrophages.

Grain counts on cultured cells, obtained from animals injected with ³H-thymidine 1 hr before obtaining marrow, provided the following observations (Table 1). At day 1, the blast cells plus promonocytes comprised essentially all the labeled cells. By day 4 label was seen in monocytes and A macrophages as well. Recognizing the limitations of calculating a precise number of cell divisions from a fall in median grain count,¹⁴ we could still estimate that the cells labeled on day 1 and still labeled on day 4 had gone through at least three divisions. Analysis of the data by the method of Maloney et al.,¹⁵ in which the grain count of the nth most heavily labeled cell determined by the initial position of the median cell was followed, also led to the conclusion that labeled cells had gone through at least three divisions during the first 3 days of culture.

In another series of experiments, a 1-hr pulse of ³H-thymidine was given to previously unlabeled cells on the third or fourth day of culture. The following observations were made. Between days 3 and 9, the labeled population had gone through at least four divisions. Between days 9 and 12 there was little further division, an observation consistent with the few cells entering S phase during this period. Therefore, it appeared that a major conversion of A to B macrophages after day 9 occurred without further cell division.

**Functional Characteristics**

*Glass Adherence:* Blast cells did not adhere to glass, whereas cells at or beyond the promonocyte stage were adherent. However, less than 5% of promonocytes of day 2–4 cultures attached to glass, and these showed little tendency to spread out over a 6–8-hr period. Whether there are distinct subpopulations of adherent and nonadherent promonocytes or whether these functional differences reflect cells at different levels of maturation is not known.

*Phagocytosis:* Blast cells were never observed to be phagocytic of C. albicans, Staph. aureus, or mouse IgG-coated sheep erythrocytes. Promonocytes

### Table 1. Distribution of Grain Counts Among Cultured Bone Marrow Cells Labeled in Vivo by ³H-Thymidine

<table>
<thead>
<tr>
<th>Days of Culture</th>
<th>Median Grain Count/Cell</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Monoblast and Promonocyte</td>
</tr>
<tr>
<td>1</td>
<td>30.1</td>
</tr>
<tr>
<td>4</td>
<td>3.1</td>
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</table>
BONE MARROW MACROPHAGE PRECURSORS

rarely phagocytized microorganisms and were never observed to ingest erythrocytes. They were conspicuously less phagocytic cells than monocytes and macrophages in the same culture. The latter cells were usually full of ingested particles, whereas less than 1% of promonocytes had phagocytized a single yeast or a few bacteria.

Surface Receptors for Immunoglobulin: The adherence and phagocytosis of sheep erythrocytes coated with mouse IgG immunoglobulin was used to demonstrate the presence on cells of surface receptors for immunoglobulin. Blocking of erythrocyte adherence by purified mouse myeloma proteins was taken as evidence of competition by proteins of a specific subclass.

As in previous studies of mouse peritoneal macrophages and bone marrow-derived macrophages arising in agar colonies, 70–90% of monocytes and A and B macrophages arising in liquid culture formed rosettes with sheep erythrocytes sensitized with IgG. Adherence was blocked by IgG2a and some IgG1 paraproteins, indicating subclass specificity of the surface receptor. Between 16 and 40% of promonocytes formed red cell rosettes, whereas undifferentiated blasts did not form such rosettes. From these observations, we conclude that the promonocyte has surface receptors for immunoglobulin, although not in as high a frequency as the more mature cells of the series.

DISCUSSION

In 1970 Van Furth et al. published descriptions of a cellular precursor of the monocyte that they called promonocyte. From 3H-thymidine studies, these investigators concluded that the promonocyte was a proliferating precursor of the monocyte and macrophage, whereas monocytes and macrophages were nonproliferating.

Most investigators generally have agreed that monocytes and macrophages have a very limited proliferative capacity in vitro and probably in vivo. On the other hand, occasional reports have suggested that under appropriate stimuli, such as intense antigenic stimulation, monocytes and macrophages are capable of DNA synthesis and replication. The present study indicates that mononuclear phagocytes, through the stage of morphologically immature A macrophages, are capable of DNA synthesis and nuclear replication, provided a stimulatory agent is present in the culture medium. Our results are very similar in this regard to those of Virolainen and Defendi, who observed proliferation of mouse macrophages obtained from the peritoneal cavity in the presence of factors contained in certain conditioned media.

As expected, those functional characteristics that are used to identify macrophages were least well developed in the earliest identifiable cells of the series and appeared progressively with maturation. These characteristics included glass adhesiveness, phagocytosis, and surface receptors for IgG immunoglobulins. The only exception to this sequence was the previously noted disappearance of peroxidase activity beyond the monocyte stage.

Based on the observations reported in this study, our current concept of the sequence of appearance of differentiated characteristics within the monocyte series is given in Table 2.
Table 2. A Concept of the Change in Certain Functional Characteristics of Mouse Mononuclear Phagocytes with Maturation

<table>
<thead>
<tr>
<th>Function</th>
<th>Undifferentiated Blast Cell</th>
<th>Promonocyte</th>
<th>Monocyte</th>
<th>&quot;A&quot; Macrophage</th>
<th>&quot;B&quot; Macrophage</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA synthesis and mitosis</td>
<td>2+</td>
<td>2+</td>
<td>+</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Peroxidase activity</td>
<td>2+</td>
<td>2+</td>
<td>2+</td>
<td>3+</td>
<td>3+</td>
</tr>
<tr>
<td>Glass adherence</td>
<td>0</td>
<td>+</td>
<td>2+</td>
<td>3+</td>
<td>3+</td>
</tr>
<tr>
<td>Phagocytosis</td>
<td>0</td>
<td>±</td>
<td>3+</td>
<td>3+</td>
<td>3+</td>
</tr>
<tr>
<td>Receptors for IgG</td>
<td>0</td>
<td>+</td>
<td>2+</td>
<td>3+</td>
<td>3+</td>
</tr>
</tbody>
</table>

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REFERENCES


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