Colony Formation In Vitro by Mouse Blood Monocytes

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Mouse blood monocytes were induced to proliferate and form discrete colonies of mononuclear phagocytes in liquid culture. The proliferation of these cells in vitro required a factor or factors present in medium conditioned by L cells. For this class of colony-forming cells, the value of D₀ to gamma irradiation in vitro was 195 rads.

Monocytes are the only known mononuclear phagocytes present in blood. Derived from rapidly proliferating promonocytes in bone marrow, they leave the circulation randomly and enter various tissues to become macrophages. Blood monocytes, under normal steady-state conditions, have not been known to replicate in blood; the transformation of monocytes to macrophages can occur in vitro and in vivo without mitotic division. It does not necessarily follow that monocytes are simply immature macrophages, unable to proliferate. In fact, the replication of monocytes when transferred to inflamed subcutaneous areas has been well documented.

Following recent success in using tissue culture techniques to identify several classes of mononuclear phagocytes having extensive proliferative capacity, we have initiated a study to evaluate the proliferative potential of blood monocytes in vitro. In this report mouse monocytes are shown to proliferate and form discrete colonies in vitro under appropriate culture conditions.

MATERIALS AND METHODS

Blood mononuclear cells. Peripheral blood from 3-mo-old C3H/He or AKR mice (Jewish Hospital, St. Louis, Mo.) was obtained by cardiac puncture. Mononuclear cells were obtained by a Ficoll Hypaque technique described by Boyum. About 1.5 x 10⁸ mononuclear cells per ml of whole blood was routinely recovered. Between 1% and 10% of the cells in this fraction could be identified morphologically as monocytes. Less than 0.2% of cells in the preparation were polymorphonuclear cells; the ratio of red cells to nucleated cells was less than 1:2.

Culture technique. Cells in 1 ml of growth medium were plated in 35-mm Falcon tissue culture dishes. The growth medium used, essentially the same as that used to grow peritoneal exudate colony-forming cells in liquid culture, consisted of 10% fetal calf serum, 5% horse serum, and 10% L cell-conditioned medium in alpha medium. All dishes were incubated at 37°C in a humidified incubator continuously flushed with 10% CO₂ in air. Cells were also grown in soft agar medium containing 0.3% Noble agar (Difco Laboratory, Detroit, Mich.) in addition to all the ingredients present in the liquid medium.

Media conditioned by L cells or other cell types and serum from endotoxin-treated animals were prepared as described previously. Ascites fluid was induced in C57BL/6 mice by injecting...
1.5 ml of Freund's adjuvant intraperitoneally and was processed according to the method described previously.11

IgG receptor. The presence of IgG receptor on cell surfaces was detected by exposing cells to antiserum-coated sheep red blood cells (SRBC). Antiserum to SRBC was prepared by injecting C3H/He mice intraperitoneally with 10⁸ washed SRBC. Ten days later, mice were bled and serum was prepared. Just before the test, SRBC were incubated with an equal volume of antiserum for 30 min at room temperature; 50 µl were then added to the 1-ml cultures. After 30 min of incubation, the culture dishes were washed three times with media.

Irradiation. Cell suspensions (10⁶ cells/ml) were irradiated with a ¹³⁷Cs unit having a dose rate of 112 rads/min.12

RESULTS

Growth of mononuclear cells in vitro. Cells in dishes containing 10⁴ nucleated cells began to replicate between days 4 and 7 and formed discrete colonies containing 50 or more cells within 14 days (Fig. 1). The length of the delay before replication commenced was dependent on the number of cells originally plated. Cells cultured at high concentrations had a tendency to begin replicating earlier. After 16–18 days, cells in the colonies began to come off the dishes. The plating efficiency was 1.9% (0.8%–4.6%). A linear relationship was observed between the number of cells plated and the number of colonies formed (data not shown). It should be noted that the mononuclear cell fraction obtained from the blood also contained hemopoietic colony-forming units in culture (CFU-C).13–14 They were nonadherent cells15 and could form large colonies within 7 days in liquid culture. Since the incidence of CFU-C was low, on the order of 1–10/10⁵ nucleated cells, they did not usually interfere with the study of this new class of colony-forming cells unless more than 10⁴ cells were plated.

Since the growth kinetics of these colony-forming cells in liquid culture resembled those of peritoneal exudate colony-forming cells,11 we decided to grow blood mononuclear cells in soft agar medium. For agar culture, the number of colonies was scored on day 28. In sharp contrast to peritoneal exudate colony-forming cells, which formed colonies equally well in both liquid and agar

Fig. 1. Photomicrograph of a 14-day-old colony. x 100.
cultures, blood mononuclear cells did poorly in agar medium. The plating efficiency of these cells in agar culture was only about 5%-15% of that in liquid culture (Table 1).

The next question was whether or not the colony-forming cells were monocyte-like cells. Monocytes are known to be adherent.\textsuperscript{2} To show that these colony-forming cells were adherent cells, groups of culture dishes containing $10^5$ cells were prepared. At various times after incubation at 37°C, medium containing cells that had not attached firmly to the bottom of the dish (the nonadherent cell fraction) was transferred by pipet to an empty dish. The original dish containing adherent cells was washed twice and incubated further with 1 ml of fresh growth medium. The colonies in these dishes were scored 14 days later. Table 2 shows the pooled results from four experiments. By 2 hr after preparation of the initial cultures, almost all of the colony-forming cells in the original suspension were retained, indicating that the colony-forming cells were adherent.

\textit{Characterization of cells in colonies.} When groups of dishes were stained with Giemsa and examined at various times after culture, we found only large mononuclear cells. No mature granulocytes were ever observed in these colonies. To study whether or not cells present in 14-day-old colonies were phagocytic, heat-killed yeast particles were added to the cultures along with 0.1 ml of guinea pig complement. After 1 hr of incubation at 37°C, more than 70\% of cells in each colony had phagocytosed yeast particles. The presence of IgG receptors was studied by adding IgG-coated SRBC to the dishes. Almost all the cells present in colonies were covered with red cells and formed rosettes. When these dishes were incubated longer than 30 min, some cells also phagocytosed SRBC. These results suggested that the cells present in these colonies were mononuclear phagocytes.

<table>
<thead>
<tr>
<th>Time After Incubation</th>
<th>Adherent Cells</th>
<th>Nonadherent Cells</th>
</tr>
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<tbody>
<tr>
<td>5 min</td>
<td>16 $\pm$ 31</td>
<td>176 $\pm$ 24</td>
</tr>
<tr>
<td>30 min</td>
<td>154 $\pm$ 24</td>
<td>27 $\pm$ 10</td>
</tr>
<tr>
<td>2 hr</td>
<td>189 $\pm$ 20</td>
<td>2 $\pm$ 1</td>
</tr>
<tr>
<td>4 hr</td>
<td>173 $\pm$ 24</td>
<td>0</td>
</tr>
</tbody>
</table>

Control, no separation 192 $\pm$ 18

\textsuperscript{*}10\textsuperscript{5} mononuclear cells were originally plated per dish.
\textsuperscript{†}Mean $\pm$ SEM.
Table 3. Comparison of Colony-stimulating Activity of Substances Obtained From Various Sources

<table>
<thead>
<tr>
<th>Source of Colony-stimulating Substance</th>
<th>Colonies/10^4 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td></td>
</tr>
<tr>
<td>Medium conditioned by L cells</td>
<td>213 ± 24*</td>
</tr>
<tr>
<td>Embryo fibroblasts (C3H)</td>
<td>202 ± 30</td>
</tr>
<tr>
<td>Serum from endotoxin-treated mice</td>
<td>184 ± 15</td>
</tr>
<tr>
<td>Ascites fluid</td>
<td>98 ± 16</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
</tr>
<tr>
<td>Medium conditioned by embryo fibroblasts</td>
<td>0</td>
</tr>
<tr>
<td>Serum from endotoxin-treated rat</td>
<td>0</td>
</tr>
<tr>
<td>Hamster: medium conditioned by</td>
<td></td>
</tr>
<tr>
<td>Baby hamster kidney cells</td>
<td>0</td>
</tr>
<tr>
<td>Chinese hamster fibroblasts (V79)</td>
<td>0</td>
</tr>
</tbody>
</table>

*Mean ± SEM.

Requirement of a growth factor. Cells did not replicate when L cell-conditioned medium was deleted from the growth medium; the optimum concentration of this substance for colony formation was about 10%. As in growing peritoneal exudate colony-forming cells and CFU-C,

Radiosensitivity of colony-forming cells. The effect of ionizing radiation on the proliferative capacity of colony-forming cells was studied. The pooled results of seven experiments are shown in Fig. 2. The regression curve fitted to the data by the method of least squares gave a D₀ value of 195 rads; the extrapolation number was 1.14.

Fig. 2. Dose–response curve of colony-forming cells to gamma irradiation in vitro. Vertical bars denote SEM. D₀ = 195 rads, n = 1.14.
DISCUSSION

These results show that adherent blood mononuclear cells, most likely monocytes, are capable of extensive proliferation and can form discrete colonies in liquid culture. The poor growth of these colony-forming cells in agar culture distinguishes them from other classes of mononuclear phagocyte colony-forming cells present in peritoneal exudates, pleural effusions, and alveolar spaces. Moreover, these cells are significantly less sensitive to gamma irradiation than hemopoietic CFU-C and colony-forming cells present in peritoneal exudates and pleural effusions.

The relationship between various classes of mononuclear phagocyte colony-forming cells on the one hand and these colony-forming cells and CFU-C on the other is not clear at present. However, they all require a factor or factors present in L cell-conditioned medium for replication and they all fail to respond to media conditioned by cells of hamster or rat origin.

Monocytes from experimental animals and man have been shown to mature or develop into macrophages without replication in vitro. Even in long-term culture, only a very small fraction of these cells could be observed replicating at any time. The major difference between our culture system and previous ones is the presence in ours of L cell-conditioned medium, which serves as a source of growth or colony-stimulating factor. In the presence of this factor and a hard surface to which they can adhere, blood monocytes can replicate and produce clones of mononuclear phagocytes. Our finding is consistent with an earlier observation made by Ryan and Spector in vivo. Their study showed that a significant fraction of mouse monocytes adherent to coverslips begins to divide not immediately but two days after they are transferred into inflamed areas. Although no exogenous colony-stimulating factor is provided in the experiment, recent studies by others, as well as the data presented here, have confirmed the presence of a similar growth factor in inflammatory exudates. The system presented here appears to be a useful in vitro model for studying the factors that control the replication and differentiation of monocytes.

ACKNOWLEDGMENT

The author thanks Scott Sauerbrunn, Barbara Devaraj, and Michael Kurtz for expert technical assistance.

REFERENCES

8. Lin H, Freeman PG: Peritoneal exudate
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cells IV. Characterization of colony forming cells. J Cell Physiol (in press)


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