CONCISE REPORT

Identification of Megakaryocytes, Macrophages, and Eosinophils in Colonies of Human Bone Marrow Containing Neutrophilic Granulocytes and Erythroblasts

By A. A. Fauser and H. A. Messner

Pluripotent hemopoietic progenitors in human bone marrow can be identified by their ability to give rise in culture to colonies that contain more than one lineage of hemopoietic differentiation. Growth of these mixed colonies is supported by media conditioned by leukocytes in the presence of phytohemagglutinin (PHA-LCM) and erythropoietin. They can be readily recognized by direct microscopic observation because of their composition of colorless cells and cells with a red appearance typical for hemoglobin. Seventy-three individual mixed colonies from 6 individuals were prepared by cytocentrifugation for further cytological examination. In addition to neutrophilic granulocytes and erythroblasts, megakaryocytes were present in 16 of the 73 colonies, as identified by positive reaction for acid phosphatase. Macrophages were found in 24 and eosinophils in 6 of the 73 colonies.

RECENTLY we have reported that human bone marrow, peripheral blood, and cord blood contain progenitors that give rise in culture to colonies containing both neutrophilic granulocytes and erythroblasts.1,2 Such colony formation in culture is dependent on media conditioned by leukocytes in the presence of phytohemagglutinin (PHA-LCM) and erythropoietin. Evidence for the single-cell origin of mixed colonies was obtained in experiments where cells from males and females were cocultivated. Mixed colonies in such cultures contained either male or female cells but not both, as determined by the presence of Y bodies using fluorescence microscopy.

We now report that some mixed colonies, recognized by their content of neutrophilic granulocytes and erythroblasts, may also contain other lineages including eosinophils, megakaryocytes, and macrophages.

MATERIALS AND METHODS

Patients

Bone marrow samples were obtained from 3 normal bone marrow transplant donors and 3 bone marrow transplant recipients after stable engraftment.

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Submitted January 15, 1979; accepted January 19, 1979.

Supported by the Medical Research Council of Canada, by the University of Toronto, and by the Banting Research Foundation, Toronto. Dr. Fauser was financially assisted by Deutsche Forschungsgemeinschaft.

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Blood, Vol. 53, No. 5 (May), 1979 1023
Preparation of Cell Suspensions

The samples were aspirated into heparinized syringes. Mononuclear cells of density less than 1.077 g/cc were obtained after centrifugation in LSM solution (Litton Bionetics, Kensington, Md.). The cells were subsequently washed in alpha medium, counted by hemocytometer, and plated as outlined below.

Culture Conditions

The culture system has been described previously in detail. Briefly, mononuclear bone marrow cells were mixed with 30% fetal calf serum, 5% PHA-LCM, alpha medium (Flow Laboratories), and methyl cellulose as viscous support to yield a concentration of 0.9% (w/v). Precursors of mixed colonies were present at low frequency in bone marrow or peripheral blood samples. In order to obtain adequate numbers of colonies, eight Petri dishes (35 mm, Lux) were routinely plated at a cell density of $2 \times 10^6$ nucleated cells per milliliter. From the number of mixed colonies in all dishes, containing $1.6 \times 10^6$ nucleated cells in total, the frequency of mixed colonies was calculated. The cultures were incubated at 37°C in humidified atmosphere at 5% CO₂. After 4 days 2.5 units of erythropoietin (Connaught, step III) in a volume of 0.1 ml were added to the cultures by micropipette. The cultures were examined with an inverted microscope after a further incubation period of 10 days. At that time colonies were recognized that contained cells with the red color typical for hemoglobin admixed with colorless cells. Occasionally, cells of significantly larger size could be recognized. Individual colonies of this description were removed by micropipette from the cultures, and slides were prepared by cytocentrifugation (Shandon Southern Instruments Ltd.). In every case where a colony was identified as mixed by direct observation in the culture dish, further cytological analysis revealed the presence of granulocytes and erythroblasts.

Staining Procedures

Slides of individual mixed colonies were routinely stained by Wright stain and examined by light microscopy for neutrophilic granulocytes and erythroblasts. In addition, each colony was screened for megakaryocytes, macrophages, and eosinophils. For positive identification of megakaryocytes the slides were stained for acid phosphatase after fixation with 10% formalin in 0.1-M acetate buffer, pH 5.0, for 20 sec at 0–4°C.

RESULTS

In total, 73 mixed colonies that contained neutrophilic granulocytes and erythroblasts were examined for the coexistence of other hemopoietic lineages (Table 1). The sizes of these colonies available for cytological examination varied from 200 to 2000 cells.

Megakaryocytes were identified in 16 of the 73 mixed colonies. They were recognized by their size, their multilobulated nuclei, and their reticular cytoplasm

<table>
<thead>
<tr>
<th>Patients</th>
<th>Diagnosis</th>
<th>Granuloerythrocytic Colonies</th>
<th>Number of Colonies Containing</th>
<th>Megakaryocytes</th>
<th>Macrophages</th>
<th>Eosinophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>J.M.</td>
<td>Bone marrow donor</td>
<td>10</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td></td>
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<tr>
<td>R.McG.</td>
<td>Bone marrow donor</td>
<td>9</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>S.P.</td>
<td>Bone marrow donor</td>
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<td>2</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>J.P.</td>
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<td>8</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>D.J.</td>
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<td>10</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>R.H.(1)</td>
<td>Bone marrow recipient</td>
<td>10</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>R.H.(2)</td>
<td>Bone marrow recipient</td>
<td>16</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>73</td>
<td>16</td>
<td>24</td>
<td>6</td>
<td></td>
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</table>

*Total number of colonies per $1.6 \times 10^6$ nucleated cells.
Fig. 1. Cells from mixed colonies prepared by cytocentrifugation. A. Megakaryocyte and erythroblasts stained by Wright stain. B. Megakaryocyte identified by positive acid phosphatase reaction. C. Macrophage and myeloperoxidase-positive granulocyte.

(Fig. 1A). In addition, cells of this morphology stained positive for acid phosphatase (Fig. 1B). The number of megakaryocytes in individual colonies varied greatly and ranged between 5 and 30. Macrophages were seen in 24 of the 73 colonies. They were identified by their characteristically vacuolated cytoplasm (Fig. 1C). Again it was noted that their numbers varied greatly from colony to colony. Eosinophilic granulocytes were observed in varying numbers in 6 of the 73 colonies. They were usually recognized by their typically eosinophilic coarse granulation.
While megakaryocytes and macrophages were found in colonies derived from each of the bone marrow specimens, eosinophilic granulocytes were seen in only 3 of the 7 samples. In some of the mixed colonies elements representing four lineages of hemopoietic differentiation were observed. None of the colonies available for examination contained all five described cell types.

**DISCUSSION**

The presence of PHA-LCM and erythropoietin in cultures of human hemopoietic cells promotes the growth of colonies that contain more than one lineage of hemopoietic differentiation. Primary identification of these mixed colonies was based on direct microscopic observation. Within individual colonies, cells characterized by their red color were admixed with translucent cells. Detailed cytological analysis revealed the presence of neutrophilic granulocytes and erythroblasts in all colonies identified by these criteria. However, in addition, some colonies contained megakaryocytes and macrophages and/or eosinophils. The observation of as many as four different hemopoietic lineages in individual colonies supports the view that pluripotent hemopoietic progenitors (CFU-GEMM) exist in man.

The growth of pluripotent murine hemopoietic progenitors in culture was reported by Johnson and Metcalf. These investigators were able to identify five different hemopoietic lineages in individual colonies derived from fetal liver cells. Colony formation was promoted by media conditioned by spleen cells in the presence of pokeweed mitogen. A major difference between our observations and those of Johnson and Metcalf is that the erythropoietic components of murine fetal-liver-derived colonies do not require erythropoietin. Recently, Johnson and Metcalf have suggested that their system promotes colony formation by cells with fetal characteristics. If progenitors detected by our method follow adult differentiation programs, the difference would be explained.

In addition to these assays for pluripotent human and murine hemopoietic progenitors, culture conditions have been reported that promote growth of mixed colonies containing erythroblasts and megakaryocytes. The initial observation by McLeod et al. for murine hemopoiesis was confirmed by Hara and Ogawa and subsequently described for human peripheral blood cells by Vainchenker et al. The association of erythroblasts and megakaryocytes in individual colonies has led to the question whether or not a common precursor exists for both lineages. Our own data for human cells obtained under different culture conditions do not support this view. In our culture system erythroblasts were more frequently associated with granulocytes than with megakaryocytes. It is conceivable that differing culture conditions, while promoting the growth of certain hemopoietic lineages, may fail to support the development of others. From this point of view, culture conditions, rather than the potential of the cells, determine the cell classes associated together most frequently in mixed colonies.

**ACKNOWLEDGMENT**

The authors wish to express their thanks to Nazir Jamal for technical assistance.

**REFERENCES**


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