Interacting Cell Populations Affecting Granulopoietic Colony Formation by Normal and Leukemic Human Marrow Cells

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Marrow from 28 nonleukemic individuals was separated by adherence to glass or plastic into nonadherent (NA) and adherent populations. The NA populations were found to be more dependent for colony formation in culture on added colony-stimulating activity (CSA) than unseparated marrow suspensions, and therefore proved useful for CSA assays. Quantitative reconstitution procedures were used to assay CSA-producing cells. Either increasing numbers of irradiated unseparated marrow, or adherent cells derived from varying numbers of marrow cells, were used to restore colony-forming efficiency to NA populations. Assay procedures for CSA-producing cells were applied to four patients with acute leukemia prior to treatment. In all four instances, a defect in CSA-producing cells was demonstrated.

COMMITTED PROGENITORS of granulopoiesis in human marrow form colonies in viscous or semisolid media; colony formation is influenced by the presence, in the cultures, of molecules derived from other hemic cells. These active materials are heterogeneous in respect to molecular size and may be collectively termed "colony-stimulating activity" (CSA). In human marrow, CSA-producing cells coexist with CSA-responsive granulopoietic colony-forming cells (CFU-C); accordingly, the growth of individual marrow specimens in culture is dependent not only on concentration of CFU-C but also on both CSA-producing cells and the amount of exogenous CSA in the cultures. The value of the culture system would be enhanced if the various interacting factors could be assessed independently. Haskill et al. have attempted such assessment using density centrifugation to separate human marrow into responding, stimulating, or inhibiting cell populations. In the studies reported in the present paper, CFU-C were separated from CSA-producing cells by the technique of adherence to glass or plastic. Using this method it was possible to improve the assay for human CSA, to develop quantitative methods for CSA-producing cells, and to provide preliminary evidence that the latter cell class may be abnormal in acute leukemia.
MATERIALS AND METHODS

Patients

Marrow was obtained from 28 nonleukemic individuals as part of clinical assessment. In one instance the marrow was obtained from a normal volunteer who was to serve as a white cell donor using an IBM cell separator. The patient material is summarized in Table 1. It may be seen from the table that marrows from patients with a variety of nonleukemic conditions were included, but that none of the diagnoses had a specific effect on colony formation in culture. Accordingly, the material from these patients was used as it became available; the results did not indicate any bias from the inclusion of all patients, selected only on the basis of absence of a diagnosis of leukemia. In addition, marrow from four patients with acute leukemia was studied prior to the initiation of therapy. The hematologic findings for these patients are listed in Table 2.

Assay for CFU-C

A modification of the method of Iscove et al. was used to measure granulopoietic progenitors in culture (CFU-C). Briefly, marrow cells obtained from buffy coats of aspirated suspensions were plated in final concentrations of 0.8% methyl cellulose, 20% FCS, and alpha medium. Routinely, the cells were plated with and without CSA. CSA was produced by layering culture medium with 20% FCS over peripheral leukocytes immobilized in agar and incubating the cultures for 7 days. In sensitivity studies, using unseparated control suspensions or nonadherent cells as target cells, CSA was added in increasing concentrations up to 30%. After 12 14 days of incubation, colonies in excess of 20 cells were scored using an inverted microscope. The counts were performed on at least duplicate plates. Three morphologically distinct colony types are seen in methyl cellulose cultures of human marrow. The cellular composition of each type has been determined by examining cells from individual colonies stained either with Wright's stain or peroxidase. The majority of colonies (60% - 90% of the total) contained peroxidase-positive neutrophils at various stages of maturation. Approximately 10% contained peroxidase-positive cells with large eosinophilic granules. Up to 30% contained large vacuolated mononuclear cells that are peroxidase negative. Such mononuclear cell colonies are usually excluded from the counts because their loose arrangement often makes them difficult to distinguish from background. Further, the significance and origin of these colonies are unknown; unlike morphologically similar colonies from mouse

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No of Patients</th>
<th>Mean Col. No. per 10^5 Cells</th>
<th>Lowest Col. No. per 10^5 Cells</th>
<th>Highest Col. No. per 10^5 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron def. anemia</td>
<td>7</td>
<td>38</td>
<td>23</td>
<td>67</td>
</tr>
<tr>
<td>Polycythemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rubra vera</td>
<td>2</td>
<td>33</td>
<td>25</td>
<td>41</td>
</tr>
<tr>
<td>Erythrocytosis</td>
<td>1</td>
<td>57</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Thalass. min.</td>
<td>1</td>
<td>39</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Bronchogenic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ca.</td>
<td>5</td>
<td>19</td>
<td>11</td>
<td>26</td>
</tr>
<tr>
<td>Ca. prostate*</td>
<td>3</td>
<td>21</td>
<td>11</td>
<td>28</td>
</tr>
<tr>
<td>Lymphomas</td>
<td>5</td>
<td>33</td>
<td>17</td>
<td>60</td>
</tr>
<tr>
<td>Cirrh. hep.</td>
<td>2</td>
<td>19</td>
<td>10</td>
<td>28</td>
</tr>
<tr>
<td>Rheum. arthr.</td>
<td>1</td>
<td>20</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Normal</td>
<td>1</td>
<td>36</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Morphological examination of the bone marrow specimens did not reveal any metastatic involvement of the bone marrow in any of the patients.
† Morphological examination of the bone marrow specimens showed minimal infiltration with lymphocytes in one of the patients. Colony growth in this individual yielded 60 colonies per 10^6 nucleated cells.
Table 2. Hematological Findings and Colony Formation in Four Patients With Acute Leukemia

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Peripheral Blood</th>
<th>Marrow Differential Count (%)</th>
<th>CFU-C/10^5 Nucleated Marrow Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WBC x 10^3/µl</td>
<td>Blasts</td>
<td>Promyelocytes</td>
</tr>
<tr>
<td>AML</td>
<td>6.0</td>
<td>4.8</td>
<td>14</td>
</tr>
<tr>
<td>AML</td>
<td>7.0</td>
<td>3.1</td>
<td>65</td>
</tr>
<tr>
<td>AML</td>
<td>8.8</td>
<td>1.5</td>
<td>43</td>
</tr>
<tr>
<td>AL*</td>
<td>10.0</td>
<td>155</td>
<td>85</td>
</tr>
</tbody>
</table>

*Acute leukemia, type undetermined.
marrow, their origin from granulocyte colonies has not been observed. Granulocyte colonies in human marrow appear to retain their characteristic feature until they lyse after 20 days in culture. This counting convention has also been used by Brown et al.  

Separation by Adherence

A modification of the technique of Mosier  was used to separate marrow cell suspensions on the basis of their adherence to glass or plastic. Nonadherent (NA) cells were separated from marrow suspensions by incubating \(2 \times 10^7\) nucleated marrow cells and not more than \(1 \times 10^8\) total cells suspended in 10 ml of alpha medium with 20% FCS in 100-mm glass petri dishes for 30 min. The incubation was carried out at 37°C under humidified conditions and a mixture of air and CO\(_2\) to keep the pH constant at levels of 7.2-7.3. The supernatant, containing cells that had not adhered, was removed and incubated for 2 hr in a second petri dish. Cells in the supernatant after this incubation were considered to be NA cells. Usually between 50% and 80% of input cells were recovered in the NA cell preparation. Adherent cells were prepared by incubating from \(10^5\) to \(10^7\) marrow cells in 1 ml of alpha medium with 20% FCS in 35-mm Falcon plastic tissue culture dishes (TC 3001) for 30 min. The supernatant was decanted and discarded, and the adherent layer washed three times with alpha medium. The layer was then used as adherent cells. Usually between 2% and 10% of input cells were recovered as adherent cells.

Irradiation Procedure

Cells were irradiated in a \(^{137}\)Cs irradiator designed by Cunningham, Bruce, and Webb.  

RESULTS

Separation of Human Marrow by Adherence

Nonadherent (NA) populations were prepared from marrow suspensions obtained from 28 patients. For a single experiment, the plating efficiency of the NA populations is compared with that of unseparated marrow in Fig. 1, where the mean number of colonies observed is shown as a function of cell number. It is evident that a high proportion (80%) of the original suspension was recovered in the NA populations; the adherence procedure did not usually yield populations greatly enriched for CFU-C. Further, like the original suspension, in the presence of 20% CSA, the NA population yielded a linear relationship between cell number plated and colony formation. This finding is consistent with the view that the separation procedure did not change the cultural requirements for colony formation.

Figure 2 shows the colony-forming efficiency of the original marrow suspen-
NORMAL AND LEUKEMIC HUMAN MARROW CELLS

Fig. 2. (A) Colony formation in unseparated marrow cells suspensions from 28 individuals used as sources of adherent and nonadherent cells. The number of colonies obtained in the presence of 20% CSA (open circles) and the absence of CSA (closed circles) is plotted as a function of cell number. (B) Colony formation by nonadherent marrow cells. The number of colonies in the presence (open circles) and absence of CSA (closed circles) is plotted as a function of cell number.

sions (panel A) and the NA cells (panel B) for all of the specimens plated with or without CSA. The wide spread of values observed is evident from the plot and may reflect the heterogeneous patient population. However, for unseparated marrow, colony formation was obtained without added CSA in almost every instance (panel A); in contrast, the addition of CSA was almost always required for colony formation by NA cells (panel B). Although not included into the counts, macrophage colony formation was also low or absent in NA cell suspensions. It is concluded that the separation procedure was effective in removing a cellular source of CSA from the marrow suspensions while yielding an acceptable recovery of CFU-C.

An Assay for CSA

The availability of populations of NA cells, markedly reduced in colony-forming ability without added CSA, but yielding numerous colonies in its presence, provided the basis for an improved assay for CSA. The assay is illustrated in Fig. 3; in the experiment unseparated and NA cells were plated in the presence of increasing concentrations of CSA. For the unseparated cells, colony number was insensitive to added CSA, although colony size increased. For the NA cells, however, a linear increase in colony number was observed with increasing concentrations of CSA. Accordingly, NA cells are more useful than whole marrow for the assessment of CSA.

The value of the technique is illustrated in Fig. 4. In the experiments of Fig. 4A, four different preparations of CSA were added to cultures of unseparated marrow cells at varying concentrations; in those of Fig. 4B, the same preparations were tested on $3 \times 10^3$ NA cells. It is evident from the figure, that the test
on NA cells ranked the four preparations, while the test on unseparated cells was successful only in identifying a preparation with very low activity.

Assay for CSA-Producing Cells

Just as NA cells provide a suitable basis for an assay for CSA, they may also be used to assay CSA-producing cells. We have used two approaches to the problem. In the first, heavily irradiated (900 rads) marrow cells were added to $2 \times 10^5$ NA cells and the mixture tested for colony-forming capacity. The results of two such experiments are presented in Fig. 5, and a third experiment is shown in Fig. 8, below. A linear relationship was found between the number of added irradiated marrow cells and colony formation; since the dose of radiation was sufficient to abolish colony formation by the marrow cells (see Fig. 5) these experiments are interpreted as providing a measure of CSA-producing...
capacity of the marrow under test. As a control for each point, 20% CSA was added to the cell mixture; no cumulative effect of CSA and irradiated bone marrow cells was observed.

In a second approach, varying numbers of marrow cells were plated in Falcon tissue culture dishes and nonadherent cells removed as described in the materials and methods section. To the dishes, now containing adherent cells derived from varying amounts of marrow, were added suspensions of $3 \times 10^5$ nonadherent cells in methyl cellulose, 20% fetal calf serum, and alpha medium. The dishes were incubated for 12-14 days and colony formation assessed. The results of one experiment of this design is presented in Fig. 6 and four further experiments are shown in Fig. 7. Colony formation from $3 \times 10^5$ NA cells increased linearly with the number of marrow cells used as a source of adherent cells; for values in excess of $10^6$ original marrow cells, colony formation reached a plateau at levels in the range of that found for NA cells plated with 20%, CSA.

Both the experimental designs described above consist of reconstitutions in which NA populations, containing CFU-C, were mixed with populations containing CSA-producing cells but devoid of colony-forming capacity. In the experiments shown in Figs. 5 and 6 the reconstitutions were made with populations derived from the same original marrow aspirate. In addition, in Fig. 5 a reconstitution is demonstrated with homologous irradiated peripheral blood cells (open triangles), indicating that the procedure may be applied to adherent and NA cells from different sources.
CSA-Producing Cells in Four Patients With Acute Leukemia

Assays for CSA producing cells were made on the marrow of four patients with acute leukemia prior to treatment. The procedure was the second of those described above; that is, varying numbers of marrow cells from the leukemic patients served as a source of adherent cells in plastic tissue culture dishes and $2 \times 10^5$ nonadherent cells from a normal individual were plated on top of the adherent layer in methyl cellulose, fetal calf serum, and alpha medium. As a control, adherent cell layers were also prepared from the marrows used as sources of NA cells. The results are shown in Fig. 7. In all four cases, leukemic marrows were less effective than normal marrows in promoting colony formation by NA cells of the normal individual. In one of the cases described in Fig. 7 both assays for CSA-producing cells were performed. The mixture of irradiated unseparated leukemia marrow with normal NA cells yielded no colonies, while complete reconstitution was achieved with autologous irradiated marrow (Fig. 8). These results are similar to those found when NA cells were plated on adherent cells from leukemic patients.
DISCUSSION

The results presented in this paper are consistent with the view that normal human marrow contains both granulopoietic colony-forming cells (CFU-C) and cells capable of producing molecules required by these progenitors for proliferation and differentiation in culture. CSA-producing cells in the peripheral blood of monkeys have been studied by Moore and Williams, and those in human peripheral blood by Chervenick and LoBuglio and by Golde and Cline. All three groups concluded that CSA production was a property of cells morphologically similar to monocytes and with the functional property of glass adherence. The present studies extend the findings to marrow, indicating that the CSA-producing cells in this site are also glass adherent.

The adherent separation procedure provides a reliable means of obtaining cell populations capable of responding to CSA but with little or no colony-forming capacity in its absence. Such populations provide the basis for quantitative assays for both CSA and CSA-producing cells. For the molecules, the assay procedure may be expected to facilitate purification and characterization of CSA from human sources. Such a procedure is required since mouse marrow cells, often used for this purpose, do not respond to all species of human CSA; for example, Price et al. have reported a CSA with low molecular weight (approx. 1300) that is active on human CFU-C but fails to stimulate mouse CFU-C.

The quantitative assays for CSA-producing cells, described in this paper, provide the basis for determining the characteristics of this population. Examination of cells that adhered to glass, either directly without fixation, or after staining, revealed large mononuclear cells without specific identifying characteristics. It is not known whether CSA production is a function of all such cells or of a specific subpopulation. However, the availability of assay procedures makes it possible to approach this question using techniques of cell separation. Analysis by velocity sedimentation of both NA cells and adherent cells is at present under way in our laboratory.

The studies of marrow from patients with leukemia are examples of how the assay procedure for CSA-producing cells may be applied clinically. A clear difference from normal was observed in the four patients investigated. Unseparated leukemic marrow failed to restore colony-forming capacity to populations of normal NA cells (Fig. 8); thus, failure to obtain reconstitution of NA populations with adherent cells from patients with leukemia (Fig. 7) cannot be attributed to a failure of CSA-producing cells from marrow of patients with leukemia to adhere normally to glass or plastic. Accordingly, in the four patients studied, we conclude that CSA-producing cells are abnormal either in number or productive capacity. Whether the abnormality is secondary to dilution of the marrow by abnormal cells or reflects part of the mechanism of defective differentiation in leukemia is unknown; however, the availability of an assay procedure makes it feasible to investigate the problem.

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REFERENCES


