Human T-Lymphocyte Products Stimulate Human Hemopoietic Progenitor Cell Proliferation in Diffusion Chambers In Vivo

By Eero Niskanen, Alan Oki, Martin J. Cline, and David W. Golde

Human myeloid colony formation in diffusion chambers in mice (CFU-DG) was enhanced following administration of a human T-cell-line-derived conditioned medium (Mo). The Mo cell line also elaborates activities stimulating human myeloid colony formation in vitro in agar (CSF) and potentiating erythroid colony formation in vitro in methylcellulose (EPA). Depletion of CSF from Mo conditioned medium by heat inactivation or gel exclusion chromatography did not affect CFU-DG formation. EPA and CFU-DG stimulating activities are heat stable and have approximately the same molecular weight. Culture of human bone marrow cells in diffusion chambers in mice for 4 days under the influence of Mo conditioned medium resulted in significant increment of BFU-E and CFU-DG as judged by subculture of diffusion chamber contents. No effect on CFU-C could be detected.

The use of in vitro culture techniques has facilitated the detection of hemopoietic factors from various sources. In addition to erythropoietin, erythropoiesis is stimulated in vitro by activities elaborated by peripheral blood leukocytes, embryonic fibroblasts, monocytes, and T lymphocytes. Some of the cell types that produce erythropoietic activities also produce colony-stimulating factors (CSF) that stimulate the formation of granulocyte-macrophage colonies in vitro (CFU-C). Under certain circumstances, T-lymphocyte products induce formation of colonies containing hemopoietic elements of several lineages, suggesting that pluripotent stem cells serve as targets for T-cell lymphokines.

A widely used technique to study the effect of humoral hemopoietic factors in vivo involves culturing bone marrow cells in diffusion chambers impermeable to cell migration implanted intraperitoneally in animals. Increased colony formation in these diffusion chambers can be observed following irradiation, cyclophosphamide administration, hypoxia, anemia, or erythropoietin injections. It is uncertain whether CSF, which is necessary for the growth of CFU-C in vitro, plays an important role in the regulation of in vivo diffusion chamber granulopoiesis. Injection of progressively increasing doses of endotoxin intravenously in host mice resulted in a lack of correlation of serum CSF or inhibitor level with diffusion chamber granulopoiesis. Most profound stimulation of diffusion chamber granulopoiesis is achieved by rendering the host mice cytopenic with irradiation or cyclophosphamide. Cyclophosphamide administration is associated with delayed and variable CSF elevations that are substantially less than those observed after endotoxin injections. The presence of high levels of CSF in diffusion chambers even in untreated mice may maximally stimulate CSF-responsive granulopoiesis, suggesting that the culture system can only be used as a probe of non-CSF-dependent granulopoiesis. In the present study, we were able to show that formation of granulocytic colonies in diffusion chambers implanted in mice (CFU-DG) only occurred under the influence of a lymphokine elaborated by a human T-lymphocyte cell line (Mo). CSF from the same cell line had no effect on CFU-DG. This is the first study identifying a factor that stimulates proliferation of granulocytic stem cells (CFU-DG) in vivo. Based on earlier studies, it is likely that the target cell population is more primitive than the CFU-C.

MATERIALS AND METHODS

Mo Conditioned Medium

The Mo T-lymphocyte cell line was derived from the spleen of a patient with a T-cell variant of hairy cell leukemia as described earlier. The cell line was maintained in α-medium (Flow Laboratories, Detroit Mich.) supplemented with 30% fetal calf serum. Conditioned medium was first tested in vitro for its ability to enhance both myeloid and erythroid colony formation and samples showing highest levels of activity were used in this study. Mo conditioned medium (0.05 ml) induced formation of 82 CFU-C and 32 BFU-E per 10⁶ human bone marrow cells, while no CFU-C and 12 BFU-E were found in the absence of this material in agar and in methyl cellulose (no erythropoietin added) in vitro, respectively.

A "normal" B-cell lymphoblastoid line has been derived from the donor of the Mo T-cell line. It is a typical Epstein-Barr virus-infected B-cell line that does not produce lymphokines. The Mo B-line is maintained under identical conditions with the T-cell line and was used to obtain control conditioned medium. None of the tested samples contained levels of endotoxin detectable by limulus assay.
Depletion of CSF From Mo Conditioned Medium

The Mo conditioned medium was immersed in a boiling water bath for 10 min, resulting in the inactivation of the bulk of the CSF activity. The denatured protein was removed by centrifugation at 400 g for 15 min and the clear conditioned medium solution was applied to an Ultralrog AcA 44 (LKB) column (2.5 x 55 cm) equilibrated with 0.15 M NaCl, 0.02 M sodium phosphate, pH 7.4. The material containing activity that potentiates erythroid colony formation in methylcellulose in vitro (EPA), eluting in a volume corresponding to a molecular weight of about 45,000, was pooled and used for our studies. This partially purified EPA preparation contained less than a few percent of the original CSF activity as judged by in vitro agar colony formation, while the recovery of erythroid-potentiating activity was nearly 100%. Addition of 0.05 ml of this material to the culture plates induced formation of 2 CFU-C and 40 BFU-E per 10^5 bone marrow cells, while no CFU-C and 17 BFU-E were detected in the absence of partially purified Mo conditioned medium.

Fractionation of Mo Conditioned Medium

Mo conditioned medium and a medium supplemented with fetal calf serum (control) was concentrated fivefold by lyophilization. The lyophilized powder was dissolved in 0.02 M sodium phosphate, 0.15 M NaCl, 8 M urea, pH 7.2. This solution (0.5 ml) was then subjected to gel exclusion chromatography on a 0.7 x 27 cm Sephacryl-S-200 column equilibrated with the same buffer. Fractions (1.0 ml) were collected, dialyzed exhaustively against 0.02 M sodium phosphate, 0.15 M NaCl, pH 7.2, and tested for ability to stimulate CFU-DG, CFU-C, and BFU-E.

Cell Preparation

Bone marrow cells were aspirated from healthy volunteers after obtaining informed consent and sedimented in 3% dextran. Then the cells were suspended in McCoy's medium containing 20% fetal calf serum.

Culture of Hemopoietic Cells in Diffusion Chambers in Mice

The diffusion chamber technique used in this study has been described in detail elsewhere. Diffusion chambers were filled with normal human bone marrow cells at a concentration of 4 x 10^6. After sealing, they were implanted in mice. The conditioned medium was dispensed by a miniosmotic pump (Alza, Palo Alto, Calif.) implanted intraperitoneally. This guaranteed a continuous flow of the Mo conditioned medium into the system. Day 0 CFU-C, CFU-DG, and BFU-E concentrations were determined from the conditioned medium into the system. Day 0 CFU-C, CFU-DG, and BFU-E concentrations were determined from the implants. After 3 hr, 1 and 4 days of implantation, 12-18 chambers were removed and placed in McCoy's medium containing 0.5% pronase (Calbiochem, La Jolla, Calif.) and 5% Ficoll (Sigma Chemicals, St. Louis, Mo.) to dissolve the clot formed in the chamber. After agitation for 80 min at room temperature, the chambers were opened and the contents were removed for determination of CFU-C, CFU-DG, and BFU-E concentrations (Fig. 1).

CFU-DG Assay

In some experiments pooled contents of individual diffusion chambers were transferred to new cyclophosphamide-pretreated mice (Fig. 1). In other experiments (Table 1, Fig. 2), diffusion chambers were filled with 4 x 10^6 human bone marrow cells and implanted in mice that were treated with Mo conditioned medium. After 7 days of culture, the diffusion chambers were transferred to new cyclophosphamide-pretreated mice (Table 1, Fig. 2). On day 14, the chambers were harvested, and the plasma clots attached to Millipore filters were fixed with 5% glutaraldehyde and stained with Harris' hematoxylin. The dried preparations were made transparent in immersion oil for microscopic examination. Routinely, the number of CFU-DG was scored at 100x magnification. Aggregates containing 20 or more granuloid cells were scored as CFU-DG.

CFU-C Assay

A double-layer agar technique was used to assay CFU-C. Samples (2 x 10^5 cells/plate) to be assayed were plated in the upper layer. Samples tested for CSF activity or 10^6 adult human peripheral blood leukocytes used as CSF source were placed in the underlayer.
Table 1. Effect of Mo Conditioned Medium on CFU-DG Before and After Depletion of CSF

<table>
<thead>
<tr>
<th>Volume (ml)</th>
<th>CSF Containing CFU-DG/Chamber</th>
<th>CSF Depleted CFU-DG/Chamber</th>
</tr>
</thead>
<tbody>
<tr>
<td>Administered to Host Mice</td>
<td>SEM</td>
<td>SEM</td>
</tr>
<tr>
<td>—</td>
<td>8 ± 3</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>0.6</td>
<td>32 ± 3</td>
<td>46 ± 4</td>
</tr>
<tr>
<td>1.8</td>
<td>83 ± 6</td>
<td>64 ± 7</td>
</tr>
<tr>
<td>3.6</td>
<td>109 ± 10</td>
<td>128 ± 14</td>
</tr>
</tbody>
</table>

*Mo conditioned medium (50 μl/ml) stimulated formation of 106 CFU-C and 84 BFU-E/2 x 10⁶ human bone marrow cells. Six CFU-C and 66 BFU-E/2 x 10⁵ cells were formed in the presence of Mo conditioned medium boiled for 10 min (50 μl/ml).

†0.3 ml/day up to the total dose.
Results from 3 experiments.

CFU-DG per chamber were observed following administration of Mo conditioned medium and α-medium (control), respectively, suggesting that factors in the Mo T-cell conditioned medium may have some species specificity in their activity. Like crude Mo conditioned medium, partially purified EPA containing no significant CSF activity caused a dose-dependent stimulation of human CFU-DG (Table 1). Administration of conditioned medium from human Mo B-cell line failed to stimulate CFU-DG formation (8 ± 2 CFU-DG/4 x 10⁵ cells), suggesting that T cells are essential for elaboration of CFU-DG stimulating activity. Injection of increasing daily doses (0.05–32 μg/mouse) endotoxin intraperitoneally did not increase the number of CFU-DG (12 ± 3 versus 12 ± 4/4 x 10⁵ cells; results from 3 experiments).

Determination of human hemopoietic progenitor cell contents in the diffusion chambers in mice by subculture revealed that under the influence of crude Mo conditioned medium containing EPA and CSF, CFU-DG and BFU-E numbers were higher on day 4 (p < 0.01) (Fig. 1). No effect on CFU-C numbers was observed (Fig. 1).

To further determine the relationship between CFU-DG stimulating activity, EPA, and CSF, we subjected Mo conditioned medium to gel exclusion chromatography and assayed the resulting fractions both in vivo and in vitro cultures. As displayed in Fig. 2, CSF was separated from other hemopoietic activities. Peak EPA and CFU-DG stimulating activities were present in the same fraction. Fractionation of culture medium (α-medium + 30% fetal calf serum) used to condition Mo T cells and Mo B-cell line conditioned medium was also fractionated and assayed for CFU-DG stimulating activity. None of the fractions increased the CFU-DG number in the chamber.
DISCUSSION

We have shown that a T-cell line, which elaborates CSF and EPA, also served as a source of activity-stimulating myeloid colony formation in diffusion chambers in mice (CFU-DG) (Table 1). Further characterization of this activity revealed that it is heat stable (Table 1) and has a molecular weight close to that of EPA (Fig. 2). Thus, we were not able to differentiate the activities that potentiate erythroid burst formation in methylcellulose (BFU-E) and myeloid colony formation in diffusion chambers in vivo (CFU-DG) (Table 1, Fig. 2), suggesting that BFU-E and CFU-DG formation may be modulated by the same or similar molecules.

Failure of the human Mo B-cell conditioned medium to stimulate CFU-DG suggests that the effect by the Mo T-cell conditioned medium was specific and not related to antigenicity of human material. Injection of fractionated α-medium supplemented with fetal calf serum did not enhance CFU-DG formation, excluding the possibility that the stimulatory activity was present in the medium but masked by a neutralizing inhibitor.

A major concern was to exclude the possibility of endotoxin causing the enhanced CFU-DG formation. Therefore, we assayed conditioned media with limulus assay and were unable to detect any endotoxin contamination. However, it is possible that during the implantation of diffusion chambers, endotoxin contamination was introduced to the peritoneal cavity and therefore we also studied the direct effect of endotoxin by using an injection schedule of increasing doses of endotoxin that maximally stimulate CSF production in the host mice. Lack of CFU-DG stimulation suggests that the human hemopoietic cells in the diffusion chambers do not respond to endotoxin directly or CSA elaborated from the host. The finding is also consistent with the concept that diffusion chamber granulopoiesis is controlled by factors different from CSF.

As in earlier studies, a rapid decrease in the number of cells capable of giving rise to colonies in vitro as judged by subculture was observed following implantation of diffusion chambers intraperitoneally in mice. Under the influence of Mo conditioned medium elaborated from the osmotic minipumps, increase in the number of BFU-E and CFU-DG was observed between day 1 and 4. This may reflect increased self-replication of the precursor cells triggered by Mo conditioned medium. Alternatively, Mo conditioned medium acted on very primitive progenitor cells, causing an efflux to BFU-E and CFU-DG compartments.

The present study indicates that an effect of CSF cannot be detected by the in vivo diffusion chamber culture technique. Depletion of CSF by heat inactivation and gel filtration chromatography did not decrease CFU-DG formation (Table 1). Following gel exclusion chromatography, the fraction with peak CSF concentration had only modest effect on CFU-DG (Fig. 2). Additionally, no change in CFU-C numbers in diffusion chambers was observed following administration of CSF (Fig. 1). Analogous findings have been reported earlier. Symann et al. injected large amounts of exogenous CSF into mice bearing diffusion chambers without observing an effect on granulopoiesis. Rothstein et al. suggested that the humoral factors stimulating diffusion chamber granulocyte production is not CSF.

The difference in responsiveness of CFU-C and CFU-DG to lymphokines is in accordance with earlier studies, indicating that these two colony-forming units do not represent the same stem cell population. The relationship between CFU-DG and BFU-E has not been established. It is possible that CFU-DG and BFU-E represent closely related stem cells with the differentiation pathway being determined by the particular culture system utilized. In that case, the stimulatory effect we detect by an in vitro methylcellulose and in vivo diffusion chamber technique (Figs. 1 and 2) could be caused by a single species of molecules.

Earlier, we observed increased erythroid colony formation from normal murine bone marrow in diffusion chambers in mice in the presence of PHA-stimulated T cells, while no effect on (CFU-DG) granulocytic colony formation was observed. In the present study, conditioned medium from the Mo T-cell line only stimulated granulocytic colony (CFU-DG) formation (Table 1) without effect on erythroid colony formation in diffusion chambers in mice. The results from these studies are not necessarily contradictory. Target populations were from different species with different growth characteristics in diffusion chamber cultures in mice. Thus far, investigators have been unable to demonstrate erythroid colony formation from adult human bone marrow in diffusion chambers in mice. As judged by high CFU-DG count in normal hosts, myeloid colony (CFU-DG) formation from murine marrow is intrinsically highly stimulated and it may be hard to demonstrate stimulatory effect. On the other hand, expression of CFU-DG from human marrow requires extrinsic stimulation by host treatment. In the present study, this was achieved by administration of the Mo T-cell conditioned medium.

ACKNOWLEDGMENT

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