Autocrine growth mechanisms of leukemic blast progenitors in acute myeloblastic leukemia (AML) were investigated. Colony formation of leukemic blast progenitors was observed in 14 of 17 patients tested when purified blast cell fraction depleted of both T cells and monocytes was plated in methylcellulose without any colony-stimulating factor (CSF). However, there existed a minimal cell density required to initiate blast progenitor growth with marked patient-to-patient variation. To clarify the role of cell density on the spontaneous growth of blast progenitors, we tested whether leukemic cells produced and secreted some stimulatory humoral factor(s). Production of colony-stimulating activity (CSA) by blast cells was observed in 17 of 18 patients tested. Following further depletion of monocytes, the CSA levels decreased markedly in 14 patients, indicating that blasts with monocytic differentiation were responsible for CSA production. We also confirmed the CSA levels decreased markedly in 14 patients.

Autocrine growth of malignant cells is an attractive model to explain the malignant growth. Cancer cells can produce and respond to their own growth factors and such cells could become autonomous of external growth factors in a manner that would lead to malignant transformation. However, the precise role of CSFs on the growth of blast progenitors still remains to be determined.

Acute myeloblastic leukemia (AML) is a highly lethal malignant disease with progressive accumulation of leukemic cells. The accumulation of leukemic cells in AML is maintained by a small subpopulation, leukemic blast progenitors; they may renew themselves and/or undergo terminal divisions with a limited differentiation. To elucidate the mechanism by which leukemic cells proliferate, we added the fundamental role played by specific growth factors in controlling the proliferation of leukemic blast progenitors must be established. Previous data observed that the leukemic blast progenitors were, like normal hematopoietic cells, dependent on the addition of colony-stimulating factor (CSF) for their proliferation in vitro. However, the precise role of CSFs on the growth of blast progenitors still remains to be determined.

In this study, we examined whether there is an autocrine growth mechanism of leukemic blasts in AML. For this purpose, we cultured purified leukemic cell fraction in the absence of any growth factor and we also tested the production of colony-stimulating activity (CSA) by leukemic cells. The results show that blast progenitors grew even without an addition of growth factor when cultured at a high cell density. We also show that leukemic cells with monocytic differentiation were responsible for CSA production. The data suggest that leukemic blast progenitors are stimulated by CSA produced by fraction of leukemic cells with adherence. The proposed mechanism may explain the growth advantage of leukemic clone over preexisting normal hematopoiesis in vivo.
procedures described above, T-cell-depleted and nonphagocytic mononuclear cells were obtained.

**Preparation of conditioned medium.** CM was prepared by cultivating leukemic cells at 10^6/mL in a-MEM containing 5% FCS for five days. CM was harvested, filtered through Millipore membrane (45 μm, Millipore Japan Ltd, Tokyo), and stored at −20°C until use. The leukemic cells used were purified by removing T cells (T blasts) or both T cells and monocytes (T-Mo blasts) as described above.

**Biological reagents.** Recombinant human granulocyte colony-stimulating factor (rhG-CSF) was provided by Chugai Pharmaceutical Co, Tokyo, Japan. cDNA for the genes of G-CSF has been cloned from the human squamous cell line CHU-2 secreting G-CSF. The rhG-CSF was obtained as a medium conditioned by monkey COS cells transfected with the expression vector containing cDNA clones for G-CSF. Monoclonal antibodies (MoAbs) for hG-CSF was a gift from Dr M. Ono, Chugai Pharmaceutical Co. MoAb 9-16 specifically inhibits G-CSF bioactivity, whereas MoAb 7-13 does not block the activity and was used as control IgG. Recombinant human granulocyte macrophage colony-stimulating factor (rhGM-CSF) was obtained from Sumitomo Pharmaceutical Co, Hyogo, Japan. rhGM-CSF cDNA was isolated from a library constructed using mRNA from human monocyctic cell line U937 by screening candidate clones with a nick-translated probe derived from the coding sequences of murine GM-CSF and expressed in *Escherichia coli*. Recombinant gibbon interleukin-3 (IL-3) was a gift from Dr S. Clark, Genetics Institute, Cambridge, MA. Anti–GM-CSF sheep serum, also provided by Dr Clark, was prepared against purified rhGM-CSF and reacted with a single broad band (apparent molecular weight [mol wt] of 19 to 26 kD) by Western blot analysis.

**Clonogenic assay in methylcellulose culture.** Leukemic blast cells or normal marrow cells depleted of T cells and monocytes were plated at various concentrations in 0.1 mL of α-MEM with 0.8% methylcellulose and 20% FCS in the presence or absence of rhCSF in 96-well microplates (Linbro, Flow Lab, McLean, VA) as described by Hoang et al. After seven days incubation at 37°C in a humidified atmosphere of 5% CO2 in air, colonies of more than 20 cells were counted under an inverted microscope. In some experiments, adherent leukemic cells were similarly cultured in the presence of rhCSF.

**Assay for CSA.** Secretion of CSF by leukemic cells was determined by assaying medium conditioned by leukemic cells for the presence of CSA for normal BM myeloid progenitors, colony forming unit-granulocyte macrophage (CFU-GM). Briefly, 10^4 phagocyte-depleted BM mononuclear cells were plated in 35-mm Lux Petri dishes in α-MEM containing 5% FCS assay for this study was 30 pg/mL. Standard or sample 0.2 mL in duplicate were dispensed into anti-G-CSF antibody coated tubes and incubated for two hours at room temperature. Peroxidase-antibody conjugate (0.1 mL) prepared by the method of Hashida et al was added and incubated for two hours at room temperature. The tubes were washed three times with 20 mmol/L Tris-HCl buffer (pH 6.0) containing 0.015% hydrogen peroxide, and then 3 mg/mL o-phenylenediamine-HCl was added. After one hour incubation at room temperature, the reaction was stopped with 1 mL of 4 N H2SO4 and the absorbance was read at 492 nm. The assay is specific for G-CSF. It does not recognize GM-CSF, M-CSF, or IL-3. The limit of sensitivity of the assay for this study was 30 pg/mL.

**Statistical Methods.** Data were presented as the mean ± SE of four replicate cultures.
RESULTS

Blast colony formation without stimulation. It is great concern for us whether clonogenic blast cells from AML patients could proliferate in the absence of exogenous CSF. We tested whether clonogenic blast progenitors grew when purified blast cell fraction, depleted of both T cells and monocytes, was plated at different cell densities in the absence of added growth factors (Fig 1). We found a significant number of colony-formation in 14 of 14 patients tested. However, several features of proliferation were noted. First, their growth was cell density-dependent, and there existed a minimal cell density required to initiate proliferation with patient-to-patient variation. Second, as cell concentrations were increased, colony-forming efficiency increased progressively. Although a linear-logarithmic relationship was observed between the number of cells plated and the number of colonies developing, the slope of this line exceeded 1:1. The slope was similar among the patients. The results suggest that purified blast cell fraction could produce factor(s) affecting the growth of autologous blast progenitors.

Production of CSA by leukemic cells. To further clarify such autostimulatory mechanisms, we measured CSA in media conditioned by leukemic cells using normal marrow cells as targets. Table 1 summarizes CSA levels produced by leukemic cells in 37 AML patients. In general, media conditioned by T cell-depleted leukemic cells from patients with FAB subtypes of M4 or M5 contained higher CSA levels compared with those from patients with FAB subtypes of M1 through M3. Media conditioned by T cell-depleted or both T cell- and monocyte-depleted leukemic cells from patient 35 showed high CSA. However, the further analysis of this CM could not be performed in the following experiments. In patients with higher CSA levels, the growth of blast clonogenic cells occurred at lower cell densities with some exceptions (Fig 1). In contrast, patients with no or minimal CSA levels needed higher cell densities to permit blast cell growth.

To clarify the nature of CSA(s) produced by leukemic cells, we examined the morphologic types of colonies in human marrow cultures after 14 days of stimulation in agar by CM from ten patients and 100 ng/mL of rhG-CSF (Table 2). CM from three patients (patient nos. 5, 28, and 33) stimulated granulocyte (G), macrophage (M), granulocyte-macrophage (GM), and eosinophil (Eo) colony formation. However, in the remaining seven patients CM, like G-CSF, preferentially stimulated the formation of granulocyte colonies, but not of Eo colonies.

G-CSF and GM-CSF levels in CM. To further characterize the growth factor(s), we quantitated G-CSF and GM-CSF using specific immunologic assays (Table 3). We found that all seven CM tested contained varying levels of

![Fig 1. Blast colony formation in methylcellulose in the absence of added growth factor. Purified blast cells depleted of both T cells and monocytes were cultured at the indicated cell densities and colonies were counted after seven days of incubation. The number of blast colonies is plotted on a logarithmic-logarithmic scale. Each point represents the mean of four replicate cultures. Patient numbers are indicated on the right end of the points.](image)
G-CSF, and that following depletion of macrophages the levels decreased markedly. We demonstrated that CM from three patients (patient nos. 5, 28, and 33) stimulated Eo colony-formation on normal marrow cells (Table 2) and contained apparent GM-CSF. Therefore, CM from patient no. 28 revealed to contain both G-CSF and GM-CSF.

**Table 2. Morphology of Colonies in Human Marrow Cultures After 14 Days of Stimulation in Agar**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Colonies/10^4 Marrow Cells</th>
<th>Morphology, % of Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML 5-CM</td>
<td>15</td>
<td>G 8 M 26 GM 0 GEo 5</td>
</tr>
<tr>
<td>AML 14-CM</td>
<td>18</td>
<td>G 5 M 11 GM 0 GEo 0</td>
</tr>
<tr>
<td>AML 27-CM</td>
<td>120</td>
<td>G 4 M 12 GM 0 GEo 0</td>
</tr>
<tr>
<td>AML 28-CM</td>
<td>138</td>
<td>G 64 M 17 GM 0 GEo 8</td>
</tr>
<tr>
<td>AML 29-CM</td>
<td>78</td>
<td>G 84 M 12 GM 0 GEo 0</td>
</tr>
<tr>
<td>AML 30-CM</td>
<td>86</td>
<td>G 73 M 12 GM 15 GEo 0</td>
</tr>
<tr>
<td>AML 32-CM</td>
<td>81</td>
<td>G 89 M 2 GM 8 GEo 1</td>
</tr>
<tr>
<td>AML 33-CM</td>
<td>104</td>
<td>G 56 M 9 GM 5 GEo 27</td>
</tr>
<tr>
<td>AML 34-CM</td>
<td>28</td>
<td>G 95 M 1 GEo 4</td>
</tr>
<tr>
<td>AML 37-CM</td>
<td>18</td>
<td>G 91 M 0 GEo 9</td>
</tr>
<tr>
<td>Control medium</td>
<td>0</td>
<td>-  -  -  -  -</td>
</tr>
<tr>
<td>rhG-CSF (100 ng/mL)</td>
<td>166</td>
<td>G 93 M 6 GEo 1</td>
</tr>
</tbody>
</table>

Medium conditioned by T cell- (patient nos. 27-30, 32-34, and 37) or both T cell- and monocyte-depleted leukemic cells (patient nos. 5 and 14) was assayed for CSA activity using normal marrow CFU-GM as targets. The results are expressed as mean of two to three replicate cultures.

**Table 3. Secretion of G-CSF and GM-CSF by Leukemic Cells**

<table>
<thead>
<tr>
<th>Sample</th>
<th>G-CSF (ng/mL)</th>
<th>GM-CSF (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T' CM</td>
<td>T' Mo' CM</td>
</tr>
<tr>
<td>AML 5-CM</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>AML 14-CM</td>
<td>185</td>
<td>75</td>
</tr>
<tr>
<td>AML 27-CM</td>
<td>1512</td>
<td>510</td>
</tr>
<tr>
<td>AML 28-CM</td>
<td>3392</td>
<td>180</td>
</tr>
<tr>
<td>AML 29-CM</td>
<td>1448</td>
<td>&lt;30</td>
</tr>
<tr>
<td>AML 30-CM</td>
<td>4240</td>
<td>&lt;30</td>
</tr>
<tr>
<td>AML 33-CM</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AML 34-CM</td>
<td>199</td>
<td>&lt;30</td>
</tr>
<tr>
<td>AML 37-CM</td>
<td>88</td>
<td>&lt;30</td>
</tr>
<tr>
<td>Control Medium</td>
<td>&lt;30</td>
<td>&lt;3</td>
</tr>
</tbody>
</table>

G-CSF and/or GM-CSF in media conditioned by T cell- (T' CM) or both T cell- and monocyte-depleted leukemic cells (T' Mo' CM) were quantitated as described in Materials and Methods.

*ND, not done.

**Fig 2. Effects of anti-GM-CSF sheep serum on blast colony formation.** Blast cells were plated at a concentration of 2 x 10^6 (patient no. 27), 4 x 10^6 (patient nos. 5, 28-30, 32, and 33), or 8 x 10^6 (patient nos. 8, 22, and 34) per well in the presence or absence of anti-GM-CSF serum without stimulation. Control sheep serum or anti-GM-CSF serum was incorporated into the culture system at a final concentration of 1:500. Preimmune sheep serum or IgG at the same dilution had no effect on colony growth. Each observation was the mean ± SE of four replicate cultures.

**Effects on anti-GM-CSF and anti-G-CSF antibodies on blast colony-formation.** We showed that clonal growth of leukemic cells from all 14 patients tested were dependent on cell density in the absence of CSF. Our results also indicate that leukemic cells release CSF(s) into the culture medium, suggesting that clonogenic blast cell growth in the absence of added growth factors is due to low levels of CSF(s) produced by leukemic cells themselves. Therefore, we tested whether addition of antibody for CSF to the culture affects the blast progenitor growth to prove that leukemic cells are secreting CSF into the culture medium, and that external CSF interaction is required for blast progenitor growth. Three patterns of responses to added anti-GM-CSF serum were observed (Fig 2). Colony-formation was almost completely inhibited by specific antiserum in all three patients with CM that was proved to contain GM-CSF (patient nos. 5, 28, and 33). In four patients (patient nos. 22, 27, 29, 32), moderate but significant (P < .05) inhibition was seen, and in the remaining three patients (patient nos. 8, 30, and 34) no significant adding effect was observed.

Addition of anti-GM-CSF serum showed dose-dependent inhibition of blast progenitor growth for two patients (patient nos. 28 and 32), and exogenously added GM-CSF abrogated the effects of anti-GM-CSF serum (Fig 3A and B). Addition of MoAb for G-CSF inhibited blast colony-formation in a dose-dependent manner in three patients tested (patient nos. 8, 27, and 32) (Fig 4). Both antibodies for G- and GM-CSF were inhibitory with patient nos. 27 and 32, and antibody for G-CSF but not for GM-CSF was inhibitory with patient no. 8. These data demonstrate that CSF(s) produced by leukemic cells themselves and stimulatory for autologous blast progenitors are G-CSF and/or GM-CSF.

**Origin of blast progenitors.** Both adherent blast cell fraction and nonadherent, nonphagocytic blast cell fraction from AML patients were cultured in methylcellulose with optimal concentrations of G-CSF, GM-CSF, and IL-3 to test which fraction mainly contains blast progenitors. Two representative experiments are shown in Table 4. As has been noted by previous investigators,^30^ blast progenitors revealed to be present in nonadherent, nonphagocytic cell fraction, whereas adherent cell fraction contained none of them.

**Growth factor(s) other than CSF.** Finally, we tested whether CSF(s) are the sole factors needed for the growth of blast progenitors. Blast progenitor growth stimulated with a combination of optimal concentrations of G-CSF, GM-CSF, and IL-3 was still cell density-dependent, although initiation of the growth occurred at lower cell density with shift of the
curves to the left (Fig 5). Further addition of IL-1 of up to 100 ng/mL showed no effect on the blast progenitor growth (data not shown). The results suggest that factor(s) other than G-CSF, GM-CSF, IL-3, or IL-1 produced by leukemic cells themselves are indispensable for optimal blast cell growth. In contrast, normal myeloid progenitors showed clonogenic growth independent of cell density.

**DISCUSSION**

Like normal myeloid progenitor cells, leukemic blast progenitors are dependent on exogenous CSFs for proliferation. Until recently, four CSFs have been identified and cloned. Three of them, namely GM-CSF, G-CSF, and IL-3, have been shown to stimulate the growth of blast progenitors in vitro. Previous studies have made it clear that T lymphocytes, monocytes, fibroblasts, BM stromal cells, and endothelial cells produce various CSFs and other regulatory factors such as IL-1 and it may be that leukemic cells proliferate in microenvironments that contain such growth factors. Recently, Young et al reported AML cases in which GM-CSF mRNA was detected. In some cases, biologically active CSF could be detected in media conditioned by leukemic cells and autonomous in vitro proliferation of leukemic progenitor cells were observed. However, the mechanisms of CSF supply to the leukemic cells are not fully understood. It is of interest to determine which of the cells mentioned above has the most intimate contact with leukemic cells and affects the growth of blast progenitors.

We reported here proliferation of clonogenic blast progenitors in methylcellulose in all AML cases tested when purified blast cells, depleted of both monocytes and T cells, were cultured at various concentrations in the absence of any growth factor. However, their growth was cell density-dependent. To investigate this point further, media conditioned by leukemic cells were tested for the presence of CSF using bioassays and specific immunologic assays. It is shown, in all cases tested, that T cell-depleted leukemic cells release varied levels of CSF into the culture medium without apparent stimulation and, following further depletion of monocytes, the levels of CSF markedly decreased. This indicates that leukemic cells with monocytoid differentiation are

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Adherent Cells</th>
<th>Nonadherent Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>27</td>
<td>0</td>
<td>23 ± 4</td>
</tr>
<tr>
<td>33</td>
<td>0</td>
<td>115 ± 6</td>
</tr>
</tbody>
</table>

Blast cells were cultured in methylcellulose with G-CSF (10 ng/mL), GM-CSF (1 ng/mL), and IL-3 (1×10^4). The input cell number per well was 2.5 × 10^2 and 2.5 × 10^3 in patient nos. 27 and 33, respectively.
mainly responsible for the production of CSF. Leukemic cells were cultured in the presence of FCS, and the FCS may have some stimulation to increase factor production. We have preliminary data that similar blast colony-formation was observed in seven of eight cases tested in serum-free culture, although some differences in colony-size and configuration were seen (Y. Maruyama et al., manuscript in preparation). Other investigators also reported blast colony formation without CSFs in serum-free cultures. Therefore, FCS does not seem to play an important role on the production of CSF by leukemic cells.

Growth of leukemic blast progenitors was observed in the absence of any growth factor. This may be due to CSF(s) produced by leukemic blasts themselves. It is unlikely that the factor is produced by the very small number of contaminated normal monocytes in the culture. Previous studies have shown that adherent blast cells are generated in parallel with changes in the frequency of clonogenic blast cells in short- and long-term suspension cultures of blast cells in the presence of adequate growth factor. The adherent cells retain blast morphology and the surface markers were similar to those seen on blast cells, except that the marker Mo1 markedly increased. We confirmed the finding that these adherent cells lacked the capacity to form colonies. These results show that leukemic blast progenitors produce cells capable of self-renewal, and a subpopulation that lacks proliferative potential but supports the blast progenitors via the production of some growth factor(s) such as CSF. Close contact of these two blast cell subpopulations may result in sustained and sufficient CSF supply to leukemic blast progenitors.

The growth properties of blast progenitors raise the possibility that CSF produced by leukemic cells may be interacting with receptors on the cell surface after secretion into the culture medium. However, in sharp contrast to normal myeloid progenitors, their growth is still cell density-dependent in the presence of an excess concentration of CSFs. It is also noted that blast cells from some patients with no or negligible ability of CSF production could grow in the absence of added growth factor. These results indicate that blast cells produce factor(s) other than CSF, which potentiate the effect of CSF on the growth of autologous blast progenitors. The mechanisms of cell-to-cell interaction remain to be clarified.

The frequency of the CSF gene expression in leukemic cells from AML patients is variable among the reports. We have investigated the mRNA gene expression for GM-CSF and G-CSF in 29 AML patients. The gene expression was noted in only one patient in whom both G- and GM-CSF mRNA were expressed (unpublished data, 1988). It is not known whether or not the discrepancy may be due to the heterogeneity of AML. However, it seems likely that low percentage of the CSFs messages expression does not necessarily mean that the CSFs are never autocrined.

Growth factor(s) required to permit the optimal growth of blast progenitors must be defined. Further studies on the mechanisms of CSF supply to the leukemic blast progenitors and the relationship of CSF-receptor-cellular response in leukemic blasts are needed to explain the growth advantage of leukemic clone over preexisting normal hematopoiesis in vivo.

ACKNOWLEDGMENT

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Autocrine growth mechanisms of the progenitors of blast cells in acute myeloblastic leukemia

I Murohashi, S Tohda, T Suzuki, K Nagata, Y Yamashita and N Nara