Antitumor Effects of Human Recombinant Interleukin-6 on Acute Myeloid Leukemia in Mice and in Cell Cultures

By Tamar Givon, Shimon Slavin, Nechama Haran-Ghera, Rita Michalevicz, and Michel Revel

Interleukin-6 (IL-6) has been shown to inhibit growth and induce differentiation of several myeloid leukemia cell lines. In this work, two in vivo models of acute myeloid leukemia (AML) in mice have been used to test the therapeutic potential of recombinant human IL-6. In mice inoculated by a transplantable AML tumor, IL-6 injections inhibited the development of leukemia and increased survival. The effect was related to dose and length of treatment. In a model of radiation-induced leukemogenesis in SJL/J mice, administration of low-dose IL-6 for 10 days, 4 months after irradiation, reduced the incidence of leukemia observed during 1 year, whereas granulocyte-macrophage colony-stimulating factor (GM-CSF) increased the incidence of leukemia. In vitro liquid cultures of leukemia blood cells obtained from AML patients showed that IL-6 slowed growth and decreased the proportion of blasts with an increase in more mature myeloid elements in 72% of M1, M2, M4 AML cases. In contrast, GM-CSF less often produced differentiation but stimulated leukemic cell growth in liquid cultures, without synergism by IL-6.

The multifunctional cytokine interleukin-6 (IL-6) mediates responses of the organism to infections and inflammation, such as induction of liver acute-phase proteins, potentiation of hematopoietic activity with production of mature myeloid and megakaryocytic cells, stimulation of B- and T-lymphocyte differentiated functions with increased antibody secretion, and T-cell cytotoxicity. As found with other cytokines, IL-6 affects the growth of various cell types in opposite ways. IL-6 promotes growth, sometimes in an autocrine mode, of some plasmacytomas and myelomas, Epstein-Barr virus (EBV)-transformed B cells, T- and B-cell lymphomas, Kaposi sarcoma-derived cells, or keratinocytes. Growth inhibition by IL-6 was observed in several breast carcinoma cell lines, alone or in combination with other cytokines, induces growth-arrest and resumption of terminal differentiation in myeloleukemic cells. Murine IL-6 is, in fact, the monocytic-granulocytic differentiation factor for normal and leukemic progenitors, known as MGI-2. Clones of the murine myeloleukemia M1 line exposed to IL-6 undergo growth-arrest and macrophage differentiation with induction of surface markers (Mac-1, Mac-3, Fc-receptors, e-fms/macrophage colony-stimulating factor [M-CSF] receptor, major histocompatibility complex [MHC] class I) and of typical cellular activities (nonspecific esterase, lysozyme, 2'-5' A synthetase, phagocytosis). Among human myeloid cell lines, growth of U937 histiocytic lymphoma cells is inhibited by IL-6, and differentiation is induced by IL-6 combined with IL-1, with interferon-γ (IFN-γ), or with granulocyte-macrophage CSF (GM-CSF), which all synergize with IL-6. In human ML-1 myeloblastic cells, IL-6 synergizes the differentiation-inducing effects of tumor necrosis factor (TNF). In THP-1 acute promonocytic leukemia cells, some differentiation is induced by IL-6 plus IL-1, and in HL-60 promyelocytic leukemia IL-6 synergizes with GM-CSF to suppress colony growth in agar. AML-193 monoblastic leukemia cells, which grow slowly with IL-6, undergo some granulocytic differentiation. In an M4 AML line OCI/AML-6, IL-6 reduces the G-CSF-dependent growth, inducing some differentiation.

Despite the above effects of IL-6 on myeloleukemic cell lines, the in vivo activity of IL-6 on acute myeloid leukemia (AML) has not been reported. It was argued that use of IL-6 in AML may be detrimental because of its synergism with IL-3, GM-, or G-CSF for growth of granulocyte/myelocyte progenitors, which is also seen in AML blast colony formation from blood cells of certain (but not all) AML patients. In this study, we have used two models of murine AML to investigate the in vivo effects of human recombinant IL-6 (rIL-6). The results indicate antitumor activity of IL-6 on AML, as has been recently found also for murine sarcoma metastases and erythroleukemia models. The possibility of using IL-6 in human AML is discussed by reexamining, in both liquid and semisolid cultures, the in vitro action of IL-6 on growth and differentiation of AML blast cells from individual patients.

MATERIALS AND METHODS

Cytokines. Recombinant human IL-6 (rhIL-6) was from Chinese hamster ovary (CHO) cells prepared and purified as described. The specific activity determined in comparison with standard 88/514 (National Institute for Biological Standards and Control, Potters Bar, UK) by a plasmacytoma growth assay, was 10^7 reference units/mg protein. Certain experiments were also performed using Escherichia coli produced rIL-6 as described. Both rhIL-6 contained less than 0.1 ng endotoxin per milligram protein. rGM-CSF, 1.2 x 10^7 U/mg, was a gift from Sandoz (Basel, Switzerland), rCSF, 2 x 10^8 U/mg, was a gift from Dr M. Moore (Memorial Sloan-Kettering Cancer Center, New York, NY) and from Amgen (Thousand Oaks, CA), rM-CSF, 10^8 U/mg was from Genzyme. Human IFN-α, 5 x 10^8 U/mg, and IFN-γ, 10^8 U/mg (both CHO-derived) were prepared and purified as previously described.

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Transplantable AML in mice. SJL/J mice were injected in the tail vein with $5 \times 10^3$ transplantable mAML-1 cells (described in the text) and treated by rIL-6 in the indicated doses and schedules. Control mice received similar phosphate-buffered saline (PBS) injections. Blood was drawn weekly (weeks 6 to 10) from the retro-orbital vein using heparinized glass capillaries, and red blood cells (RBCs) were lysed with 2% acetic acid. White blood cells (WBCs) were counted, and values over $20 \times 10^9$/mL were considered as overt leukemia. AML incidence and survival of the mice was followed. Statistical probabilities were calculated by the two-tailed Student's t-test.

Radiation-induced AML in mice. Three-month-old female SJL/J mice (Jackson Labs, Bar Harbor, ME) were exposed to 350 rads subcutaneous (s.c) injection of dexamethasone (0.5 mg), as described.20 Starting at day 140 after irradiation, cytokines were injected for 2 courses of 5 days per week. Incidence of overt leukemia was followed for 8 months after treatment.

Human AML cell studies in vitro. We studied a group of 23 untreated AML patients with French-American-British (FAB) classification and percentage of blasts in the blood samples indicated in the text. Liquid cultures of peripheral blood mononuclear cells (PBMC) were performed at 0.5 to 1 $10^6$ cells/mL in Iscove-modified Dulbecco medium (IMDM; GIBCO, Grand Island, NY) with 10% fetal calf serum and the indicated cytokines. After 5 days at 37°C, cells were counted and slides were Giemsa-stained. The number of blasts, promyelocytes, metamyelocytes, bands, and segmented myeloid elements and monocytes were compared after culture with and without cytokines. The few lymphocytes were not included in the calculations. The growth of blast colonies in methyl-cellulose was performed as described.23

RESULTS

Effects of IL-6 on transplantable AML in mice. In this first model, SJL/J mice were inoculated intravenously (IV) by mAML-1 tumor cells freshly taken from leukemic mice. These cells were originally obtained from radiation-induced AML in SJL/J mice2526 and maintained by passage in vivo. The newly injected mice develop overt leukemia (i.e., WBC counts over $20 \times 10^9$/mL) in about 6 to 7 weeks (Table 1). We first tested several doses of rIL-6 (CHO-produced) administered from day 1 to 5 after mAML-1 cell inoculation. Daily doses of 1, 5, 25, and 120 pg/d were retested on larger groups of 32 mice and with 3 courses of 5 days per week. Incidence at both doses (P < .05) and 100% survival at 72 days compared with 25% in the control and 25 pg/d groups.

The effective dose and the first ineffective dose (120 and 25 pg/d) were retested on larger groups of 32 mice and with a more prolonged treatment, ie, 3 courses of 5 d/wk for the 3 first weeks after mAML-1 inoculation. At 8 weeks, 75% of the control mice had leukemia, and both doses of rIL-6 reduced the incidence of disease, by 50% (P < .025) at 25 pg/d and by 62.5% (P < .02) at 120 pg/d (Table 2). At 10 weeks, there was still a 35% reduction of leukemia incidence at both doses (P < .05, not shown). The 3-course treatment seems essential for response at 25 pg rIL-6/d (compare the two experiments in Table 2). Follow-up for 115 days shows (Fig 2) that with the longer treatment schedule, rIL-6 at 25 pg/d prolonged survival (P = .02) as well as at 120 pg/d (P < .01). Similar reductions in AML incidence were seen whether rIL-6 was administered i.p. or s.c., or if the treatment was started only in the second week after mAML-1 cell inoculation and continued for 2 weeks (not shown).

Table 1. Effect of IL-6 Injections on Transplanted AML Development in SJL/J Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peripheral Blood WBC x 10^9/mL at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 wk</td>
</tr>
<tr>
<td>PBS</td>
<td>72</td>
</tr>
<tr>
<td>IL-6 1 pg/d</td>
<td>39</td>
</tr>
<tr>
<td>IL-6 5 pg/d</td>
<td>20</td>
</tr>
<tr>
<td>IL-6 25 pg/d</td>
<td>13</td>
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<tr>
<td>IL-6 120 pg/d</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>8</td>
</tr>
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</table>

Mice treated with the indicated daily dose of rIL-6 divided in 3 i.p. injections, from day 1 to 5 after IV inoculation of mAML-1 cells. Mean WBC in PBS vs rIL-6 120 pg/d: 105 ± 81 vs 11.5 ± 4 (P < .05) at 7 weeks; 162 ± 90 vs 15 ± 8.5 (P < .025) at 8 weeks. Abbreviation: D, death from leukemia.

Fig 1. Survival of SJL/J mice inoculated by mAML-1 and treated 5 days by IL-6. Mice (four per group) were treated with rIL-6, 25 pg or 120 pg per day divided in three i.p. injections, from day 1 to 5 after IV inoculation of mAML-1 cells. For time of leukemia onset, see Table 1. Survival was followed for 80 days after treatment. Mean day of death: control and 25 pg/d rIL-6, 65 ± 11; 120 pg/d rIL-6, 79 ± 6 days (P < .05).
We also tested a pretreatment in which mice received rIL-6 (25 and 120 μg/d in three i.p. injections) from days −3 to −1 before being injected IV with mAML-1 cells. At 7 weeks, eight of eight mice in both IL-6 groups had developed leukemia versus seven of eight in the control group. There was also no beneficial effect of rIL-6 on survival and incidence can be increased to about 50% with a mean latency of 280 days, when a single injection of dexamethasone would have no interference with the rIL-6 treatment. On day 140, one group of preleukemic mice (20 animals) received rIL-6 (CHO) at 1 μg/d/mouse in two i.p. injections for 10 days (5 days per week for 2 consecutive weeks). Another group received rGM-CSF (0.15 μg/d) with the same schedule and a third group (25 mice) was used as control. In bone marrow collected from the rIL-6–treated mice, 14 days after the first injection, karyotyping already indicated a significant reduction in the percentage of cells carrying chromosome 2 deletions (9% ± 7% with rIL-6 v 28% ± 12% in the control group, P < .001). No rIL-6–treated mouse had more than 30% cells with the deletion, whereas this was the case in 40% of the control mice (not shown). Therefore, rIL-6 pretreatment is not inducing a stable resistance to AML without the tumor cells are present.

Effects of rIL-6 on radiation-induced AML. A model of myeloid leukemogenesis was used to test the effect of rIL-6 administration on the slow de novo development of AML in mice. In this model, young SJL/J mice subjected to x-irradiation at 350 rads develop radiation-induced AML (RI-AML) with an incidence of about 20% at 1 year. This incidence can be increased to about 50% with a mean latency of 280 days, when a single injection of dexamethasone is given with the irradiation. With this protocol, the latency of 280 days, when a single injection of dexamethasone would have no interference with the rIL-6 treatment. On day 140, one group of preleukemic mice (20 animals) received rIL-6 (CHO) at 1 μg/d/mouse in two i.p. injections for 10 days (5 days per week for 2 consecutive weeks). Another group received rGM-CSF (0.15 μg/d) with the same schedule and a third group (25 mice) was used as control. In bone marrow collected from the rIL-6–treated mice, 14 days after the first injection, karyotyping already indicated a significant reduction in the percentage of cells carrying chromosome 2 deletions (9% ± 7% with rIL-6 v 28% ± 12% in the control group, P < .001). No rIL-6–treated mouse had more than 30% cells with the deletion, whereas this was the case in 40% of the control mice (not shown). Therefore, rIL-6 treatment leads to a rapid decrease in the putative preleukemic cells. Figure 3 shows the cumulative incidence of overt myeloid leukemia during the 8 months after the short cytokine treatment. In the rIL-6–treated mice, the incidence of overt of leukemia at 1 year after leukemogenesis was 23% versus 48% in the control group (P < .05) and the mean time of leukemia onset was delayed (P = .01). In contrast, with rGM-CSF the incidence of leukemia was increased to 77% (P < .025 v control) and the mean leukemia onset was earlier (P < .001). In other experiments, M-CSF was found, like GM-CSF, to accelerate the rate of RI-AML and increase its incidence, with both CSF acting most probably by enhancing growth of preleukemic AML clones.

Effects of IL-6 on growth of human AML blasts in vitro. In view of the inhibitory effect of rIL-6 on the two murine AML in vivo models, it was important to consider if IL-6 could be used at all in human AML. IL-6 has been suspected to promote growth of human AML blast by synergism with GM-CSF, but IL-6 also contributes to differentiation of a number of myeloleukemic cell lines. To evaluate what rIL-6 does to fresh ex vivo human AML blasts, we performed not only blast colony assays in methylcellulose as done before but also liquid cultures of PBMC from AML patients with 33% to 89% of undifferentiated blasts. The number of leukemic cells and their differentiation status were measured after 5 days of liquid cultures without or with rIL-6, GM-CSF, or IFNs (example shown in Table 3). Table 4 gives the statistical analysis of growth: in 22 AML cases studied with rIL-6, average growth was 15% lower than in cultures without cytokine (P < .001), similar to what is seen with IFN-β. In contrast, rGM-CSF stimulated growth, yielding an average 50% cell increase (P < .001). In liquid cultures, the combination of rIL-6
with rGM-CSF did not increase the growth effect of GM-CSF (Table 4, last column).

The proportion of blasts compared with more differentiated cells (myelocytes, bands, and segmented, see Table 3) changed during the 5-day liquid cultures. We calculated a differentiation index (percent of myelomonocytes divided by that of blasts) and the ratio of this index in cultures with cytokines over cultures without them. Table 5 shows these ratio values, with the FAB class and percentage of blasts (before culture) of the AML patients studied. Considering first only M1, M2, M4 AML, ratios of 2 to 10 (ie, significant decreased blasts v more differentiated elements) were produced by rIL-6 in 72% of cases (13 of 18), by IFN-β in 73% (8 of 11), by both in 78% (7 of 9), but with rGM-CSF in only 28% (5 of 18, P = .003 v rIL-6). When GM-CSF was added to rIL-6, the differentiation responses were 27% (4 of 15), much lower than with IL-6 alone (67%, 10 of 15) in the same cases (Table 5). We also scored cases with ratios <1 (indicating increased blast proportion): in M1, M2, M4 AML there were 11% (2 of 18) with rIL-6, and 33% with rGM-CSF alone or with rIL-6 (6 of 18 and 5 of 15), indicating that IL-6 does not enhance the risk of GM-CSF to increase blasts. The same holds true if one includes the M5 and M0 (stem cell) AML (Table 5). M5 AML showed no differentiation with rIL-6. From these liquid cultures it appears that GM-CSF but not IL-6 stimulates AML cell growth, and is more likely than IL-6 to increase blasts. IL-6 does not synergize these GM-CSF effects. While the differentiation effects of IL-6 are often prevented by GM-CSF, the two together are not worse than GM-CSF alone.

In the AML blast colony assay on methylcellulose, rGM-CSF was also a potent growth factor, whereas rIL-6 alone was not (Table 6). But, unlike the liquid cultures, the semisolid colony assay indicates that rIL-6 can sometimes increase the GM-CSF effect. In line with previous studies,22,23 such synergism was apparent only in some patients; in this study it was in three out of eight AML cases (Table 6).

**DISCUSSION**

In vivo antitumor activity of human rIL-6 on AML in mice was observed in two experimental settings. First, in
mice inoculated with transplantable murine mAML-1 cells, treatment with rIL-6 during 1 to 3 weeks after inoculation reduced the incidence of leukemia development and increased survival. The effect appears to be dose-dependent. At high doses (120 μg/d) a 5-day treatment was efficient, but at lower doses (25 μg/d) the treatment had to be prolonged. IL-6 therapy could be started 1 week after AML inoculation but pretreatment was not efficient, suggesting that IL-6 acts when the leukemic cells are growing in the animal. The second model used was in vivo leukemogenesis, in which high-incidence AML develops slowly after irradiation combined with a single dexamethasone injection.30,31 rIL-6 was administered 4 months later, when preleukemic cells, with chromosome 2 deletions, are known to accumulate in the bone marrow and lymphoid organs.30 In this model, a low-dose rIL-6 treatment (1 pg/d for 10 days) reduced the rate and incidence of overt leukemia in mice inoculated with transplantable murine mAML-1 cells,30,31 thereby decreasing the development and incidence of leukemia from 23% to 61% at 140 days after rIL-6 treatment. Several mechanisms could be invoked to explain these in vivo effects of rIL-6 on murine AML, including direct effects on tumor cells and indirect effects on host defenses, such as immune effects related to IL-6 being a coactivator of T and B lymphocytes.2 An involvement of T cells was found to be required for the antimetastatic effects of IL-6 on sarcoma.25 Similarly, rIL-6 induced specific CD8+ cytotoxic lymphocytes (CTL) against murine erythroleukemic FBL-3 cells, and maintained survival 100 days after tumor inoculation, with the mice showing immunity to the tumor.26 However, in this study IL-2 also induced this CTL response, but was much less efficient than IL-6 to prolong survival. This may indicate that CTL induction is not the only mechanism involved in IL-6 action, or may be due to the toxic effects of IL-2. Whereas the mice appear to tolerate high doses of rIL-6, such treatment with IL-2 can cause vascular leak syndrome, neurologic toxicity, and often death.25

It is more difficult to assess whether rIL-6 in vivo could also decrease AML cells by some effect on their differentiation and growth control as observed in several human and murine myeloleukemic cell lines with rIL-6 alone or in combination with other cytokines.12,22 If the outcome of IL-6 treatment in vivo was influenced by the growth response of the leukemic cells, one would expect that leukemias of B cells for which IL-6 is a growth factor24 would not respond to in vivo IL-6. Indeed, we found that rIL-6 did not delay leukemia in mice inoculated with murine B-lymphocytic leukemia BCL-1 cells (T.G. and S.S., unpublished observation, November 1990). Similarly, in AKR mice thymectomized at age 1 to 3 months, rIL-6, administered at 1 year (1 μg/d for 10 days) increased the incidence of B-cell leukemia from 23% to 61% at 140 days after IL-6, and in the same system IL-2 produced 100% leukemia within 70 days (N.H.-G. and A. Peled, unpublished observation, March 1990). Moreover, the fact that GM-CSF accelerated notably the rate of RI-AML also supports some relation between in vivo effects on AML and leukemia cell growth control.

### Table 5. In Vitro Response of Leukemia Cells From 23 AML Patients to Cytokine Induced Differentiation

<table>
<thead>
<tr>
<th>AML Type</th>
<th>Blasts</th>
<th>Ratio of Differentiation Index Relative to Control Cultures</th>
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<tbody>
<tr>
<td></td>
<td>%</td>
<td>IL-6</td>
</tr>
<tr>
<td>M1</td>
<td>40</td>
<td>3.5</td>
</tr>
<tr>
<td>M2</td>
<td>89</td>
<td>3.2</td>
</tr>
<tr>
<td>M3</td>
<td>83</td>
<td>3.1</td>
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<tr>
<td>M4</td>
<td>74</td>
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<td>74</td>
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<tr>
<td>M6</td>
<td>50</td>
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<td>M7</td>
<td>44</td>
<td>4.5</td>
</tr>
<tr>
<td>M8</td>
<td>50</td>
<td>0.6</td>
</tr>
<tr>
<td>M9</td>
<td>75</td>
<td>2.8</td>
</tr>
<tr>
<td>M10</td>
<td>36</td>
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<td>M17</td>
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<td>M18</td>
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<td>60</td>
<td>1.6</td>
</tr>
<tr>
<td>M23</td>
<td>85</td>
<td>5.6</td>
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</table>

PBMC from AML patients with indicated FAB type were incubated for 5 days with IL-6, 5 ng/mL (CHO-produced unless otherwise indicated), rIFN-β 100 U/mL, rGM-CSF 5 U/mL, or without cytokines. The percentage of blasts before incubation is shown (mean 64% ± 20%). The differentiation index is the percentage of myelomonocytes divided by that of blasts, computed from data as in Table 3. The ratio of the differentiation index after incubation with the indicated cytokine over that in control cultures is shown (ratio > 2 is defined as differentiation response).

*E cell/IL-6 used.
†Bone marrow cells instead of PBMC.

### Table 6. Blast Colony Formation on In Vitro Semisolid Culture of AML Cells With IL-6 and GM-CSF

<table>
<thead>
<tr>
<th>AML Type</th>
<th>None</th>
<th>IL-6</th>
<th>GM-CSF</th>
<th>IL-6 + GM-CSF</th>
<th>IFN-β</th>
<th>IFN-β + IL-6</th>
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<tbody>
<tr>
<td>M1</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>21</td>
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<td>ND</td>
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<td>M2</td>
<td>55</td>
<td>38</td>
<td>87</td>
<td>89</td>
<td>40</td>
<td>41</td>
</tr>
<tr>
<td>M2</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>22</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>M2</td>
<td>240</td>
<td>193</td>
<td>300</td>
<td>286</td>
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<td>183</td>
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<tr>
<td>M4</td>
<td>6</td>
<td>5</td>
<td>48</td>
<td>78</td>
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<tr>
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<td>51</td>
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<td>95</td>
<td>96</td>
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<td>5</td>
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Monocyte- and T-cell-depleted PBMC from AML patients with indicated FAB type were grown for 15 days in methyl cellulose with rIL-6, 5 ng/mL, rGM-CSF 5 U/mL or rIFN-β 100 U/mL.
Several studies have been concerned with the role that IL-6 could play in human AML. First it was observed that IL-6 is produced by human AML cells, although only the more differentiated M4 and M5 AML cells produce significant amounts, and the production is not constitutive in fresh ex vivo cells but only seen after in vitro incubation. Nevertheless, IL-6 was suspected to act as a growth factor for AML blasts, which would limit its use in human AML. In fact, as confirmed here, IL-6 alone does not significantly stimulate growth of AML blast colonies in semisolid medium, although it can synergize the growth activity of GM-CSF, G-CSF, IL-3, or IL-4. However, this synergistic action of IL-6 on blast colony growth is found only in a subset of AML cases, while in others the effect is absent or even suppression is observed. Recently, it was reported that CD34+ AML rarely show synergism of IL-6 with IL-3 or IL-4, whereas it is seen in greater than 80% of CD34+ AML. In our group of AML patients, three of eight showed some synergism of IL-6 with GM-CSF. In addition, our study shows that in liquid cultures IL-6 does not enhance the growth stimulatory effect of GM-CSF. Like IFNs, IL-6 was even able to induce a decrease in the percentage of blasts with an increase in partially differentiated myeloid elements after 5 days of liquid culture, in over 70% of M1, M2, M4 (but not M5) AML cases. GM-CSF produced similar effects in only 28% of cases and often inhibited IL-6 differentiative action.

These in vitro activities of IL-6 will have to be taken into account when considering how to use rIL-6 in human AML. Liquid cultures and blast colony assays may be useful to select subsets of patients for treatment. However, considering the beneficial effects of rIL-6 in the experimental AML models in mice, it appears that the administration of rIL-6 alone in human AML should not be prohibited by the risk of enhancing blast colony growth through some synergistic action. The mechanism by which rIL-6 exerts in vivo an antitumor effect on murine AML could be complex, and our data suggest that trials of IL-6 in the treatment of certain human AML cases may be warranted.

ACKNOWLEDGMENT

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