Effects of Interleukin-11 on the Proliferation and Cell Cycle Status of Myeloid Leukemic Cells

By J.P. Hu, A. Cesano, D. Santoli, S.C. Clark, and T. Hoang

Interleukin-11 (IL-11) is a pleiotropic cytokine with effects on many different targets. Within the hematopoietic system, the effects of IL-11 are largely manifest only through combination with other cytokines, including IL-3 and Steel factor (SF). In the present study, we addressed the question of IL-11 responsiveness within the different types of human leukemic cells, as well as the mechanism of action of IL-11 at the cellular level. Analysis of a panel of samples from different patients with acute myeloblastic leukemia (AML) and myeloid leukemic cell lines indicated that IL-11 alone was ineffective in supporting myeloid leukemic cell growth but frequently enhanced growth supported by IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), or SF. In contrast, three acute pre-B lymphocytic leukemia (pre-B-ALL) and two acute T lymphocytic leukemia (T-ALL) lines failed to respond to IL-11 alone or when combined with other cytokines. The growth enhancement of IL-11 among the AML patient samples was dose dependent and remarkably constant with half-efficient concentrations in the range of 0.3 to 0.4 ng/mL. The thymidine suicide studies with the patient samples revealed that 40% to 50% of the blast cells were in S-phase when exposed for 16 hours to IL-3 and this level was increased to 70% to 90% in response to either IL-11 or IL-6. Our data suggest that the latter two interleukins act synergistically with the direct mitogenic factor, IL-3, in triggering AML blast-cell proliferation. Detailed analysis with several patient samples further revealed that SF and IL-11 both enhance IL-3-supported clonogenic growth of AML blasts and the combination of all three growth factors yields optimal growth. In contrast, IL-6 does not further enhance the effect of IL-11. These results indicate that SF and IL-11 enhance IL-3-dependent clonogenic growth through two distinct pathways, whereas IL-6 and IL-11 may trigger the same pathway.

INTERLEUKIN-11 (IL-11), a novel lymphohematopoietic cytokine, was identified in medium conditioned by a primary stromal cell line, PU-34. Although IL-11 was originally discovered as a mitogen for the plasmacytoma cell line, T1165, in general it has not proved to be a growth factor for either plasmacytomas or hybridomas. Instead, it has proved to have a variety of biologic effects in different systems: Within the hematopoietic system it enhances the proliferation of factor-dependent growth of early multipotent progenitors as well as later progenitors committed to either the erythroid or megakaryocyte lineage; within the immune system, it promotes the T-cell–dependent development of immunoglobulin-producing B cells; within the hepatic system, it promotes production of hepatic acute-phase proteins; and within the fibroblast/adipocyte system, it inhibits adipogenesis. As a growth factor with normal target cells in the hematopoietic system, IL-11 has generally acted as a synergistic factor serving to augment the responses to primary growth factors, particularly IL-3 and Steel factor (SF), the ligand for the c-kit receptor. With primitive normal bone marrow cells, IL-11 enhances the frequency of IL-3-dependent colony formation and the rate at which colonies emerge in culture. As previously been shown with IL-6 and granulocyte colony-stimulating factor (G-CSF), the accelerated emergence of colonies results from shortening the dormant or G0-phase of the cell cycle of the quiescent primitive progenitor cells. In the present study, we have addressed the question of the mechanism of action of IL-11 at the cellular level, as well as the target cell specificity of IL-11. To this end, we have used populations that were less heterogeneous than normal hematopoietic cells; ie, the blast cells of acute myeloblastic leukemia (AML) and a panel of leukemic cell lines. As with normal cell populations, several myeloid leukemic cell lines displayed growth responses to IL-11, but only in combination with primary growth factors such as IL-3 or SF. Various lymphoid cell lines of pre-B-cell and T-cell origin failed to respond to IL-11. With primary samples of acute myeloblastic leukemia (AML), growth factor combinations showed that IL-3-dependent proliferation can be triggered by two different pathways, one responsive to IL-11 or IL-6, and one responsive to SF. These data indicate that clonogenic AML blasts may provide a useful model for studying growth factor regulation of the cell cycle of hematopoietic progenitors.

MATERIALS AND METHODS

Source of cells and growth factors. AML blasts were isolated by centrifugation of peripheral blood cells on a Ficoll-Hypaque gradient (Pharmacia Fine Chemicals, Uppsala, Sweden). The cells were cryopreserved as described previously and stored frozen before use. The French American British (FAB) classification of the different patient samples is shown in Table 1. The cell line M07-E, derived from a megakaryoblastic leukemia, was a kind gift of Dr G.C. Avanzi. The cells were maintained in the presence of 8 U/mL of IL-3. The myeloid cell lines M44-11 and AML 193 were maintained in the presence of GM-CSF. T-lymphoid cell lines included T-ALL-103/22 and T-ALL-104, whereas pre-B-cell lines included ALL-1, ALL-2, and ALL-3. Two cell lines with myeloid characteristics originally derived from patients with T-ALL included T-ALL-101 and T-ALL-103/3. Purified human IL-11 (2.5 x 106 U/mg), IL-6 (4 x 106 U/mg), IL-3 (4 x 106 U/mg), and GM-CSF (9 x 106 U/mg) were prepared from

From the Clinical Research Institute of Montreal, the Department of Medicine, University of Montreal, Quebec, Canada; the Wistar Institute, Philadelphia, PA; and the Genetics Institute, Cambridge, MA.

Submitted July 7, 1992; accepted November 10, 1992.
Supported in part by a grant from the National Cancer Institute of Canada (T.H.) with funds from the Canadian Cancer Society, a fellowship from the Canadian Association for International Development (J.P.H.), and a grant from the National Institute of Health (CA-47589) (D.S.). T.H. is a scholar of the N.C.I.C.
Address reprint requests to T. Hoang, PhD, Laboratory of Hemopoiesis and Leukemia, Clinical Research Institute of Montreal, 110 Pine Ave West, Montreal, Quebec, Canada H2W 1R7.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1993 by The American Society of Hematology.
as described previously. Recombinant human SF was a generous gift of Dr K. Zsebo (Amergen, Thousand Oaks, CA) and was higher than 95% pure by sodium dodecylsulfate/polyacrylamide gel electrophoresis (SDS/PAGE). Serum-free conditioned medium from the bladder carcinoma cell line 5637 (5637 CM) was harvested after 1 week of incubation and stored at 4°C as detailed previously. The neutralizing monoclonal antibody (MoAb) against human GM-CSF HGM 2/3.1.6 was a generous gift of Dr E. M. Alderman (Genetics Institute, Cambridge, MA).

Culture conditions. Culture conditions were chosen in order to minimize colony formation in the absence of growth factors. AML blasts were plated in 96-microwell plates (Linbro; Flow Lab; McLean, VA) in 100 μL of Isove’s modified Dulbecco’s medium (IMDM; Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Gibco) and 1% methylcellulose (Fluka, Ronkonkoma, NY) as described previously.

Where indicated, serum-free cultures were performed in the presence of bovine serum albumin (BSA; 20 mg/mL; Sigma Chemical Co, St Louis, MO), iron-saturated transferrin (30 μg/mL; Hoechst, Pharmaceuticals Inc, Heidelberg, Germany), and insulin (1.7 μg/mL; Upstate Biotechnology Institute, Lake Placid, NY). Titration curves for the various cytokines were analyzed with the program ALLFIT, using a nonlinear regression curve fitting routine.

For proliferation assays, leukemic cell lines were seeded at 5 x 10⁴ cells in 200 μL of serum-free IMDM. Cultures were maintained for 3 days in the presence of the different growth factors as indicated. Cells were pulsed with tritiated thymidine (1 μCi/well; Amersham, Arlington Heights, IL) overnight before harvesting and counting.

Cell separation. AML blasts were labeled with a mouse MoAb against CD34, anti–HPCA-1 (10 μg/10⁶ cells/100 μL) (Becton Dickenson, Mountain View, CA) and a fluorescein isocyanate (FITC)-tagged goat anti-mouse IgG (10 μg/10⁶ cells) (Amersham, Arlington Heights, IL). As negative controls, cells were labeled with the second antibody alone. Gating for sorting of CD34+ cells was chosen such that 99% of cells in the negative control were excluded, as well as dead cells and cell aggregates as described previously.

Thymidine suicide determinations. The proportion of blast progenitors in DNA synthesis was determined by the thymidine suicide technique as described by Iscove et al. Briefly, cells were exposed to IL-3 or IL-11 combined with IL-3 in suspension culture for 16 hours, washed twice, and exposed to [3HTdR, 20 μCi/mL, specific activity, 45 Ci/mmol; Amersham]. After 20 minutes at 37°C, the cells were washed twice and plated in the presence of 10% FCS, which has been shown previously to contain a mixture of G-CSF, GM-CSF, and IL-3 that is optimal in stimulating blast colony formation. Control cells were exposed to the same conditions without tritiated thymidine or in the presence of unlabeled thymidine. Clonogenic cells surviving the exposure to [3HTdR were expressed as survival fraction of control groups.

RESULTS

Dose-dependent enhancement of AML blast colony formation. AML blasts were plated in the presence of optimal concentrations of IL-3 or GM-CSF and varying concentrations of IL-11 ranging from 0.03 ng/mL to 100 ng/mL. To minimize cell interactions and spontaneous growth, each sample was plated at different cell concentrations in the presence or absence of IL-3 or GM-CSF (data not shown). The minimal cell concentrations required for colony formation were chosen for further studies (Table 1). Colony formation in the presence of IL-3 or GM-CSF was observed in 13 of 14 AML samples tested. Addition of IL-11 to GM-CSF–containing or IL-3–containing cultures resulted in a twofold to fourfold increase in colony count in these samples, whereas IL-11 alone had little if any effect (Table 1). Only when there was spontaneous colony formation did IL-11 appear to increase growth by itself (AML 28R, AML 36, and AML 39). The half-efficient concentration (EC₅₀) was strikingly constant in 11 of 14 samples and was in the range of 0.3 to 0.4 ng/mL. Only samples AML 35 and AML 39 required higher concentrations of IL-11, 0.7 and 2.3 ng/mL, respectively. AML 43 grew very poorly and could not be assessed accurately in culture.

To assess the serum requirement for colony formation by AML blasts, FCS was replaced by a combination of BSA, transferrin, and insulin. In all seven patient samples tested, EC₅₀ values estimated from IL-11 dose-response curves in serum-free cultures were essentially the same as those obtained from serum-containing cultures (Table 2). Finally, IL-11 significantly enhanced IL-3–supported colony formation from CD34+ cells (Table 3). Taken together, these data indicate that the synergistic effects of IL-11 with IL-3 or GM-CSF does not require the presence of accessory cells or of additional serum “co-factors.” All subsequent experiments with AML blast cells were performed in FCS-containing medium.

IL-11 enhances the growth of the myeloid cell line M07-E. In addition to testing IL-11 for the ability to stimulate colony formation by AML blasts, we also have screened numerous established myeloid cell lines for responsiveness to IL-11, IL-11 on myeloid leukemic cells.
this cytokine. Three pre-B-ALL (ALL-1, ALL-2, and ALL-3) and two T-ALL (T-ALL-103/2 and T-ALL-104) cell lines did not respond to IL-11 alone or in combination with IL-2, IL-3, or GM-CSF (data not shown). Two T-ALL lines with myeloid phenotype, T-ALL-101 and T-ALL-103/3, also failed to respond. In contrast, several myeloid cell lines, including M07-E, MV4-11, and AML 193, displayed enhanced rates of proliferation in the presence of cytokine combinations supplemented with IL-11 (data for M07-E shown in Fig 1). As with the AML samples, none of these cell lines responded to IL-11 alone but all three displayed enhanced proliferation in the presence of IL-3 or GM-CSF. Of the three myeloid cell lines, M07-E displayed the greatest response, particularly in combination with IL-3. In the presence of optimal concentrations of IL-3 (20 ng/mL) or SF (20 ng/mL), IL-11 induced a 4.5-fold and 2.5-fold increase in incorporation of [3H]thymidine by the M07-E cells, respectively, whereas the enhancement of GM-CSF–dependent proliferation was only approximately 50%. With both the MV4-11 and AML 193 cells, the enhanced proliferation by IL-11 was never more than 50% of the proliferation achieved with IL-3, SF, or GM-CSF alone (data not shown). In the case of the M07-E cells, the EC50 for IL-11 enhancement of IL-3–dependent proliferation was 8 ng/mL, significantly higher than the EC50 values observed for growth of the primary AML blasts.

We have also addressed the question of whether IL-11 may decrease the requirement in IL-3 for colony formation in M07-E cells and in AML blasts, as shown previously for SF.22 There was an 8-fold decrease in EC50 for IL-3 in only one sample (AML 44), whereas in the other samples, the difference in EC50 was within experimental variation (Table 4).

IL-11 increases the proportion of AML clonogenic cells in S-phase in association with IL-3. With normal cells, IL-11 has been shown to reduce the time of appearance of single colonies in culture. We have, therefore, determined the kinetics of appearance of blast colonies in the presence of IL-3 alone or in combination with IL-11. Data shown in Fig 2 indicate that the presence of IL-11 shortens the time of appearance of colonies from 5 to 6 days to 3 days in both AML samples tested. Based on similar observations on normal cells, it was suggested previously that IL-11 may reduce the G0 residence time of hematopoietic precursors. To directly address this question with AML blasts, cells were exposed to IL-3 in the presence or absence of IL-11 for 24 hours and their cycling status was analyzed by a 20-minute exposure to tritiated thymidine, and subsequent plating in clonogenic cultures, as described in the Materials and Methods (Fig 3).

### Table 2. Effect of IL-11 on AML Blast Colony Formation in Serum-Free Cultures

<table>
<thead>
<tr>
<th>Cells</th>
<th>Colony Count Control</th>
<th>IL-3</th>
<th>IL-11</th>
<th>IL-3 + IL-11</th>
<th>EC50 (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML28R</td>
<td>10 ± 2</td>
<td>30 ± 3</td>
<td>18 ± 5</td>
<td>61 ± 3</td>
<td>0.28 ± 0.03</td>
</tr>
<tr>
<td>AML 29</td>
<td>0</td>
<td>47 ± 1</td>
<td>0</td>
<td>105 ± 4</td>
<td>0.27 ± 0.06</td>
</tr>
<tr>
<td>AML 34</td>
<td>0</td>
<td>70 ± 3</td>
<td>ND</td>
<td>110 ± 4</td>
<td>0.30 ± 0.03</td>
</tr>
<tr>
<td>AML 35</td>
<td>0</td>
<td>20 ± 1</td>
<td>2 ± 1</td>
<td>74 ± 1</td>
<td>0.77 ± 0.3</td>
</tr>
<tr>
<td>AML 36</td>
<td>4 ± 1</td>
<td>31 ± 2</td>
<td>9 ± 2</td>
<td>99 ± 3</td>
<td>0.36 ± 0.06</td>
</tr>
<tr>
<td>AML 39</td>
<td>17 ± 2</td>
<td>43 ± 2</td>
<td>28 ± 4</td>
<td>86 ± 3</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>AML 44</td>
<td>5 ± 2</td>
<td>106 ± 5</td>
<td>ND</td>
<td>207 ± 4</td>
<td>0.32 ± 0.03</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

Serum-free cultures were performed as described in Materials and Methods. Cell and growth factor concentrations were the same as in Table 1. EC50s were calculated through curve fitting of full IL-11 titration curves performed in serum-free medium, covering the concentration range of 0.03 ng/mL to 100 ng/mL (data not shown).

### Table 3. Colony Formation by CD34+ Cells

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Colony Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>IL-3</td>
<td>9 ± 4</td>
</tr>
<tr>
<td>IL-11</td>
<td>1 ± 2</td>
</tr>
<tr>
<td>IL-3 + IL-11</td>
<td>44 ± 13</td>
</tr>
</tbody>
</table>

Cells from sample AML 33 were labeled with HPCA (anti-CD34) and a fluoresceinated goat antimouse antibody. Doubled and dead cells were gated out. Positive cells were collected and reanalyzed before culture. 98.3% of the sorted cells were positive for CD34. Cells were plated at a final concentration of 7,000 cells/well in the presence of IL-3 (20 ng/mL), IL-11 (10 ng/mL), or both.

### Table 4. Concentration of IL-3 Required for Half-Maximal Stimulation of Colony Formation

<table>
<thead>
<tr>
<th>Cells</th>
<th>EC50 (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M07-E</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>AML 29</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>AML 35</td>
<td>6.0 ± 2.7</td>
</tr>
<tr>
<td>AML 44</td>
<td>5.0 ± 1.6</td>
</tr>
</tbody>
</table>

Cells concentrations were as indicated in Table 1. EC50s were derived by nonlinear regression analysis of IL-3 titration curves that were performed in the range of 0.2 to 60 ng/mL.
IL-11 ON MYELOID LEUKEMIC CELLS

Because IL-11 and IL-6, but not SF (data not shown), were found to act similarly on the cycling of the AML 44 blasts, we compared these three different growth factors for their effects on IL-3–dependent colony formation (Fig 5). Each of the three cytokines induced a 1.5-fold to 2-fold increase in the cloning efficiency of the AML cells relative to that achieved in the presence of IL-3 alone. Combination of IL-11 and IL-6 together with IL-3 did not result in a further increase in cloning efficiency. In contrast, coaddition of SF and IL-11 with IL-3 resulted in a fourfold enhancement of colony formation relative to that supported by IL-3 alone. These data are consistent with the model that there are two distinct pathways for growth factor enhancement of clonogenic cell proliferation: one mediated by either IL-6 or IL-11 and the other by SF. Simultaneous growth factor stimulation through both pathways yields even greater proliferation than is achievable through either alone.

DISCUSSION

As has been found with normal target hematopoietic cells, IL-11 alone had little effect on a variety of different leukemic cell populations tested. However, in combination with other growth factors, including IL-3, GM-CSF, and SF, IL-11 frequently acted to enhance the proliferation of various myeloid leukemic cell populations. In a survey of samples from 14 patients with AML, all but one who could not be assessed accurately were found to respond to IL-11. In addition, three different myeloid leukemic growth factor–dependent cell lines, M07-E, AML 193, and MV4-11, all showed enhanced proliferation when IL-11 was combined with the primary growth-supporting factor IL-3 or GM-CSF. Of these three cell lines, the effects of IL-11 were greatest with the M07-E cells, perhaps reflecting their megakaryoblastic phenotype and the responsiveness of normal megakaryocytic progenitors to the growth-enhancing effects of IL-11. 13 In our survey of lymphoid cell lines, no responsiveness to IL-11 was detected among three pre-B-ALL and two T-ALL lines tested. This is consistent with the relative lack of direct effects of IL-11 on cells of the lymphoid lineages. In the mouse, IL-11 in combination with SF has been shown to expand primitive cells in primary culture ultimately capable of yielding either myeloid or B lymphoid cells in secondary culture. 24 However, development of pre-B cells from these expanded cell populations depends on IL-7 and SF and does not require IL-11. The failure of pre-B–cell lines to respond, therefore, may reflect loss of responsiveness to IL-11 as the cells become committed to the B-lymphoid lineage.

Several growth factors, including G-CSF, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), SF, and IL-6 have been shown to act synergistically with GM-CSF or IL-3 in augmenting the proliferation of AML blasts, each through different mechanisms. G-CSF recruits a distinct subpopulation of AML blasts, whereas TNF-α has been shown to act on the same population of target cells as GM-CSF. 26 At the molecular level, TNF-α has been shown to upregulate GM-CSF receptor expression on AML blasts, whereas G-CSF does not affect GM-CSF binding to hematopoietic cells, suggesting distinct mechanisms of action. The synergistic effect of IFN-γ with IL-3 or GM-CSF is mediated through...
induction of TNF-α.\textsuperscript{29} In contrast to TNF-α, SF increases the responsiveness of AML blasts to GM-CSF at a level distal to GM-CSF receptor expression.\textsuperscript{22} Our results indicate that IL-3-dependent proliferation of AML blasts is enhanced through a common pathway by IL-6 and IL-11 and through a separate pathway by SF.

We have used various AML cell samples for studying the effects of the different growth factors, both alone and in combinations, on the cell cycle status of individual cells. With normal steady-state bone marrow cells, most of the primitive precursors are quiescent in G\textsubscript{0} and these cells divide infrequently to yield actively proliferating multipotential progenitors. Through mapping and kinetic studies of the appearance of individual blast colonies in the mouse, G-CSF, IL-6, and IL-11 have all been identified as early-acting cytokines that accelerate the appearance of colonies in culture, suggesting a shortening of the G\textsubscript{0} period of the cell cycle.\textsuperscript{30,31} That the accelerated transition of cells from G\textsubscript{0} depends on the simultaneous presence of two cytokines was demonstrated with IL-3 plus IL-6 using 2-day exposure in suspension culture to both cytokines followed by 1-hour exposure to \textsuperscript{3}H]thymidine to kill cells in S-phase before plating in clonogenic cultures.\textsuperscript{30} Only when both IL-3 and IL-6 were present in the 2-day suspension culture was there evidence for triggering of the cycling of the G\textsubscript{0} cells. Our results with human clonogenic AML cells show some similarities and some differences in comparison with these results. In agreement with the murine system, analysis of six different AML samples showed that the greatest fraction of S-phase cells was always found in the presence of a combination of IL-3 and IL-11. However, in contrast to the murine cultures, 40% to 60% of AML clonogenic cells were in S-phase when treated with IL-3 alone for 16 hours, as measured by thymidine suicide immediately after the culture period. Whether these differences reflect variations between species or if the direct effect of IL-3 on the cycling of the clonogenic AML blast cells is a consequence of the leukemic state remains to be determined. Equivalent experiments with normal human cells will be required to resolve this issue.

In M07-E cells and in two of three AML samples, IL-11 does not significantly increase the responsiveness of AML blasts to GM-CSF or IL-3, whereas SF consistently results in a threefold to fourfold decrease in half-efficient concentrations of GM-CSF.\textsuperscript{22} Interestingly, IL-11, but not SF,\textsuperscript{22} can enhance
of two distinct pathways for enhancement of IL-3-dependent growth, one triggered by IL-11 and the other by SF.

IL-6 and IL-11 have previously been shown to have overlapping biologic effects that include the shortening of the time of appearance of blast colonies in culture of normal bone marrow cells and the induction of the acute-phase response in hepatocytes. Our data suggest that IL-6 and IL-11 trigger the same pathway in AML blasts for two reasons. First, IL-6 was as effective as IL-11 in triggering cells into S-phase as shown by the thymidine suicide experiment. Second, both IL-6 and IL-11 enhanced GM-CSF or IL-3-dependent colony formation by AML blasts, but the combination of both did not further enhance colony formation. Together, these data suggest that IL-6 and IL-11 may trigger the same pathway in target cells as has been shown previously for IL-3 and GM-CSF, two hematopoietic growth factors with overlapping biologic activities, whereas IL-11 and SF act through distinct pathways.

Finally, it will be interesting to compare normal cell populations with the AML cells to determine if the effect of IL-11 in increasing S-phase cells might be generally applicable to hematopoietic cells or if these effects of growth factors represent characteristics of the leukemic state. If there are differences between normal and leukemic cells, IL-11 might find use clinically in bringing AML blasts synchronously into cycle prior to exposure to S-phase-specific chemotherapeutic agents. Even without differences, IL-11 should prove useful in manipulation of the cycling status of primitive stem cells for both clinical and experimental purposes.

REFERENCES


Effects of interleukin-11 on the proliferation and cell cycle status of myeloid leukemic cells

JP Hu, A Cesano, D Santoli, SC Clark and T Hoang