Interferon-γ Enhances Growth Factor-Dependent Proliferation of Clonogenic Cells in Acute Myeloblastic Leukemia

By I. Murohashi and T. Hoang

Interferon-γ (IFN-γ) has been reported to antagonize the stimulatory effect of various conditioned media on the growth of normal hematopoietic progenitor cells and clonogenic blasts from patients with chronic myelogenous leukemia (CML) and acute myeloblastic leukemia (AML). In the present study, using purified recombinant cytokines and homogenous cell populations, we provide evidence for a synergistic or additive effect of IFN-γ with recombinant human (rhu) hematopoietic growth factors in the stimulation of clonogenic blasts from most AML patients examined. Under conditions of limiting cell concentration, rhuIFN-γ alone showed little effect on blast proliferation, whereas in conjunction with recombinant human interleukin-3 (rhuIL-3), IFN-γ significantly enhanced colony formation in 13 of 15 AML cases. Maximal stimulation was obtained at low concentrations of IFN-γ (2 to 20 pmol/L) in four cases and at higher concentrations (700 to 7,000 pmol/L) in the remainder. IFN-γ also synergized with recombinant human granulocyte-macrophage colony-stimulating factor (rhuGM-CSF) in 9 of 13 cases. Within 1 hour of exposure, IFN-γ induced a twofold to fourfold accumulation of tumor necrosis factor α (TNFα)-specific transcripts in AML blasts and several AML cell lines that include HL-60 and OCI-AML 1. Further, the synergy between IFN-γ and IL-3 on AML blasts was partially or completely abrogated by a TNFα neutralizing antibody, suggesting that growth enhancement by IFN-γ may be mediated through TNFα production in AML blast culture. Exposure of normal precursors (burst-forming unit-erythroid [BFU-E] and colony-forming unit granulocyte-macrophage [CFU-GM]) to IFN-γ also resulted in significant growth enhancement, suggesting that the proliferative response elicited by IFN-γ was not limited to AML blasts. Finally, in M07-E, an IL-3-dependent human "megakaryoblastic" cell line, IFN-γ also significantly enhanced IL-3-supported colony formation, much in the same way as in primary AML blasts. In contrast, IFN-γ inhibited growth of all CSF-independent leukemic cell lines tested. This inhibition was partially alleviated by anti-TNFα antibody. In summary, our data indicate that IFN-γ can enhance or antagonize cell proliferation, depending on the cell type. Further, TNFα appears to mediate the biologic effect of IFN-γ either in growth stimulation or growth inhibition.

© 1991 by The American Society of Hematology.

MATERIALS AND METHODS

Cells. Blast cells were separated from the peripheral blood (PB) of AML patients at diagnosis (D) or in relapse (R) by centrifugation on a Ficoll-Hypeaque gradient (d = 1.077) (Pharmacia Fine Chemicals, Uppsala, Sweden). The cells were cryopreserved in fetal calf serum (FCS; Gibco, Grand Island, NY) containing 10% dimethyl sulfoxide and stored in liquid nitrogen until use. A programmed freezing rate of 1°C/min ensured a 95% recovery in viable cells upon thawing. Cell morphology and clinical

From the Laboratory of Hemopoiesis and Leukemia, Clinical Research Institute of Montreal, Montreal, Quebec, Canada.

Submitted October 17, 1990; accepted April 23, 1991.

Supported in part by grants from the National Cancer Institute of Canada and the Cancer Research Society. T.H. is a scholar of the National Cancer Institute of Canada.

Address reprint requests to T. Hoang, PhD, Laboratory of Hemopoiesis and Leukemia, Clinical Research Institute of Montreal, 110 Pine Ave W, 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.

© 1991 by The American Society of Hematology.

0006-4971/91/7804-0033$3.00/0
data are shown in Table 1. The KG-1 myeloblastic cell line was a

gift from Dr J. Gasson (UCLA School of Medicine, Los Angeles,

CA) and the myeloblastic cell line OCI-AML 1 was a gift from Dr

E.A. McCulloch (Ontario Cancer Institute, Toronto, Ontario,

Canada). The cell line IRCM-8 was established from long-term
culture of patient sample AML 8. The M07-E human megakaryo-

blastic cell line is IL-3-dependent and was a gift from Dr S.C.

Clark (Genetics Institute, Cambridge, MA).

Biologic reagents. rhuIFN-γ was obtained from Genzyme (Bos-

ton, MA). The specific activity of IFN-γ was 2.5 × 10⁷ U/mg. The

level of endotoxin was 0.008 ng/μg protein (less than 1.10⁶).
rhuIFN-ω was from Amgen Biologicals (Thousand Oaks, CA).
rhuTNFα was a generous gift from Dr Palladino (Genentech, CA).
rhuGM-CSF and rhuIL-3 were generous gifts from Dr S.C. Clark.
Endotoxin levels were less than 1.68 EU/mg GM-CSF, 0.13 EU/mg
TNFα, and 0.13 EU/mg IL-3. The sheep antiserum to huIL-1α and
IL-1β were generously provided by Dr A. Shaw (Glaxo IMB,
Geneva, Switzerland). The polyclonal antibodies were non-toxic
in culture, and did not cross-react with IL-3 or G-CSF. A dilution
of 1:50,000 was sufficient to neutralize 1 pg/mL of the correspond-
ing growth factor. The goat anti–G-CSF and anti–IL-6 were
generously provided by Dr S.C. Clark. Final dilutions of 1:1,000
were sufficient to neutralize 5 ng/mL of the corresponding cyto-

cine. The neutralizing rabbit antiserum against huTNFα and that

generated by Dr D. Hankins (Armed Force Radiobiological Insti-
tute, Bethesda, MD).

Cell separation. Mononuclear cells were prepared from the PB

or bone marrow (BM) of healthy donors through centrifugation on

a Ficoll-Hypaque gradient. Monocytes and T and B lymphocytes

were removed by complement-mediated lysis with the monoclonal

antibody (MoAb) against CDw52, Campath-1, as described previ-
sously (30 μg/10⁶ cell). Following a depletion in plastic adherent

cells, nonadherent cells were labeled with the mouse MoAb against
CD34, anti–HPCA-1 (1 μg/10⁶ cells) (Becton Dickinson, Mountain
View, CA) and a fluorescein isothiocyanate (FITC)-tagged goat anti-
mouse IgG (10 μg/10⁶ cells) (Amersham, Arlington Heights, IL). As negative controls, nonadherent, non-T-non-B
cells were labeled with the second antibody alone. FITC-labeled
cells were sorted using the FACSSTAR (Becton Dickinson). Dead
cells were eliminated on the basis of red fluorescence with
propidium iodide and cell aggregates were excluded using forward
and 90° scatter properties, as detailed previously. Blast colony
formation. AML blasts were plated in 100 μL of Iscove’s modified Dulbecco’s medium (IMDM) supplemented with
10% FCS, and cocultivated with methylcellulose (1% wt/vol; Fluka,
Buchs, Switzerland) in 96-microwell plates (Lux, Miles Scientific,
Napierville, IL), as described previously. Cell concentrations
were selected to yield a minimum number of colonies in the
absence of added growth factor.

Serum-free cultures were supplemented with deionized bovine
serum albumin (BSA; 20 mg/mL, Sigma, St Louis, MO), iron-
saturated human transferrin (300 μg/mL; Hoechst, Behringwerke,
Marburg, Germany), and purified porcine insulin (1.7 × 10⁻⁶
mol/L; Novo Nordisk, Novo Allé, Bagsvaerd, Denmark) as de-
scribed by Migliaccio and Migliaccio. Normal BM cultures.

Nonadherent mononuclear cells were plated in 35-mm culture dishes (Lux) in 1 mL of IMDM supple-
cmented with 10% FCS, iron-saturated transferrin (300 μg/mL;
Hoechst), deionized BSA (20 mg/mL; Sigma), α-thioglycerol
(7.5 × 10⁻⁵ mol/L, Sigma), and methylcellulose, as described previ-
ously. Preparation of total cellular RNA and Northern blot analysis.

Blast cells were incubated in IMDM with 10% FCS in the presence
or absence of growth factor(s) for various time periods as indi-
cated. Total cellular RNA was prepared according to Chomczynski
et al. After glyoxilation, equal quantities of samples (10 μg) were
size-fractionated on agarose gels and blotted onto Nytran mem-
branes (Schleicher and Schuell Inc, Keene, NH). Membranes were
hybridized overnight at 65°C with 10⁶ cpm/mL DNA using DNA
probes radiolabeled by random priming with the Klenow fragment of
DNA polymerase to a specific activity of 0.5 to

1.5 × 10⁶ cpm/μg DNA. Blots were autoradiographed on Kodak
XAR film (Eastman Kodak, Rochester, NY) at ~80°C with an
intensifying screen. To ascertain that equal amounts of messenger
RNA (mRNA) were loaded in individual lanes, dot blots were
prepared with serial dilutions of RNA samples and hybridized to
dot-blot probes. Northern blots and dot blots were quantified by
scanning densitometry (Model 520, BioRad, Richmond, CA).

Abbreviations: FAB, French-American-British; ND, not done.

Table 1. Clinical Data on the AML Patients Studied

<table>
<thead>
<tr>
<th>Patient</th>
<th>FAB Classification</th>
<th>Blasts (%)</th>
<th>% Blasts Promyelocytes After Cell Separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML 3</td>
<td>M5b</td>
<td>38</td>
<td>ND</td>
</tr>
<tr>
<td>AML 8</td>
<td>M2</td>
<td>80</td>
<td>97</td>
</tr>
<tr>
<td>AML 9</td>
<td>M4</td>
<td>90</td>
<td>100</td>
</tr>
<tr>
<td>AML 10</td>
<td>M2</td>
<td>43</td>
<td>87</td>
</tr>
<tr>
<td>AML 17</td>
<td>M1</td>
<td>91</td>
<td>100</td>
</tr>
<tr>
<td>AML 18</td>
<td>M4</td>
<td>94</td>
<td>98</td>
</tr>
<tr>
<td>AML 19</td>
<td>M6b</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>AML 20</td>
<td>M1</td>
<td>61</td>
<td>94</td>
</tr>
<tr>
<td>AML 25 (R)</td>
<td>M2</td>
<td>65</td>
<td>100</td>
</tr>
<tr>
<td>AML 26</td>
<td>M2</td>
<td>32</td>
<td>93</td>
</tr>
<tr>
<td>AML 28 (R)</td>
<td>M1</td>
<td>92</td>
<td>95</td>
</tr>
<tr>
<td>AML 29</td>
<td>M1</td>
<td>90</td>
<td>99</td>
</tr>
<tr>
<td>AML 33</td>
<td>M4</td>
<td>40</td>
<td>84</td>
</tr>
<tr>
<td>AML 34</td>
<td>M1</td>
<td>93</td>
<td>99</td>
</tr>
<tr>
<td>AML 36</td>
<td>M3</td>
<td>96</td>
<td>ND</td>
</tr>
<tr>
<td>AML 39</td>
<td>M6</td>
<td>96</td>
<td>ND</td>
</tr>
</tbody>
</table>

RESULTS

Effects of IFN-γ on the proliferation of blast clonogenic
cells. Blasts from 13 AML patients were cultured in
methylcellulose in the presence of increasing concentra-
tions of IFN-γ alone or in combination with IL-3 and
GM-CSF. To assess the effect of exogenous growth factors,
cultures were performed under conditions that minimize
cell interaction. AML blasts were therefore plated at 7,000
to 10⁶ cells per well. Under such conditions, colony forma-
tion was entirely dependent on the addition of exogenous
growth factors. Further, optimal cell proliferation required
the presence of more than one growth factor. Thus, IFN-γ had little effect by itself (data not shown) and IL-3 or
GM-CSF showed weak stimulatory activity in most samples
(Table 2).
IFN-γ ENHANCES LEUKEMIC CELL GROWTH

Actual colony counts are shown in Table 2. Colony counts in cultures containing IL-3 and IFN-γ were normalized as the ratio of that observed in the presence of IL-3 in 8 of 13 samples. However, IFN-γ stimulated colony formation in synergy with IL-3 in 12 of 13 AML samples (Fig 1 and Table 2). Optimal synergy was seen with concentrations as low as 7 to 20 pmol/L IFN-γ (right panel), whereas some samples required higher concentrations (700 to 2,000 pmol/L, left panel). In one sample, designated AML 18, plateau level growth was not attained by addition of up to 7,000 pmol/L IFN-γ. The effects were striking, with a twofold to 23-fold increase in colony count over that induced by IL-3 alone. The only exception was sample AML 26, which showed a dose-dependent inhibition of growth with increased concentrations of IFN-γ (right panel). As shown in Fig 2 and Table 2, IFN-γ also synergized with GM-CSF in the stimulation of blast colonies in 9 of 13 patients. The response pattern to IFN-γ in the presence of GM-CSF was comparable with that observed in the presence of IL-3 in 8 of 13 samples. Thus, samples AML 8 and 10 responded to low concentrations of IFN-γ (Fig 2, right panel), whereas samples AML 18 through 20, 28, 29, and 33 (Fig 2, left panel) responded to high concentrations of IFN-γ, as observed in the presence of IL-3. However, IFN-γ enhanced colony formation in samples AML 3, 9, 17, and 25 (R) in the presence of IL-3 only and, in sample AML 26, in the presence of GM-CSF only.

The response of AML blasts to IFN-γ was abrogated by a neutralizing antibody against human IFN-γ. This antibody did not affect colony formation in the presence of IL-3 alone, suggesting that the growth enhancing effect shown in Figs 1 and 2 was due to IFN-γ itself, rather than a potential contaminant. To determine whether serum growth factors may modulate the biologic effects of IFN-γ on AML blasts, cultures were performed in serum-free medium (Table 4). IFN-γ significantly enhanced AML blast colony formation supported by IL-3 in all three samples tested, suggesting that growth enhancement by IFN-γ does not require the presence of serum growth factors.

Effect of IFN-γ on CD34+ cells. To further address the effect of IFN-γ on blast progenitors, AML blasts were separated on the basis of CD34 expression, using the fluorescence-activated cell sorter (FACS) (Table 5). In both samples (AML 29 and 33), IL-3 showed a weak

![Fig 1. Synergistic effects of IFN-γ and IL-3 on the proliferation of AML clonogenic cells. AML blasts were plated at 7,000 to 10^6 cells per well in the presence of IL-3 (1 nmol/L). IL-3 titration curves were performed separately and a concentration of 1 nmol/L was found to be optimal. IFN-γ titration curves for individual AML samples are shown (left panel, AML 17 through 20, 25 (R), 28, 29, and 33; right panel, AML 3, 8 through 16, and 26). Data were the mean of five replicate cultures and were normalized as the ratio of colony counts in cultures containing IL-3 and IFN-γ (E) over that of control cultures containing IL-3 alone (C). Actual colony counts are shown in Table 2.](image-url)
stimulatory activity only on CD34+ cells, whereas the combination of IL-3 and IFN-γ caused a significant increase in colony formation.

We (data not shown) and others45 have observed an inhibitory effect of IFN-γ on unseparated normal bone marrow cells. Because accessory cells have been shown to modulate the effect of TNFα on normal precursors,46,47 it is possible that the cellular heterogeneity of normal marrows could interfere with the biologic effects of IFN-γ. Precursors were therefore sorted with anti-CD34 before plating with IFN-γ and IL-3. In both PB and BM, there was a twofold increase in erythroid colony formation on addition of IFN-γ to IL-3-containing cultures (Table 5). Taken together, our data indicate that growth enhancement by IFN-γ may be attributed to a direct effect of IFN-γ on CD34+ cells.

Role of IFN-γ in reducing the requirement in cell interaction. Optimal cell proliferation in culture of AML blasts requires the presence of soluble growth factors48-50 and also depends on cell interaction.45 Therefore, we asked the question whether the synergistic effects of IFN-γ and IL-3 in IFN-γ-responsive cells may reduce the requirement in cell numbers. Adherent cells were removed by an overnight incubation at 37°C.46 Nonadherent cells were plated at different concentrations in the presence of IL-3, IFN-γ, or both. Representative data are shown in Fig 3 for an IFN-γ-responsive sample (AML 33). Growth enhancement by IFN-γ was more striking at low cell concentrations than at higher cell concentrations in IFN-γ-responsive cells (AML 25 (R), 29, and 33). Further, the slopes of the regression lines generated in the presence of a combination of IL-3 and IFN-γ at different cell concentrations were close to 1 for all three samples, suggesting the presence of a single limiting cell, ie, the blast clonogenic cell (Table 6). In contrast, the slopes of the regression lines generated with IL-3 alone, IFN-γ alone, or culture medium alone in the same samples were higher than 2, suggesting interacting cell populations or cell products.47,48 Taken together, our data suggest that the simultaneous presence of IFN-γ and IL-3 allows for an alleviation, at least in part, of the requirement in cell interaction for optimal growth.

Among the AML tested, sample 34 had an unusual profile because the slope of the regression line generated from data obtained with cultures stimulated by IL-3 alone was 0.9, suggesting a single limiting cell. Therefore, addition of IFN-γ did not result in a significant enhancement of IL-3–supported growth at any cell concentration.

Reversal of IFN-γ-mediated growth enhancement by anti-TNFα. AML blasts have been reported to express mRNA and/or release several cytokines that affect the growth of AML clonogenic cells.31-34,46 Further, it is well established that IFN-γ exerts significant immunomodulatory effects on numerous cell types and induces the release of various cytokines that include TNFα, IL-1, and IL-6.49,50 To determine whether the synergy between IFN-γ and CSF on AML blasts might be due to IFN-γ-induced increase in growth factor production by AML blasts, anti–IL-1, anti–G-CSF, anti-TNFα, and anti–IL-6 were assessed independently or in combination for their capacity to neutralize the synergistic stimulation (Fig 4). In samples AML 25 (R), 29,
Table 4. IFN-γ-Mediated Growth Enhancement in the Presence or Absence of Serum

<table>
<thead>
<tr>
<th>Colony Count</th>
<th>AML 25 (R)</th>
<th>AML 33</th>
<th>AML 36</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-FCS</td>
<td>+FCS</td>
<td>-FCS</td>
</tr>
<tr>
<td>0</td>
<td>24 ± 6</td>
<td>20 ± 5</td>
<td>17 ± 6</td>
</tr>
<tr>
<td>IFN-γ (0.23 nmol/L)</td>
<td>38 ± 7</td>
<td>39 ± 2</td>
<td>12 ± 5</td>
</tr>
<tr>
<td>IL-3 (1 nmol/L)</td>
<td>58 ± 2</td>
<td>50 ± 5</td>
<td>54 ± 9</td>
</tr>
<tr>
<td>IL-3 + IFN-γ</td>
<td>86 ± 5*</td>
<td>118 ± 14*</td>
<td>89 ± 8*</td>
</tr>
</tbody>
</table>

Cell concentrations were 7,000/well for samples AML 25 (R) and AML 3 and, for AML 36, 20,000/well (−FCS) and 10,000/well (+FCS). Serum-free cultures were supplemented with BSA, transferrin, and insulin as indicated in Materials and Methods. Data shown represent mean ± SD of five replicate cultures.

*P < .05 when compared with cultures containing single cytokines.

33, and 36, a combination of anti-IL-1α and anti-IL-1β significantly reduced blast colony formation supported by IFN-γ and IL-3, whereas anti-IL-6 produced a significant reduction in colony formation in samples AML 25 (R), 28, and 33. Anti-G-CSF showed no significant effect. The most striking results were observed with anti-TNFα, which caused a significant decrease in colony count in all seven samples tested. In fact, the synergy between IFN-γ and IL-3 was almost completely abrogated by anti-TNFα in four patient cells (AML 3, 25 (R), 29, 33, and 36). Finally, the combination of several antibodies that included anti-TNFα returned colony counts to basal levels obtained with IL-3 alone in all AML samples.

As controls for antibody specificities, we decided to test the antibodies in culture of normal BM cells stimulated with IL-3 and Epo (Table 7), because AML blasts have been shown to secrete several cytokines in culture and to respond to their proliferative stimuli.31,34,46 Anti-G-CSF, anti-TNFα, anti-IL-6, or a combination of these had no effect on granulocyte-macrophage progenitors (granulocyte-macrophage colony-forming units [GM-CFU]) or erythroid progenitors (burst-forming unit-erythroid [BFU-E] and CFU-erythroid [CFU-E]). In fact, some enhancement in erythroid colony formation was observed with anti-TNFα, suggesting the presence of TNFα in culture of unseparated normal BM cells. None of these antibodies had any significant effect on colony formation from HL-60 cells (Table 8 and data not shown). Taken together, our results indicated that the inhibitory effects of antibodies on the growth of AML blast clonogenic cells supported by IFN-γ and IL-3 was not due to a nonspecific toxicity of the different antibodies.

Enhanced TNFα mRNA in IFN-γ-treated AML blasts. The specific effects of anti-TNFα suggested that TNFα may act as a mediator of IFN-γ in growth stimulation of AML blasts. Sample AML 29 was, therefore, exposed to IFN-γ for different length of time and assessed for expression of TNFα-specific transcripts (Fig 5). IFN-γ induced a significant enhancement in the number of TNFα transcripts (Fig 3).
Table 6. Influence of IFN-γ and IL-3 on AML Blast Colony Formation

<table>
<thead>
<tr>
<th>Slope Factor</th>
<th>AML 25 (R)</th>
<th>AML 29</th>
<th>AML 33</th>
<th>AML 34</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-3 + IFN-γ</td>
<td>1.2 ± 0.03</td>
<td>1.2 ± 0.05</td>
<td>1.1 ± 0.01</td>
<td>0.84 ± 0.06</td>
</tr>
<tr>
<td>IL-3</td>
<td>1.9 ± 0.12</td>
<td>2.2 ± 0.01</td>
<td>2 ± 0.09</td>
<td>0.9 ± 0.04</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1.9 ± 0.1</td>
<td>NG</td>
<td>3.4 ± 0.18</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>Medium</td>
<td>2.9 ± 0.13</td>
<td>NG</td>
<td>4 ± 0.22</td>
<td>2.5 ± 0.19</td>
</tr>
</tbody>
</table>

AML blasts were plated at different cell concentrations ranging from 1,200 to 20,000 per culture (Fig 3). In sample AML 29, there was no growth (NG) in the presence of IFN-γ alone, or in culture medium alone. Data shown are the slopes ± SD of the regression lines represented in Fig 3, fitted by the least square method to the logarithms of the arithmetic means of colony counts at each cell concentration. A slope of 1 is consistent with the dependence of colony formation on a single progenitor cell. Slopes of ≥2 indicate complex bi- or multi-order interactions.*** Typical colony counts for sample AML 34 (5,000/well) are shown in Table 2.

Table 7. Effects of Antibodies Against Cytokines on Normal BM Cells

<table>
<thead>
<tr>
<th>Addition to Culture</th>
<th>Colonies per Dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-3 (1 nmol/L)</td>
<td>CFU-GM CFU-E BFU-E</td>
</tr>
<tr>
<td>+ -</td>
<td>6 ± 1 62 ± 11 15 ± 1</td>
</tr>
<tr>
<td>+ G-CSF</td>
<td>93 ± 1 72 ± 11 57 ± 0</td>
</tr>
<tr>
<td>+ IL-6</td>
<td>94 ± 7 103 ± 8 63 ± 3</td>
</tr>
<tr>
<td>+ TNFα</td>
<td>98 ± 2 60 ± 4 51 ± 4</td>
</tr>
<tr>
<td>+ G-CSF, TNFα, and IL-6</td>
<td>99 ± 3 135 ± 11 84 ± 2</td>
</tr>
</tbody>
</table>

Duplicate cultures were established with mononuclear BM cells from normal donors as described in Materials and Methods. Nonadherent BM cells were plated at 10^5/mL. Epo was added at 1 U/mL. All antibodies were added at a dilution of 1:600.

Significant and transient accumulation of TNFα mRNA in AML blasts, with a peak at 1 hour of stimulation. By 17 hours the expression of TNFα mRNA returned to basal level. There was no increase in TNFα mRNA when the cells were kept in culture medium alone. IFN-γ also caused an accumulation of IL-1β mRNA, with a kinetic that was similar to that of TNFα mRNA. In agreement with Northern blot data, the antibodies against TNFα and IL-1β caused a significant reduction in the growth enhancing effects of IFN-γ in these cells (Fig 4), suggesting that IFN-γ modulates AML blast proliferation through increased accumulation of TNFα and IL-1β-specific transcripts.

Comparison of sensitivities of different AML cell lines with IFN-γ. Several established AML cell lines were evaluated with regards to their relative sensitivity to IFN-γ (OCI-AML 1, HL-60, KG-1, IRCM-8, and M07-E). In M07-E cells, an IL-3-dependent cell line, IFN-γ stimulated colony formation in synergy with IL-3 (Fig 6). The stimulation by IFN-γ was dose-dependent, and the maximum level was achieved at a concentration of 300 pmol/L. As observed with AML blasts, neutralization of the endogenous source of TNFα also resulted in a significant reduction in the growth-enhancing effect of IFN-γ on IL-3-dependent colony formation from M07-E cells (Fig 4). Northern blot analysis of M07-E cells also indicated that exposure to IFN-γ resulted in a twofold increase in TNFα mRNA (Fig 7).

In contrast, in the other AML cell lines, OCI-AML 1, HL-60, KG-1, and IRCM-8, IFN-γ inhibited clonogenic cell growth in a dose-dependent manner, regardless of the presence or absence of IL-3 (Table 8 and data not shown). Maximum inhibition of colony formation ranged from 30% to 75%, depending on the cell type. Therefore, we asked the question whether or not the antibody against TNFα may cause a reversal of IFN-γ-mediated growth inhibition in these cell lines. As shown in Table 8, IFN-γ suppressed colony formation in all three cell lines tested. Addition of...
anti-TNFα to IFN-γ-containing cultures caused a significant increase in colony count in HL-60 and OCI-AML 1 cells, whereas that of IRCM-8 cells was not affected. In control cultures, colony formation in the absence of IFN-γ was not affected by the antibody against TNFα, indicating that the antibody did not react with an undefined culture component. Rather, the reversal of IFN-γ-induced growth inhibition by anti-TNFα may be due to neutralization of the endogenous source of TNFα.

Culture results were confirmed by the accumulation of TNFα mRNA in HL-60 and OCI-AML 1 cells treated with IFN-γ (Fig 7). The extent of IFN-γ-induced TNFα transcripts was much more important in HL-60 cells than in IRCM-8 cells, suggesting that the partial effect of anti-TNFα antibody on IFN-γ-mediated growth inhibition of HL-60 cells (shown in Table 7) could, in part, be due to insufficient antibody concentration.

Exposure to IFN-γ also resulted in an accumulation of IL-1β mRNA in HL-60 cells, whereas no increase was observed in OCI-AML 1 cells (Fig 7). However, despite the accumulation of IL-1β mRNA in HL-60 cells, the antibodies against IL-1α and IL-1β did not affect IFN-γ-mediated growth inhibition in these cells (data not shown). Taken together, our data suggest that growth stimulation by IFN-γ may be mediated in part through increased IL-1 expression in AML blasts, whereas growth inhibition in AML cell lines may be IL-1-independent.

**DISCUSSION**

Using defined growth conditions and samples selected for low or undetectable contamination with normal accessory cells, we provide evidence here that IFN-γ acts synergistically with purified GM-CSF or IL-3 in stimulating...
AML blast colony formation. Likewise, we have previously shown that TNFα potentiates the effect of GM-CSF on AML blasts, whereas previous reports based on mixed cell populations and/or growth factors have suggested the reverse. Salem et al have also reported a synergistic effect of TNFα with IL-3 on AML blast proliferation. Recently, Caux et al and Piacibella et al have documented the potentiating effect of TNFα on CSF-induced proliferation of hematopoietic progenitors after positive selection with anti-CD34 or after removal of accessory cells, whereas previous work using total BM cells has indicated an inhibitory effect of TNFα on normal precursors. Accessory cells may therefore modulate the biologic effects of a cytokine. In the present study, we have addressed the issue of accessory cells in several ways. First, we show that IFN-γ can either be stimulatory or inhibitory for cell proliferation in cloned AML cell lines (HL-60, IRCM-8, KG-1, and M07-E) in absence of accessory cells. Second, our AML samples have been chosen for high blast counts. Third, the growth enhancing effects of IFN-γ was comparable in samples containing 84% to 100% blasts, ie, with no detectable accessory cells, suggesting that the response was not mediated by normal accessory cells. Fourth, our cultures were performed at limiting cell concentration to minimize cell interaction. Fifth, growth enhancement by IFN-γ was still observed following removal of adherent cells and selection for CD34+ cells. Taken together, our data indicate that IFN-γ enhances growth of normal precursors and of clonogenic blasts in the absence of accessory cells. We have also investigated the response to IFN-γ of purified CD34+ cells from normal donors. Following selection for CD34-expressing cells, IFN-γ significantly enhanced colony formation supported by IL-3, whereas IFN-γ appeared to cause growth inhibition in unseparated BM cells (data not shown). Our data further underline the role of accessory cells in modulating the biologic effect of a cytokine.

We have also compared the effects of IFN-γ and IL-3 separately or together at different cell concentrations. In IFN-γ-responsive cells and in the presence of both cytokines, the data were described by linear regression lines with a slope of 1, suggesting that only clonogenic cells were limiting. In contrast, spontaneous growth or growth supported by single cytokines were dependent on cell concentrations and were characterized by regression lines with slopes of 2 to 4. Under such conditions, the cultures were probably limiting for more than one cell type. In summary, our data suggest that IFN-γ can alleviate, in part, the requirement for cell interaction in culture of AML blasts. However, such cell interaction is not provided by normal accessory cells. First, sample AML 25 (R) contained 100% blasts, yet the slope of the regression line observed with IL-3 alone was 2, suggesting that the accessory cell can be a leukemic blast. Further, Nara and McCulloch have previously shown that autologous blasts provide a better “supporting activity” for blast colony formation than heterologous blasts. Finally, it has been shown that blasts with “accessory” function can be physically separated from the population of clonogenic cells in AML samples. Our data also suggest that the lack of synergistic response to IFN-γ and IL-3 in IFN-γ–unresponsive” cells (AML 34) may be due to the fact that “accessory” blasts can provide for the necessary growth factor(s) or growth requirements because the slope of the regression line observed with IL-3 alone is 1. In fact, AML 34 cells spontaneously express TNFα mRNA in culture (unpublished results), which may account for the lack of response to IFN-γ.
The unusual proliferative effect of IFN-γ on AML blast progenitors prompted an investigation on the mechanism of action of the cytokine. The use of neutralizing antibodies against TNFα indicates the activation of TNFα-dependent pathway by IFN-γ-receptor, either in growth inhibition or growth stimulation. Neutralization of the endogenous source of TNFα abrogates the synergistic effect of IFN-γ with IL-3 on AML blasts and M07-E, an IL-3-dependent cell line. In addition, the antibody alleviates part of IFN-γ-mediated growth inhibition in HL-60 and OCI-AML 1 cells. These data are further supported by the accumulation of TNFα-specific transcripts upon exposure of AML blasts or cell lines to IFN-γ. The difference in response to IFN-γ between primary AML blasts and established AML cell lines appears to correlate with the dependency on an exogenous source of growth factor for proliferation. Indeed, M07-E, an IL-3-dependent cell line, is stimulated by IFN-γ, whereas HL-60, KG-1, and IRCM-8 cells that are able to grow autonomously, are suppressed by the presence of IFN-γ in culture.

At high cell concentration, primary AML blasts have also been shown to grow autonomously. Yet, our data indicate that spontaneous colony formation is not suppressed by IFN-γ in all of the samples tested (AML 25 [R], 29, 33, and 34). Although the cells do not require exogenous growth factors, several lines of evidence indicate that spontaneous growth under such conditions may be attributable to endogenously produced growth factors, and may, therefore, differ from the autonomous growth behavior of the cell lines HL-60, KG-1, and IRCM-8.

What is the mechanism of IFN-γ-mediated inhibition in cell lines that grow autonomously? It has been suggested that two enzymatic pathways are involved in the antiviral, and perhaps the antiproliferative, effect of IFN-γ. The first one involves the synthesis of the enzyme 2′-5′ oligoadenylate synthetase, with subsequent activation of endonuclease L or H. The second enzyme is the 68-Kd protein kinase, that upon activation, phosphorylates the eukaryotic initiation factor eIF2α and blocks further initiation of translation. However, pathways that govern the response of hematopoietic cells to IFN-γ are not well understood. Preliminary data suggest that the level of 2′-5′ oligo A synthetase mRNA is very low in HL-60 cells and is unaffected by exposure of the cells to IFN-γ or TNFα (Murohashi and Hoang, unpublished data). The role of the p68 kinase remains to be documented.

The contrasting response pattern to IFN-γ between primary AML blast and CSF-independent cell lines indicates that the choice of a test assay in the design of antileukemic agents is of great importance. It appears from our data that the IL-3-dependent cell line, M07-E, might be a more suitable model. Development of an animal model to study antileukemic agents in vivo may ultimately provide the most suitable assay.

IL-3 and GM-CSF have been shown to share common biologic properties and, perhaps, a common binding protein in target cells. It is, therefore, not surprising that IFN-γ (and TNFα as well) acts synergistically with both IL-3 and GM-CSF in supporting hematopoietic cell proliferation. In contrast, G-CSF, M-CSF, or CSF-1 and Epo differ markedly from IL-3 and GM-CSF. It has, in fact, been shown that TNFα antagonizes rather than enhances the effect of G-CSF on hematopoietic cells. Whether the effect of IFN-γ would be different in the presence of the other hematopoietic growth factors requires further investigation.

Both transforming growth factor β (TGFβ) and TNFα have been widely recognized as bimodulators of cell proliferation in many biologic systems, whereas IFNs have been largely described as antiviral and antiproliferative agents. Our study suggests that IFN-γ can either enhance or suppress cell proliferation, much in the same way as the other two cytokines. Our data also suggest that IFN-γ exerts a positive or negative effect on cell proliferation through the modulation of TNFα production (or gene expression) in target cells.

ACKNOWLEDGMENT

The authors are indebted to Drs F. Letendre and R. Godin (Hôtel-Dieu Hospital, Montreal), R. Bélanger (Maisononneuve-Rosemont Hospital, Montreal), and R. Pichette (Sacre-Coeur Hospital, Montreal) for providing access to clinical material, and Drs M. Trudel, J.C. Rodriguez-Cimadevilla, and R.-P. Sékaly for critical reading of the manuscript. The authors wish to thank A. Haman for expert technical assistance and F. De Coste for skilled secretarial support.

REFERENCES


27. Vellenga E, O'Stapiotz D, O’Rourke B, Griffin JD: Effects of recombinant IL-3, GM-CSF and G-CSF on proliferation of leukemic clonogenic cells in short-term and long-term cultures. Leukemia 1:584, 1987


42. Broxmeyer HE, Williams DE, Lu L, Cooper S, Anderson SL, Beyer GS, Hoffman R, Rubin BY: The suppressive influences of a human tumor necrosis factor on bone marrow hematopoietic...


51. Chapekar MS, Glazer RI: The synergistic cytolicidal effect produced by immune interferon and tumor necrosis factor in HT-29 cells is associated with inhibition of rRNA processing and (2', 5') oligo (A) activation of RNase L. Biochem Biophys Res Commun 151:1180, 1988


57. Onetto-Pothier N, Aumont N, Haman A, Park L, Clark SC, De Léan A, Hoang T: IL-3 inhibits the binding of GM-CSF to AML blasts, but the two cytokines act synergistically in supporting blast proliferation. Leukemia 4:329, 1990

Interferon-gamma enhances growth factor-dependent proliferation of clonogenic cells in acute myeloblastic leukemia

I Murohashi and T Hoang