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Enhancement of the Cytotoxicity of Cytosine Arabinoside by Interleukin-3

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We have evaluated the feasibility of enhancing the cytotoxic effect of cytosine arabinoside (ara-C) on acute myeloid leukemia (AML) cells by increasing the proliferative activity with hematopoietic growth factors. Leukemic cells from 8 persons with AML were tested. Preincubation with interleukin (IL)-3 (5 U/ml) for 3 days increased DNA synthesis as measured by tritiated thymidine incorporation and Ki67 expression in cells from 7 out of 8 persons with AML. Leukemic cells preincubated with IL-1 (10 U/ml) or IL-3 (5 U/ml) were subsequently exposed to ara-C (3 μg/ml) for the final 24 h and the activity of ara-C against clonogenic acute myeloid leukemia cells was evaluated in terms of the inhibition of colony formation in semisolid media. The exposure to ara-C inhibits the proliferation of a higher proportion of clonogenic cells in culture pretreated with IL-3 than in control or cells pretreated with IL-1. The enhanced cytotoxic effect of ara-C in the cells pretreated with IL-3 correlated with increased formation of intracellular ara-CTP. IL-3-induced recruitment of quiescent blasts into the proliferative compartment will lead to increased formation of ara-CTP in the cells, which would result in an enhanced leukemia cell kill.

Key words: Interleukin-3 — Cytosine arabinoside — Acute myeloid leukemia

Cytosine arabinoside (ara-C) is a cytotoxic agent that, after intracellular transformation into its triphosphate (ara-CTP), interferes with DNA polymerase, and is incorporated into DNA, leading to defective DNA synthesis, inhibition of cell proliferation and cell death.[1, 2] It is one of the most effective agents against acute myelogenous leukemia (AML).[3]

Chemotherapeutic regimens, including ara-C, are successful in inducing complete remission in the majority of patients, but resistance to the drugs used is frequently observed. These failures of therapy can be explained in part by the proliferative characteristics of the AML blasts. The [^{3}H]thymidine (TdR) labeling index (LI) of leukemic cells obtained at the time of first diagnosis is almost always low.[4, 5] Studies using flow cytometry have confirmed the low proportion of cells in S and G_1+M in the blood and marrow in AML.[6, 7] On average, between 10 and 25% of marrow blasts are proliferating.[8] When proliferating cells divide, they usually give rise to non-proliferating cells.[9, 10] The numbers of proliferating cells are continuously replenished by transformation of non-proliferating blasts and the system as a whole is cyclic.

Recruitment of cells from the non-proliferating into the proliferating pool has been suggested as a way of enhancing the effectiveness of subsequent chemotherapy,[11] because cells in the non-proliferating (G_0) pool are thought to be immune to the action of phase-specific drugs such as ara-C.[12] Humoral growth factors released during chemotherapy have been shown to increase the sensitivity of the blasts to ara-C either *in vitro*[13] or *in vivo*.[14] It has recently been reported that hemopoietic growth factors, which are regulators of normal hematopoiesis, can also stimulate the proliferation of AML cells both in semisolid media and suspension cultures.[15]

We have therefore assessed the feasibility of enhancing the *in vitro* cytotoxic effect of ara-C by stimulating the quiescent leukemic blasts with hemopoietic growth factors, interleukin (IL)-1 and IL-3, prior to incubation with the drug.

MATERIALS AND METHODS

Leukemic cells Heparinized peripheral blood was obtained at diagnosis from 8 patients with AML (French-American-British classification, M1, M2, M3 and M4). The clinical information on the patients is summarized in Table I. T-cell-depleted mononuclear cells from blood were obtained as described previously by using the two-cycle Ficoll-Hypaque procedure, with the second centrifugation after the formation of E-rosettes.[16] These cells were cultured in suspension or preserved by freezing in liquid nitrogen in 10% dimethyl sulfoxide and 50% fetal calf serum (FCS).

Hematopoietic growth factors and 5637-conditioned medium (5637-CM) Human recombinant IL-1β and IL-3 were obtained from the Genetics Institute (Cambridge, MA). The supernatant of the cell line 5637, used as the source of colony-stimulating factor,[17] was obtained by growing the cell line 5637 in Iscove's modified Dulbecco's medium (IMDM, Gibco Ltd, Grand Island, NJ) supplemented with 20% FCS for 3 days and was used at 10% final concentration.

Suspension culture T-cell-depleted leukemic blast cells were washed with α-minimum essential medium (α-

MEM), in Linbro multiwell plates (Flow Laboratories, McLean, VA) in 2 ml of α-MEM supplemented with 10% FCS and exogenously added hemopoietic growth factors (10 units/ml of IL-1β, or 5 units/ml of IL-3) or without growth factors. The cells were adjusted to the concentration of 10^6 cells/ml and cultured at 37°C in a fully humidified atmosphere of 5% CO_2 in air for 72 h. Ara-C (Sigma, St. Louis, MI, 3 μg/ml) was routinely added for the last 24 h. The blast cells were harvested and the number and morphology of the cells were determined. The cells were then washed with α-MEM and tested for their ability to form colonies.

Blast colony assay Blast colony formation was assessed as previously described.[18] Cells cultured in suspension were plated in 35-mm Lux culture dishes (Miles Laboratories, Naperville, IL) in 1 ml of α-MEM supplemented with 20% FCS, 0.8% methylcellulose and 10% 5637-CM at the concentration of 10^6 cells/ml and incubated for 7 days at 37°C in 5% CO_2 in air. Colonies (more than 50 cells) and clusters (less than 50 cells) were scored. In some experiments, clusters and colonies were picked up and evaluated for morphology by Giemsa staining. Four replicate plates were used to determine the number of clonogenic cells per ml.

Determination of ara-CTP pool size AML blasts depleted of T-cells ($0.5–2.5 \times 10^6$/ml) were preincubated with growth factor (10 units/ml of IL-1β or 5 units/ml of IL-3) or 5637-CM for 72 h. Then, the cells were washed and transferred to the medium free of growth factor and incubated with 1 μM [^{3}H]ara-C (24 Ci/mmol, Amersham International, Buckinghamshire, England) for 6 h. After the incubation, cultures were chilled on ice for 10 min and the cells were collected by sedimentation (500g for 10 min, 4°C), washed twice with ice-cold phosphate-buffered saline (PBS) and then extracted twice with 300 μl of 60% methanol at 4°C for 10 min. The two methanol extracts were combined and the debris was removed by sedimentation (10,000g for 10 min). Ara-CTP is stable for at least 2 months at −20°C. The ara-CTP content of the methanol extracts was determined as described before[19] by injecting 100 μl of the extract, along with the internal standard of ara-CTP (Sigma), into an LC-5A HPLC apparatus (Shimadzu, Kyoto) equipped with a 4.6 mm × 25 cm Partisil SAX/10 anion exchange column (Whatman Inc., Clifton, CA). Samples were eluted with a mobile phase of KH_2PO_4 (0.3 M, pH 3.3) under isocratic conditions at a flow rate of 1.0 ml/ml. Fractions (0.5 ml each) were collected and the radioactivity of [^{3}H]ara-CTP was determined with a liquid scintillation spectrophotometer.

Proliferation assays Proliferation of cells was assayed by [^{3}H]TdR incorporation and Ki67 intranuclear staining. For [^{3}H]TdR incorporation, cells were plated in duplicate at the concentration of 1×10^5/microwell in 200 μl of α-MEM supplemented with 10% FCS with or without growth factors. After 72 h of culture, 2 μCi/microwell of [^{3}H]TdR (15 μCi/mmol, Amersham International) was added for the last 8 h. [^{3}H]TdR uptake was determined by liquid scintillation counting.[20] Ki67 nuclear antigen is present in S, G_2 and M phases, but is absent in G_0, and immunostaining with monoclonal antibody against Ki67 provides a reliable means of rapidly evaluating the growth fraction of normal and neoplastic cell populations.[21] For Ki-67 staining, a 0.1 ml aliquot of the culture was used to prepare a cytospin slide. Slides were air-dried, fixed in acetone, incubated with an anti-Ki67 monoclonal antibody and stained by means of the alkaline phosphatase technique.[22]

Labeling index Labeling index (LI) of leukemic blasts was determined as follows: aliquots of dispersed leukemic cells were incubated with 15% FCS in α-MEM containing [^{3}H]TdR (0.1 mCi/ml, specific activity 15 μCi/mmol) for 60 min at 37°C in a humidified atmosphere containing 5% CO_2. Following incubation, cells were washed three times with PBS and cytocentrifuged on slides coated with gelatin. Autoradiographs were prepared with Kodak photographic emulsion (VTB-2), exposed for 18 days, developed and then stained with Giemsa. The LI of leukemic cells was determined by counting the number of cells per 1000.

RESULTS

The optimal concentration of IL-3 and IL-1 for the induction of AML colonies The optimal concentrations

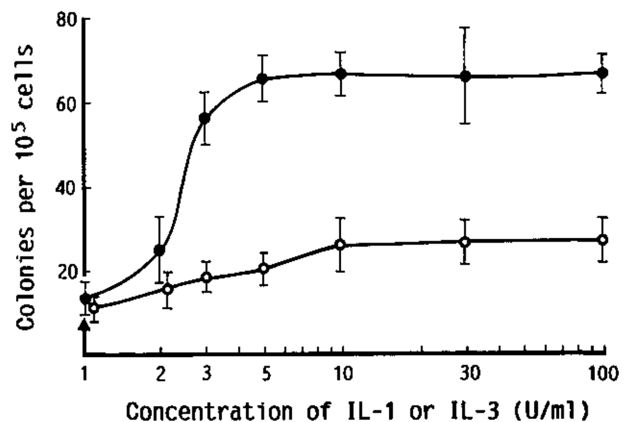

Fig. 1. Effect of pretreatment of leukemic cells with IL-1 (○) or IL-3 (●) on the leukemic colony formation. Leukemic cells were pretreated with growth factors in suspension culture for 72 h as described in "Materials and Methods." Then, the cells were seeded in semisolid media and the day-7 colonies were counted. The numbers of colonies of the leukemic cells without pretreatment are shown (▲).

Table I. Characteristics and Effect of Growth Factor Preincubation on $[^3H]$TdR Incorporation of AML Cells

Sample	FAB	LI[b] (%)	$[^3H]$TdR uptake (cpm)[a]		
			Control	IL-1[c]	IL-3[c]
AML-1	M1	16.2	2828±32	2969±125	4962±45
AML-2	M2	3.5	478±15	492±31	1892±76
AML-3	M3	3.1	2712±24	2891±65	8410±80
AML-4	M4	6.6	1520±60	1621±43	5621±92
AML-5	M4	6.2	1934±32	1989±81	4644±48
AML-6	M2	3.8	3148±56	3821±110	14145±290
AML-7	M3	4.8	4769±70	4189±98	20890±341
AML-8	M1	5.6	4181±78	3990±101	13191±211

a) $[^3H]$TdR incorporation per 1×10^5 cells after 8 h incubation. Values indicate mean±SEM of triplicate cultures performed twice using the same samples.
b) Labeling index (LI) of leukemic cells was measured as described in the text. The leukemic cells were incubated in α-MEM containing $[^3H]$TdR in the absence of growth factors.
c) The concentrations of IL-1 and IL-3 used in these experiments were 10 U/ml and 5 U/ml, respectively.

of IL-3 to induce AML colony formation were determined in dose-response experiments. AML cells were treated with various concentrations of IL-3 in suspension culture for 72 h and seeded in semisolid medium for 7 days. After culture, the AML colony cells were harvested and analyzed morphologically. Based on the data shown in Fig. 1, it was concluded that the optimal concentration of IL-3 was 5 U/ml. The optimal concentration of IL-1 to induce colony formation was determined in the same manner as that for IL-3. On the basis of the curve shown in Fig. 1, the optimal concentration of IL-1 was concluded to be 10 U/ml.

Proliferative characteristics of the AML cells In order to determine the effect of growth factors on the fraction of leukemic cells in S phase, blasts from 8 patients with AML were incubated in suspension culture for 72 h with or without growth factors and were evaluated by $[^3H]$TdR uptake measurement and Ki67 staining analysis. In Table I, FAB subtypes of AML cells, the LI and $[^3H]$TdR uptake in the presence or in the absence of growth factors are summarized. In all cases, except in AML-1, the LI was lower than 10%. The $[^3H]$TdR uptake study indicated that all 8 AML subjects demonstrated more than 2-fold increase in $[^3H]$TdR incorporation in the presence of IL-3. When Ki67 staining was used to measure the S phase fraction, 7 out of 8 cases showed a more than 50% increase in the percentage of cells in S phase in the presence of IL-3 (Table II). However, no increase was observed in the percentage of cells in S phase in the presence of IL-1. Thus, the proliferative response to the growth factors demonstrated that IL-3 was a more potent stimulator of proliferation than IL-1 in almost all cases studied. No correlation was detected between the LI and the response to the growth factors.

Table II. Effect of Growth Factor Preincubation on Ki67 Staining of AML Cells

Sample	Ki67-positive cells (%)[a]		
	Control	IL-1[b]	IL-3[b]
AML-1	10±2	11±2	35±3
AML-2	6±1	5±1	16±2
AML-3	8±1	9±2	27±3
AML-4	11±2	10±1	29±2
AML-5	14±2	13±1	34±4
AML-6	9±1	10±2	14±1
AML-7	11±1	13±1	24±2
AML-8	11±2	12±2	30±2

a) Percent of Ki-67-positive cells among 2×10^3 cells counted. Values are mean±SEM of two slides.
b) Concentrations of IL-1 and IL-3 used in these experiments were 10 U/ml and 5 U/ml, respectively.

Enhancement of growth inhibition of ara-C by addition of growth factors in AML cells Ara-C was given for the last 24 h in the suspension culture in order to allow most of the dividing cells to go through S-phase at least once and to incorporate ara-C before the cells were seeded in semisolid media. The effect of ara-C on AML blasts stimulated with growth factors is shown in Fig. 2. When no growth factor was present in the suspension culture, ara-C treatment inhibited the formation of AML blast colonies by 7 to 63% with a mean value of 36.3%. In IL-1-stimulated AML populations, the reduction of clonogenic ability in ara-C-treated samples ranged from 9 to 71%, with a mean value of 42.7%. When the AML cells were stimulated with IL-3, the inhibition of clonogenic ability by ara-C treatment ranged from 65 to 95%, with a mean value of 79.8%. Statistical analysis (Student's *t*

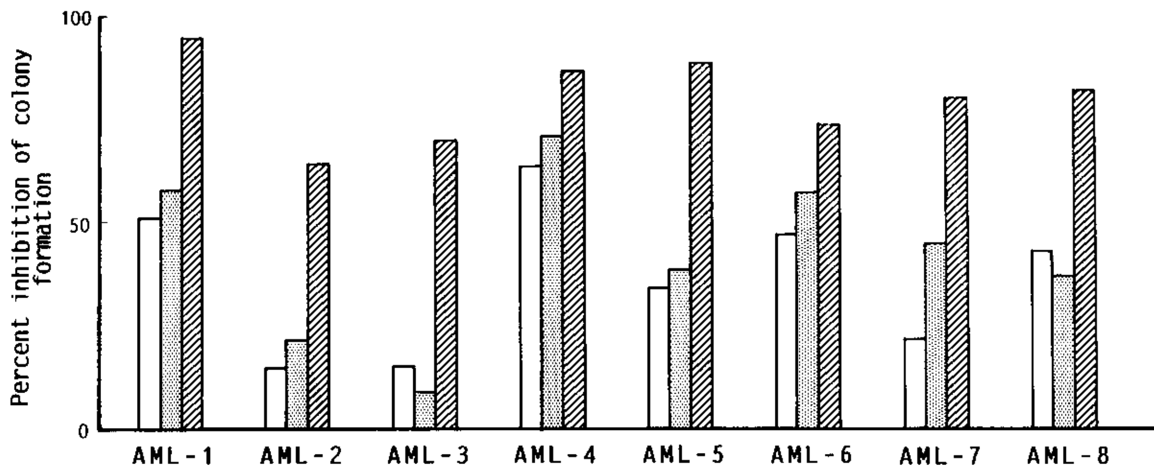

Fig. 2. Enhancement of ara-C cytotoxicity on AML blasts by IL-1 and IL-3. Leukemic blasts (1×10^6/ml) were incubated with α-MEM media containing 10% FCS (control, A □) or with the same media containing IL-1 (10 U/ml, B ▒) or IL-3 (5 U/ml, C ▨) for 72 h. The incubations were performed in Linbro multiwell plates with a final volume of 2 ml. Ara-C was used at the concentration of 3 μg/ml for each experiment. After incubation, cells were washed three times and plated in semisolid media as described in the text. The percent of inhibition was calculated by dividing the number of colonies with ara-C by the number of colonies without ara-C. Percent of inhibition (mean±SEM): A=36.3±21.7, B=42.7±23.8, C=79.8±12.1. Statistical significance: B vs. A, t=0.767, P<0.25; C vs. A, t=3.465, P<0.005.

Table III. Enhancement of Ara-CTP in AML Blasts after Pretreatment with Growth Factors

Cells	Ara-CTP formed (pmol)[a] Control ↓ [³H]ara-C	IL-1 ↓ [³H]ara-C	IL-3 ↓ [³H]ara-C
AML-1	ND		
AML-2	12.5±0.6	14.6±0.7	30.0±2.1
AML-3	14.9±1.2	13.2±0.9	25.6±1.4
AML-4	21.4±1.2	23.4±2.1	45.9±2.7
AML-5	24.3±2.1	19.4±0.9	108.8±3.5
AML-6	32.1±2.0	34.3±1.1	96.6±2.5
AML-7	ND		
AML-8	28.8±1.0	27.7±2.1	54.4±2.1

a) Each number is the amount (pmol) of ara-CTP formed/10^6 cells during incubation for 6 h with [^{3}H]ara-C. ND: Not determined.

test for paired samples) demonstrated that colony formation was inhibited significantly in IL-3-pretreated samples only.

Enhancement of ara-CTP formation in AML blasts treated with IL-3 In order to study the mechanism of increased sensitivity to ara-C of IL-3-stimulated AML blasts, we measured the formation of ara-CTP, the active form of ara-C, in AML blasts (Table III). The pretreatment of the cells with IL-1 for 72 h did not significantly increase the amount of intracellular ara-CTP formation in any of the patients studied. In contrast, the pretreatment of the cells with IL-3 for 3 days followed by incubation with ara-C for 6 h significantly increased the ara-CTP formation in the cells in all of the patients studied.

DISCUSSION

We have shown in the present study that the sensitivity of leukemic cells to ara-C can be increased by IL-3 treatment of the cells and that enhanced sensitivity is attributed to the increased intracellular accumulation of ara-CTP.

Although ara-C is the single most effective drug for acute myeloid leukemia and myeloid crisis of chronic myeloid leukemia, many leukemic cells escape its cytotoxic effects. Agents that stimulate myeloid blasts into active cycles could make S phase-specific agents such as ara-C more effective. One such agent is IL-3 or granulocyte-macrophage colony-stimulating factor (GM-CSF). The two growth factors, i.e., IL-1 and IL-3, were used to stimulate the proliferation of leukemic cells in this study. The proliferative response of AML blasts, as shown in Table I, demonstrates that IL-3 is a more potent inducer of proliferation of AML cells than IL-1. In cases 2, 3, 7 and 8, stimulation was obtained with IL-3 only.

The effect of ara-C on cells preincubated with growth factors and control cells was evaluated by enumerating

the number of residual leukemic chronogenic cells growing in semisolid media. Our results demonstrated that ara-C produced greater inhibition of proliferation of IL-3-treated cells than of control cells. It seems reasonable to assume that this effect is derived from the increased recruitment of the quiescent blast cells into the cell cycle. The increased recruitment of the cells into S-phase would lead to increased intracellular ara-CTP accumulation, because the cells in S-phase contain an increased level of deoxycytidine kinase.[23] Vierwinden and coworkers have reported that in the fraction of AML blasts enriched with cells in late G_1 or S-phase of the cell cycle, ara-C-phosphorylating activity is 1.5–8 times higher compared to the fractions with the lowest activity.[24]

Tafuri and Andreeff[25] recently reported that cytokines (GM-CSF, G-CSF and IL-3) could recruit myeloid blast cells *in vitro* and that this effect could enhance chemotherapeutic cytotoxicity. In their report, IL-3 was the most effective cytokine in recruitment induction and therefore this cytokine was most effective in the enhancement of cytotoxicity when used in combination with ara-C. Lista *et al.*[26] reported similar results using chronogenic assays in semisolid medium, comparing the activity of ara-C in the presence or absence of IL-3. Subsequently, Cannistra *et al.*[27] evaluated the activity of GM-CSF in the presence of ara-C and hydroxyurea and found a significant increase in the cytoreduction of chronogenic cells. The results of our study described here are consistent with these reports. However, the increased formation of ara-CTP in cells pretreated with IL-3 has not been reported by other authors.

With clinical trials under way, it is important to predict which patients will benefit from this cytokinetic approach and in which patients the induced increase in the leukemic cell mass will not be followed by increased cytoreduction. It also becomes important to establish the optimal dose schedule for cytokines and chemotherapy to maximize recruitment from G_0 and to optimize leukemic cell kill. In our present *in vitro* studies, we have used 48-h cytokine pretreatment to obtain the maximal increase in [^{3}H]TdR incorporation, but the *in vivo* responses may be different.

In conclusion, we have reported a novel mechanism for the increased sensitivity of leukemic cells pretreated with IL-3, i.e., enhanced accumulation of ara-CTP in the cells.

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