Cancer Chemother Pharmacol (1989) 23: 87–94

Cancer Chemotherapy and Pharmacology

In vitro evidence for dose-dependent cytotoxicity as the predominant effect of low dose Ara-C on human leukemic and normal marrow cells* **

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Summary. To determine whether cytosine arabinoside (Ara-C) has a differentiating effect in vitro, marrow cells from nine patients with acute non-lymphocytic leukemia or myelodysplastic syndrome and eight non-leukemic controls were exposed to drug concentrations comparable to those achieved in vivo with low-dose Ara-C therapy. In soft agar cultures, the predominant effect of Ara-C at concentrations between 10^{-8} *M* and 10^{-6} *M* was cytotoxicity with a dose-dependent decrement in Colony Forming Unit of the granulocyte and monocyte lineage (CFUg/m) at 14 days. Growth in liquid cultures containing Giant Cell Tumor(GCT)-conditioned media without Ara-C resulted in a significant increment in the recovery of mature cells at day 10 from the non-leukemic cultures ($P = 0.03$), while only a minor increase was found in the leukemic cultures ($P = 0.09$). All liquid cultures exposed to $\geq 10^{-9}$ *M* Ara-C showed a marked reduction in the immature proliferating cell pool, with a concomitant increase in the percentage of mature non-dividing cells at 10 days. However, the absolute number of differentiated cells recovered remained constant or decreased in all non-leukemic and eight of nine leukemic cultures, compared with cultures without Ara-C. Enhanced recovery of differentiated cells was also never observed in any culture exposed to the relatively non-toxic 10^{-9} *M* Ara-C. These in vitro findings support clinical observations suggesting that cytotoxicity rather than differentiation is the major mechanism involved in the therapeutic effect of low-dose Ara-C in acute leukemia and myelodysplasia.

Introduction

Cytosine arabinoside (Ara-C) administered in low doses of 10 to 20 mg/m^2 daily for 2–3 weeks has been reported to induce clinical remission in patients with acute leukemia and myelodysplastic syndrome [3, 7, 17, 18, 20, 29, 30, 34, 39, 41, 42]. Initially it was suggested by some investigators that these remissions were due to induction of cellular differentiation of the leukemic blasts by Ara-C [3, 7, 17, 18, 29]. However, in subsequent clinical trials, cytopenia and severe marrow hypoplasia were frequently observed [20, 30, 33, 39, 42], indicating that low-dose Ara-C was exerting a cytotoxic effect. In this in vitro study we examined the cytotoxic and differentiation effects of Ara-C on freshly isolated marrow cells from nine patients with acute leukemia or myelodysplasia and eight control subjects without myeloid abnormalities. Marrow cells were studied in both soft agar colony formation and short-term liquid cultures at Ara-C concentrations that are achieved in vivo during low-dose therapy. The predominant effect was cytotoxicity, with little evidence for induction of cellular differentiation.

Materials and methods

Soft agar culture experiments. Bone marrow cells from nine previously untreated patients with acute non-lymphocytic leukemia (ANLL) or myelodysplastic syndrome (MDS) and eight non-leukemic patients were cultured. The patients were allocated to FAB categories as follows: M1 (1), M2 (1), M4 (3), RAEB-T (2), and RAEB (2) (Table 1). The diagnoses of the non-leukemic patients included anemia resulting from chronic disease (4), idiopathic thrombocytopenia (2), stage IIA Hodgkin's disease (1), and pure red cell aplasia (1). The non-leukemic patients had never received any cytotoxic therapy and had normal peripheral white blood cell counts and normal myelopoiesis on examination of the bone marrow morphology.

Table 1. Patient characteristics

Patient no.	FAB	WBC per μl	CBC Hct %	Platelets per μl	Bone marrow % blasts	Karyotype
1	M1	35K	17	108K	96%	Normal
2	M2	115K	27	18K	50%	+8
3	M4	10K	29	70K	57%	ND*
4	M4	19K	30	64K	83%	Normal
5	M4	9K	33	101K	72%	ND
6	RAEB-T	2K	33	55K	25%	ND
7	RAEB-T	2K	28	52K	24%	+8
8	RAEB	3K	18	23K	8%	Normal
9	RAEB	8K	33	15K	14%	−Y

* ND = not done

* Supported by NIH Grant 2 S07 RR05359-23 and the Veterans Administration Research Service

** Presented in part at the American Society of Clinical Oncology meeting in Los Angeles, CA, May 6, 1986

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Marrow mononuclear cells were isolated by Ficoll-Hypaque centrifugation and suspended at a concentration of 2×10^5/ml in McCoy's medium containing 10% fetal calf serum, 0.3% agar and varying concentrations of Ara-C (0, 10^{-9} to 10^{-6} *M*). In each culture dish, 1 ml cell suspension was plated over an underlayer of McCoy's medium containing 0.5% agar (Difco), 10% fetal calf serum, and 15% GCT-conditioned medium which is a colony stimulating factor derived from a human Giant Cell Tumor culture established from a fibrous histiocytoma (Gibco). Triplicate cultures were incubated at 37° C in 7.5% CO_2. On day 14, Colony Forming Units of the granulocyte and monocyte lineage (CFUg/m aggregates containing ≥ 25 cells) and clusters (<25 cells) were scored with an inverted microscope. The entire top agar layer of each culture dish was then removed and stained with Wright-Giemsa for light-microscopic examination [35].

Liquid culture experiments. Aliquots of mononuclear cells from bone marrow aspirates of the ANLL/MDS patients described above and four non-leukemic patients were also placed in liquid culture. The four controls included one patient with iron deficiency anemia, two with anemia resulting from chronic desease, and a paid normal donor. Aliquots of 1×10^6 mononuclear cells were each suspended in a 5 ml polystyrene culture tube containing 1 ml McCoy's medium, 5% fetal calf serum, and 15% GCT-conditioned medium with Ara-C at 0, 10^{-9}, 10^{-8} or 10^{-7} *M*. Cells from three patients were also studied at 10^{-6} *M* Ara-C. Each marrow culture was incubated in triplicate at 37° C in 7.5% CO_2. On day 10, cells were pooled, counted, tested for viability by trypan blue exclusion, and then centrifuged on glass slides and stained with Wright-Giemsa. A 200-cell differential count was determined by light microscopy (× 1500).

Results

Effect of Ara-C on colony formation

In the non-leukemic control marrow soft agar cultures, incubation with Ara-C resulted in a dose-dependent reduction of CFUg/m colonies at day 14 (Fig. 1). There was complete inhibition of normal colony growth at 10^{-6} *M* and more than 50% inhibition in a majority of the cultures at 10^{-8} *M* and 10^{-7} *M*. At 10^{-9} *M*, only minimal reduction in colony formation or none at all was noted.

Colony growth was markedly abnormal in the soft agar cultures of the leukemic patients. Three patients (pts 1, 7, and 8) showed no growth in soft agar in the absence or presence of Ara-C. The cultures of the six remaining leukemic marrows uniformly demonstrated clusters without colony formation, and Ara-C exposure resulted in a further dose-dependent inhibition of cluster formation (Fig. 2). At 10^{-9} *M* the number and size of clusters were similar to those found in cultures grown without Ara-C.

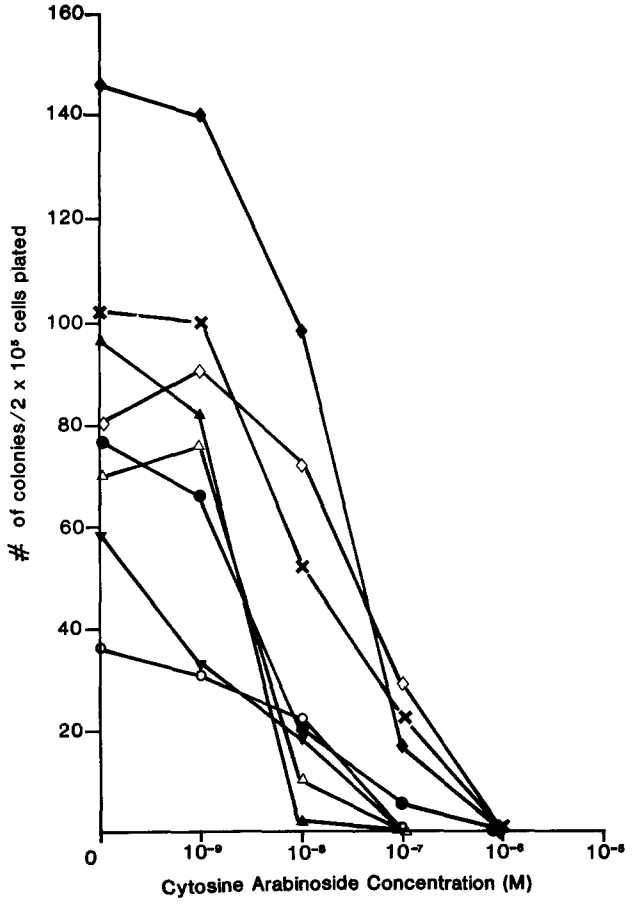

Fig. 1. Effect of Ara-C on colony formation at day 14 in soft agar cultures of marrow from eight control (non-leukemic) patients. There is a dose-dependent reduction in CFUg/m beginning at 10^{-8} *M*, with elimination of all colonies at 10^{-6} *M* Ara-C

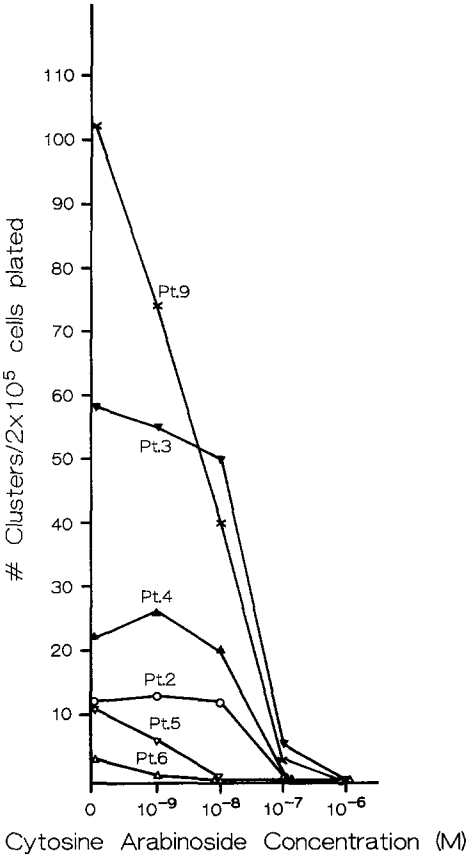

Fig. 2. Effect of Ara-C on cluster formation at day 14 in soft agar cultures of 6 ANLL/MDS marrows. There is a dose-dependent reduction of cluster formation, beginning in most instances at 10^{-8} *M*, with complete elimination of all clusters at 10^{-6} *M*. Three patients (not shown) had no cluster formation in the absence or presence of Ara-C

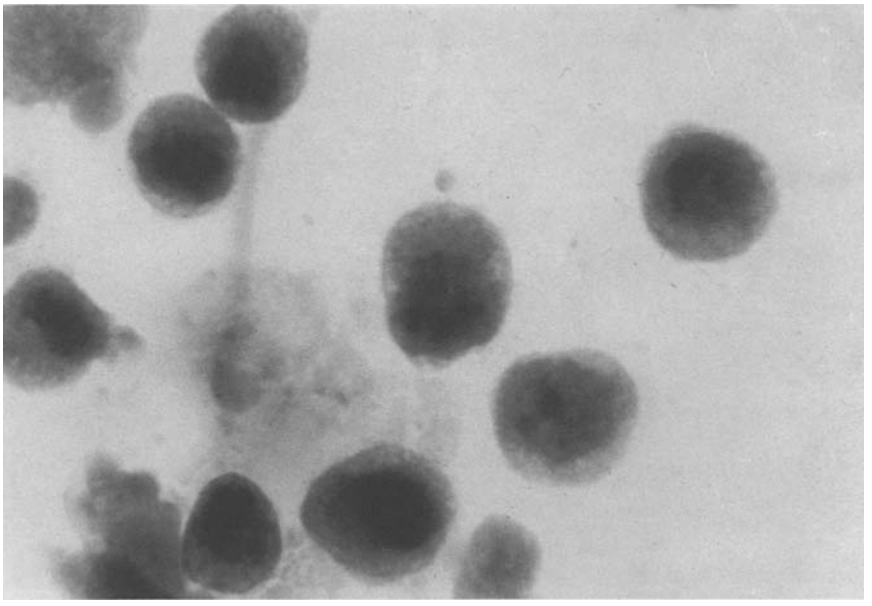

Fig. 3. Leukemic blasts (×200) from a 14-day cluster in a soft agar culture exposed to the non-cytotoxic concentration of 10^{-9} *M* Ara-C. There was no evidence of morphologic differentiation

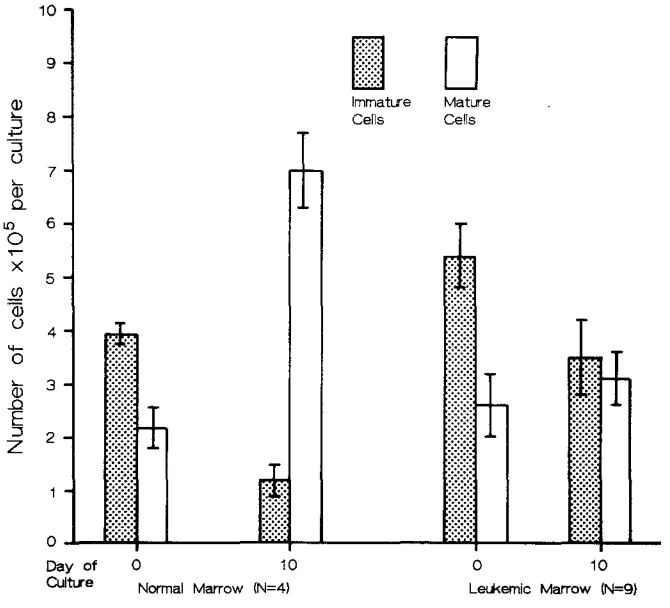

Fig. 4. Differentiation in the absence of Ara-C by normal and ANLL/MDS marrow cells in 10-day liquid cultures. Normal marrow cultures ($n=4$) showed a 70% reduction in the mean absolute number of immature cells with proliferating potential (blasts, promyelocytes, promonocytes, and myelocytes) and almost a three-fold increment in the non-dividing mature cells (metamyelocytes, bands, PMN, monocytes, and macrophages) at 10 days. While there was a 35% reduction in the mean of immature cells recovered at the end of the 10-day ANLL/MDS liquid cultures ($n=9$), there was very little increment in the recovery of mature cells (Wilcoxon signed-rank test, $P=0.09$) to suggest any significant degree of maturation of leukemic cells in the absence of Ara-C

There was no evidence of colony formation or differentiation to more mature cells within the clusters at this nontoxic dose of Ara-C (Fig. 3). At Ara-C concentrations greater than 10^{-8} *M* leukemic clusters were almost entirely eliminated, and only cellular debris was noted in the stained preparations.

Effect of Ara-C on liquid culture of marrow cells

Over the 10-day culture period non-leukemic marrow cultures grown in the absence of Ara-C showed a 70% reduction in the mean absolute number of immature cells with proliferating potential (myeloblasts, monoblasts, promyelocytes, promonocytes, and myelocytes). There was a concomitant three-fold increment in the mean absolute number of differentiated or "mature" cells (monocytes, macrophages, metamyelocytes, bands, and granulocytes) recovered after 10 days compared with the original inoculum (Wilcoxon signed-rank test, $P = 0.03$; Figs. 4, 5A). This finding indicated that normal immature cells could differentiate in this culture system in the absence of Ara-C. Exposure to concentrations from 10^{-9} to 10^{-7} *M* Ara-C led to a dose-dependent reduction in the recovery of total viable cells as well as in the percentage and absolute number of immature cells at the end of the 10-day culture period (Fig. 6) While the percentage of mature cells appeared to increase with higher concentrations of Ara-C, there was no significant enhancement in the recovery of the absolute number of cells that differentiated into mature macrophages and granulocytes. In fact, at more than 10^{-9} *M* there was also a dose-dependent reduction in the mature cell pool.

In contrast to normal marrow cells, the ANLL/MDS immature cells differentiated poorly into mature cells in liquid culture (Figs. 4, 5B). In the absence of Ara-C, there was a 35% reduction in the mean absolute number of immature cells, with only a 1.2-fold increment in the mean absolute number of mature cells recovered at the end of the 10-day culture period (Wilcoxon signed-rank test, $P = 0.09$; Fig. 4). There was also a marked variability in the recovery of viable cells at day 10, which ranged from 35% to 120% (median, 69%) in the absence of Ara-C (Fig. 7A).

Exposure to Ara-C resulted in a dose-dependent cytotoxicity in a majority of the ANLL/MDS cultures which was most clearly manifested at concentrations greater than 10^{-9} *M*. Cultures from five patients (pts 1, 3, 4, 5, and 6)

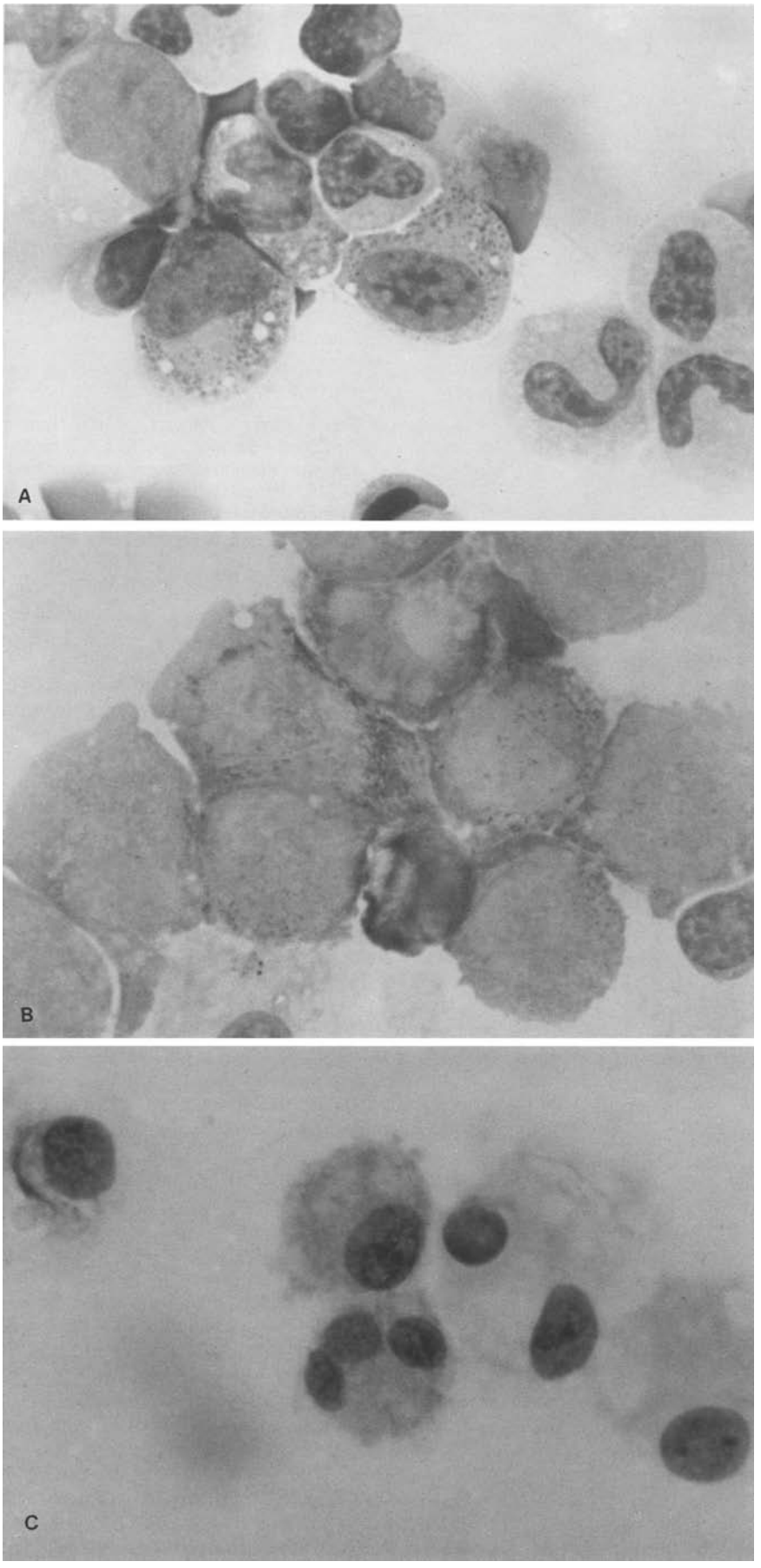

Fig. 5A–C. Cytocentrifuge preparations of normal and leukemic marrow cells from liquid cultures at day 10 (×1500) with and without Ara-C. **A** Normal marrow, no Ara-C, cellular differentiation; **B** leukemic marrow, no Ara-C, lack of cellular differentiation; **C** predominance of mature macrophages in leukemic marrow exposed to a cytotoxic concentration (10^{-7} *M*) of Ara-C

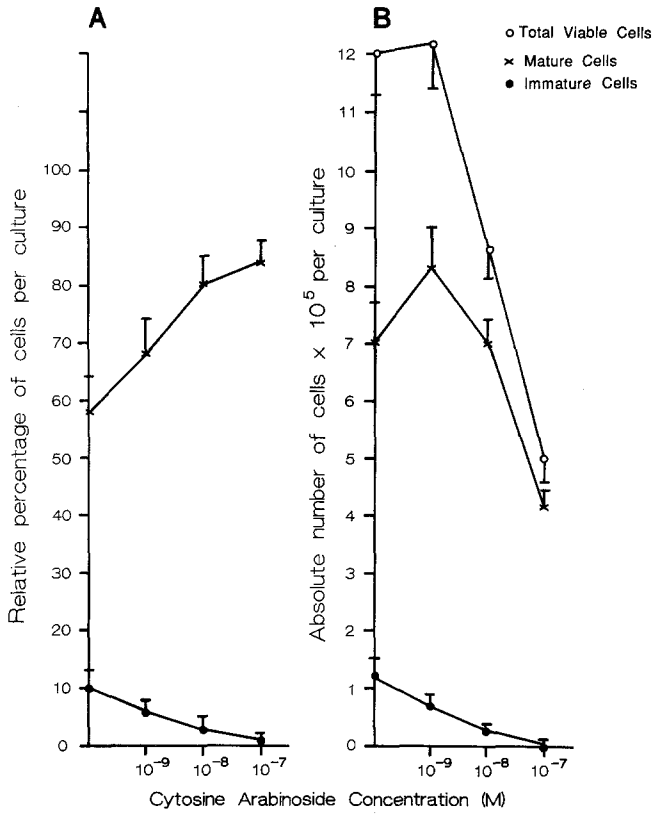

Fig. 6A, B. Effect of Ara-C on the mean total number of viable cells and the distribution between the immature and mature cell pools recovered at day 10 from liquid cultures of normal marrow ($n=4$). **A** Dose-dependent reduction in the mean relative percentage of immature cells with a concomitant increment in the relative percentage of mature cells. **B** Dose-dependent reduction of total viable cells, proliferating cells (blasts, promyelocytes, promonocytes, and myelocytes), and cells that matured into macrophages and granulocytes in liquid cultures

had greater than 60% reduction (range, 61%–78%) in the total cell recovery at 10^{-7} *M*, which was accompanied by a parallel decrease in the absolute number of immature cells. Of the four patients (pts 2, 7, 8, and 9) who had smaller reductions in cell recovery, all but one had a relatively low percentage of immature cells (Fig. 7B).

In the majority of the ANLL/MDS cultures, Ara-C treatment resulted in little change in the recovery of mature cells (Fig. 7C). At 10^{-7} *M*, three patients had moderate reductions (40%–50%) similar to those in the normal controls. In one patient (pt 3) the recovery of mature cells was enhanced 2.5-fold at the higher concentrations of Ara-C (10^{-8} and 10^{-7} *M*), despite a marked reduction in total cell recovery. At the relatively non-toxic concentration of 10^{-9} *M*, no enhancement of recovery of mature cells by Ara-C was noted in any patient sample compared with cultures not exposed to the drug.

Figure 8 summarizes the dose-response effect of Ara-C on the cellular composition of the ANLL/MDS cell cultures and the absolute cell recoveries. The rise in the percentage of mature cells (Figs. 5C, 8A) is seen to be a relative increase resulting from the absolute reduction in the immature cell pool. The mean absolute number of mature cells was not enhanced by any concentrations of Ara-C exposure (Fig. 8B).

Discussion

The concept that the biologic defect in myelodysplasia and acute leukemia involves a progressive maturation arrest of genetically transformed hematopoietic stem cells has led to the use of differentiation-inducing agents as a novel therapeutic approach in these disorders. Sachs reported the induction of cellular differentiation of murine leukemic blasts by a variety of compounds including steroids and low concentrations of chemotherapeutic agents such as cytosine arabinoside, daunomycin, hydroxyurea, methotrexate, and mitomycin C [26, 32]. Other biological and chemical agents, including interferon, retinoids, vitamin D, phorbol ester, DMSO, and *N*-methylformamide, have also been reported to induce cellular differentiation in human leukemic cell lines, particularly the HL-60 human acute promyelocytic leukemia cell line [1, 4, 6, 11, 14, 22, 31, 38].

In vivo induction of cellular differentiation of leukemic blasts was hypothesized in several early reports of low dose Ara-C therapy of acute leukemia, because bone marrow aplasia had not been observed prior to clinical remission [3, 18]. In subsequent trials with larger numbers of patients, however, severe cytopenia and bone marrow aplasia have frequently been documented [9, 20, 30, 33, 39, 42], suggesting that cytotoxicity rather than differentiation was the major mechanism. Severe bone marrow suppression with fatal sepsis has also been reported [30, 33, 39].

In our experiments with both CFUg/m agar and short-term liquid cultures, we have consistently demonstrated a dose-dependent reduction of colony formation and viability of leukemic blasts with Ara-C. At concentrations higher than 10^{-9} *M*, Ara-C was consistently cytotoxic to the proliferating pool of cells in both control and leukemic marrow cultures, and aborted colony or cluster formation in soft-agar CFUg/m cultures.

We established that 10^{-9} *M* Ara-C was relatively non-toxic in both culture systems, but we could find no evidence of enhancement in the recovery of mature cells to indicate induction of cellular differentiation at this concentration. The relative increase in the percentage of mature cells recovered in all the liquid cultures exposed to toxic concentrations of Ara-C could be interpreted as a differentiation effect of the drug. However, determination of the absolute number of cells recovered after Ara-C exposure clearly demonstrated that the relative increase in mature cells in eight of nine instances was due to a marked reduction in immature cells in association with a stable or reduced concentration of mature cells. The mature cells then represented a residual cohort of non-dividing Ara-C-resistant cells remaining after the cytotoxic elimination of the proliferating immature cell pool by Ara-C. In one patient an increase in the recovery of mature cells was observed at cytotoxic concentrations (10^{-8} and 10^{-7} *M*), but not at the non-toxic 10^{-9} *M* Ara-C. While it is possible that both cytotoxicity and differentiation occurred in this patient's cultures, the marked reduction in the immature cells in the face of a small rise in mature cells favors cytotoxicity as the major effect.

In this study, we were also able to demonstrate quantitatively a significant in vitro maturation of normal marrow cells in the absence of Ara-C in the liquid culture system with GCT-conditioned media. A minor in vitro maturation of the leukemic marrow cells was also found in the same culture system. That leukemic blasts may retain a limited

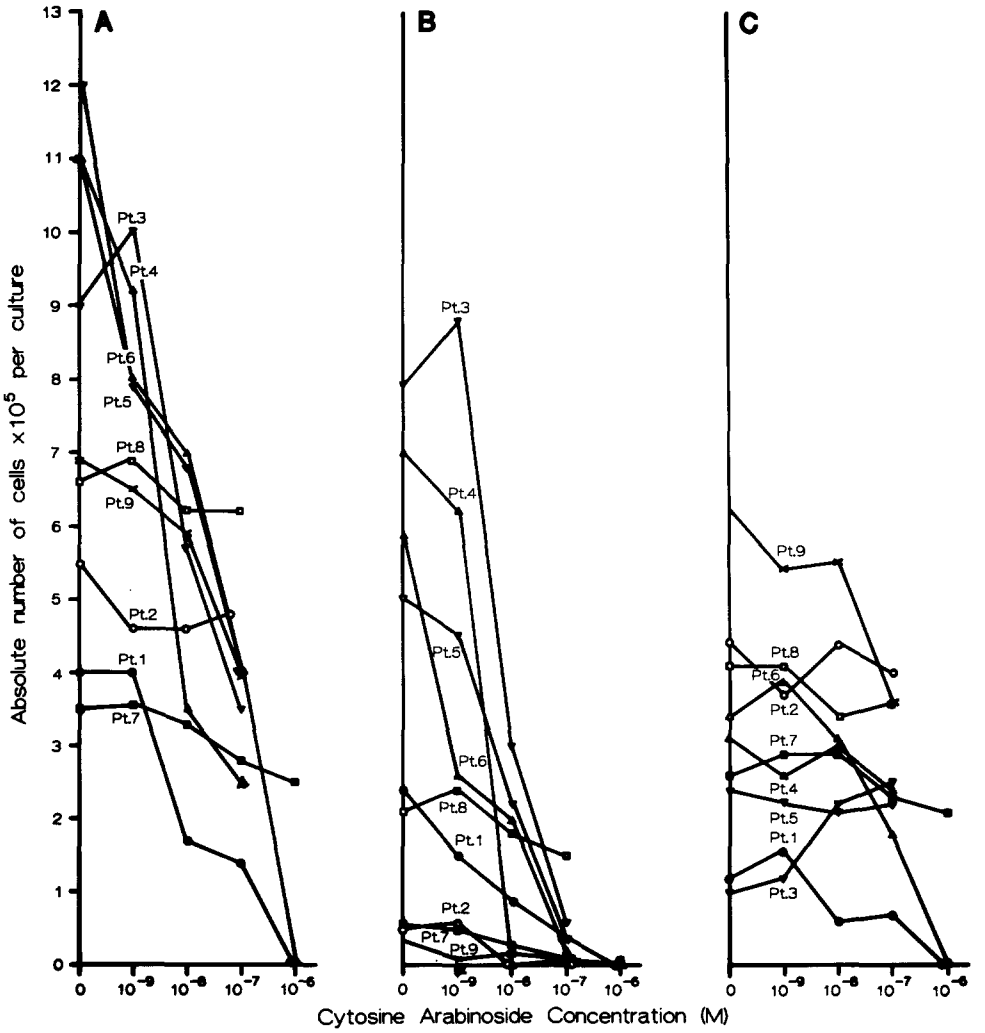

Fig. 7 A–C. Dose-response curves of the effect of Ara-C on the recovery on day 10 of total viable cells (**A**), immature cells of proliferating potential (**B**), and mature non-dividing cells (**C**) from liquid cultures of marrow from five patients with acute leukemia (patients 1–5) and four with myelodysplastic syndrome (patients 6–9). There was a dose-dependent cytoreductive effect of Ara-C on the total and immature leukemic cells, while the number of mature cells remained constant or decreased slightly with increasing concentrations of Ara-C in all but one patient. In patient 3, the absolute number of mature cells increased two-fold at 10^{-8} and 10^{-7} *M* Ara-C in the face of a marked reduction in the total number of viable cells

capacity to differentiate is supported by previous in vivo observations, such as the presence of Auer rods in mature granulocytes [2, 5] and the more recent demonstrations of monoclonality of fully differentiated cells in leukemic patients by G6PD and phosphoglucomutase-1 isoenzyme studies [13, 21, 40] and recombinant DNA polymorphism techniques [28]. As no specific tumor markers were used in these experiments we cannot distinguish whether the mature cells recovered from leukemic marrow cultures were derived from a subclone of malignant cells which retained some differentiating capacity, or from a small residue of normal cells. Nevertheless, from whatever stemline the mature cells in the ANLL/MDS cultures were derived, Ara-C exposure generally did not result in an absolute increase in the differentiated, mature cell pool.

Few in vitro studies have been performed on the differentiating effect of Ara-C on human leukemic cells in various culture systems, and those performed have yielded conflicting results [10, 12, 15, 16, 19, 20, 24, 25, 27, 37]. Ishikura et al. reported a quantitative enhancement in the recovery of mature granulocytes bearing Auer rods in short-term liquid cultures from two of eight patients whose leukemic cells were exposed to 10^{-6} to 10^{-7} *M* Ara-C without reduction in cell viability [20]. Griffin et al. reported an increase in the absolute number of mature granulocytes in cultures of HL-60 cell exposed to 10^{-8} to 10^{-6} *M* Ara-C, which were cytostatic concentrations as determined by the inhibition of H^3-thymidine incorporation [15].

In contrast to the above results, Leyden et al. [25] demonstrated a dose-dependent reduction in cell viability in HL-60 cells as well as in freshly isolated leukemic cells when these were exposed to 10^{-9} *M* to 10^{-3} *M* Ara-C. In another study, cultures of freshly isolated leukemic cells from two patients with acute leukemia exposed to a single dose of Ara-C showed an 11-fold and a 3-fold increase in the ratio of differentiated myeloid cells to leukemic blasts, which was interpreted as indicating cellular differentiation [27]. The absolute number of mature granulocytes or macrophages recovered was, however, less than twice that from the control cultures.

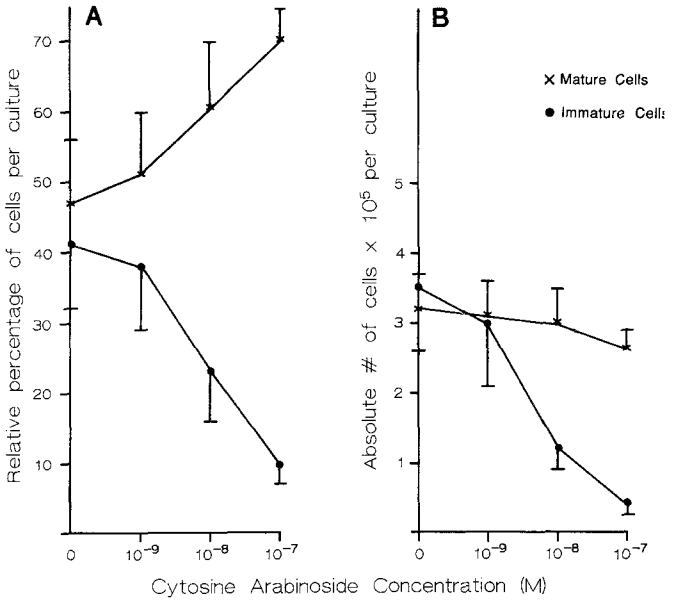

Fig. 8. A Dose-dependent effect of Ara-C on the mean percentage of immature and mature cell pools scored on 200 cell differential counts of cells recovered at day 10 from the leukemic and myelodysplastic marrow cultures ($n=9$). **B** Corresponding mean absolute number of immature and mature cells recovered at day 10

We believe that for a drug to be considered a differentiation agent in vitro or in vivo, drug exposure must result in an absolute increase in the recovery of mature cells. In the present study, we failed to detect any clear enhancement of mature cell recovery by Ara-C at the non-cytotoxic concentration. Furthermore, at higher levels of Ara-C, we demonstrated that the high percentages of mature cells recovered generally represented only relative increases attributable to elimination of the proliferating pool by the cytotoxic action of Ara-C. Presumably the mature differentiated cells found at the end of the culture represented either residual normal postmitotic hematopoietic cells or the progeny of a leukemic subclone with some ability to differentiate, neither of which would be sensitive to the antiproliferative effect of Ara-C.

Plasma levels of Ara-C in patients treated with low-dose Ara-C have been reported to range from 10^{-8} *M* to 10^{-6} *M* [20, 23, 26]. These levels coincide with the concentrations clearly found to be cytotoxic in Leyden's studies as well as our own. It is conceivable that by continuously exposing patients to low doses of this S-phase-specific agent over a 2- to 3-week period, fractions of cycling leukemic cells are eliminated daily, leaving behind the noncycling mature progeny cells. Bone marrow evaluated at an early phase of the treatment course could therefore give the impression that leukemic blasts had undergone differentiation. However, similar to the in vitro phenomenon reported in this study, bone marrow examinations at more frequent intervals have revealed that the reduction in leukemic blasts is frequently accompanied by a reduction in the overall marrow cellularity and ultimately marrow aplasia in the majority of cases [8, 9, 20, 30, 33, 39, 40]. The high incidence of severe myelosuppression suggests that this form of therapy most probably induces remission through a progressive cytoreduction mechanism, rather than by inducing cellular differentiation of the leukemic clone. However, since the adverse effects of Ara-C on normal extramedullary tissues have been limited [8], this modality may continue to have a therapeutic advantage over conventional antileukemic regimens in certain patients despite the lack of evidence for differentiation induction.

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Received March 28, 1988/Accepted June 24, 1988