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Low Dose Cytosine-Arabinoside Has Only Minimal Differentiation Inducing Capacity in HL60 Cells

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Abstract—*Cytosine-arabinoside (ARA-C) in low doses induces complete remissions in myelodysplastic syndromes and acute leukemia. Evidence is accumulating that these remissions are not reached by differentiation induction but through cytotoxicity. In HL60 cells differentiation was measured by a comprehensive panel of quantitative and qualitative markers of maturation. After exposure to ARA-C (10^{-7} M) for 4 days HL60 cells did not mature morphologically. Cell volume increased. The increase in esterase activity was small and did not reach the amount measured in normal monocytes. There was no significant difference in latex phagocytosis and NBT reduction between cultures with and without ARA-C. HL60 cells were arrested in S-phase and clonogenic capacity persisted. The observed changes after exposure to ARA-C seem to be caused by impeded cell division while synthesis of protein continues. We conclude that ARA-C in low dose exerts its effect by halting proliferation through cytotoxic effects and not by differentiation induction.*

INTRODUCTION

CURRENT TREATMENT regimens in acute myeloid leukemia (AML) are aimed at eradicating the malignant cell clone by their cytotoxic action. Cytosine-arabinoside (ARA-C) is a nucleoside analog which remains the most effective drug in the treatment of AML [1, 2]. At low concentrations of 10^{-8} to $^{10-6}$ M ARA-C has been shown to promote terminal differentiation of HL60 cells *in vitro* [3]. On the contrary, other authors found no sign of differentiation when HL60 cells were exposed to low dose ARA-C [4]. The ability of low dose ARA-C to induce human leukemic cells to undergo *in vivo* differentiation has been tested using ARA-C treatment regimens that achieve serum concentrations (10^{-8} M) comparable to the ARA-C concentration shown to be capable of inducing murine leukemic cell differentiation [5]. In several recent studies complete remissions were described in patients with AML or myelodysplastic syndromes (MDS) [6–10]. It is still controversial whether these effects are due to cytotoxic or differentiation-inducing actions of the drug. Evidence is beginning to accumulate that remission of human AML with low dose ARA-C is reached by suppression or destruction of the leukemic cells [11–13].

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The variance in data from several *in vitro* studies is probably due to the use of a limited number of differentiation markers in these experiments. In this study we describe the influence of low dose ARA-C on HL60 cells. The differentiation was measured by a comprehensive panel of quantitative and qualitative markers of maturation.

MATERIALS AND METHODS

Cells and culture

The HL60 promyelocytic leukemia cell line was maintained in suspension culture in α-MEM (Gibco, Grand Island, New York) containing 20% fetal calf serum (FCS) (Flow Lab, Rockvill, Maryland), heat inactivated at 56°C for 30 min, with penicillin and streptomycin. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air [14]. Experiments were performed by culturing HL60 cells at 0.5×10^6 cells/ml in logarithmic growth phase in the presence of 10^{-7} M ARA-C for 4 days. Dose escalation studies revealed that this is the optimal ARA-C concentration in this test system (Fig. 1). At a lower dose hardly any effect was obtained while a higher dose gave unacceptable cell kill. Each experiment was accompanied by a control culture of HL60 cells under the same conditions without exposure to ARA-C. Viability was assessed by trypan blue exclusion. Cell counts were determined with a Coulter counter. Nine separate experiments were performed unless otherwise

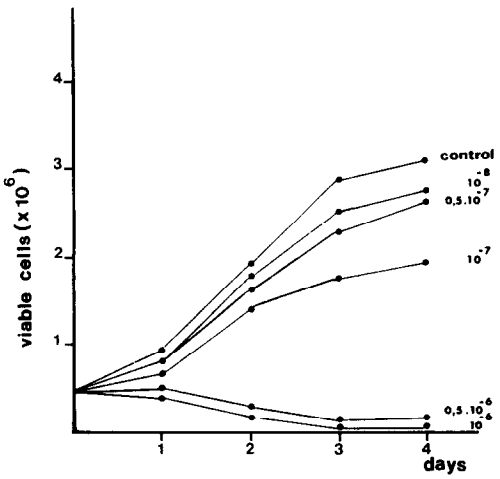

Fig. 1. Survival of HL60 cells after exposure to ARA-C in different concentrations.

stated. Tests were only performed when the cell viability was above 90%.

Cytochemistry

After 96 h in culture, cells were collected on slides using a Shandon cytocentrifuge. Cytospin slide preparations were stained according to May Grünwald Giemsa for assessment of morphology. The cells were also stained with Sudan Black, naphthol AS-D chloroacetate esterase and alpha naphthyl acetate esterase stains as previously described [15]. Differential counting of at least 100 cells was performed by two members of our study group independently. In each cytochemical stain 100 cells were judged for positivity by at least two of the investigators.

Intracellular enzyme activity

Cells were lysed by freezing them three times in liquid nitrogen. Cell fragments were spun down at 7000 ***g*** for 4 min and the collected supernatant was used for enzyme determination. Techniques included minor variations, as described in detail elsewhere [16], of standard methods for the estimation of myeloperoxidase [17], acid esterase using both alpha naphthyl acetate and alpha naphthyl butyrate as a substrate [18], and lactate dehydrogenase [19]. The amount of enzyme is expressed as units per 10^6 cells and as units per mg protein. The amount of protein in the cell lysate was determined according to the method of Lowry *et al.* [20].

Phagocytic assay

Phagocytic capacity was determined from the uptake of latex particles (Sigma, St. Louis, U.S.A.; 1.091 particle diameter). Cells were washed and resuspended in Hanks medium supplemented with 0.1% bovine serum albumin (BSA) containing latex particles. The final cell concentration was 0.5×10^6/ml. After 1 h incubation at 37°C in a water bath, cells were washed three times and resuspended in Hanks medium with 0.1% BSA. Cytospin preparations were made. Cells ingesting more than five particles were defined as phagocytosis positive.

Nitro blue tetrazolium (NBT) reduction

The reduction of NBT was measured quantitatively. One hundred microliters of cells (0.5×10^6/ml) in α-MEM containing 20% FCS were incubated at 37°C for 15 min with 100 μl NBT (0.5 mg/ml) and 2 μl 10 μM freshly diluted 12-*O*-tetradecanoyl phorbol 13-acetate (TPA). A native preparation was made in which the percentage of cells containing blue black deposits of formazan was determined by evaluating 200 cells.

Cell diameter

Cell diameters were measured in cell suspensions by means of an Elzone 80 XY Volume Counter (Particle, Data, U.S.A.), which measures and plots cell diameters according to the Coulter principle in 128 channels.

Pulse cytophotometry

HL60 cells were fixed in 70% ethanol, treated with RNase and pepsin and stained with ethidium bromide (Hoechst 33342). DNA histograms were obtained using the ICP 11 impulse cytophotometer (Phyne, F.R.G.) and percentages of G_1 cells, S-phase cells and G_2 + M cells were calculated as previously described [21].

Clonogenic survival of HL60 cells

After 96 h in culture 0.5×10^6 HL60 cells in 0.1 ml α-MEM were plated with methylcellulose (0.9%), 20% fetal calf serum (FCS), and 10% phytohemagglutinin leukocyte conditioned medium (PHA-LCM). After 7 days incubation in 5% CO_2 and 37°C, clusters containing more than eight cells and colonies containing more than 40 cells were counted. The plating efficiency of untreated cells in this system was between 20 and 30%.

Statistical methods

For comparison of the tests Student's *t* test was used.

RESULTS

Morphology

Cells grown under the influence of ARA-C for 4 days exhibited increased cytoplasmic volumes with a minimal decrease of granules. The nuclear structure became looser while nucleoli persisted. These changes were not considered to be signs of differentiation. The morphology of obviously differentiated cells deviated from normal human mature myeloid cells. It was difficult to classify these differentiated cells into either the granulocytic or monocytic pathway by morphological criteria (Figs. 2 and 3).

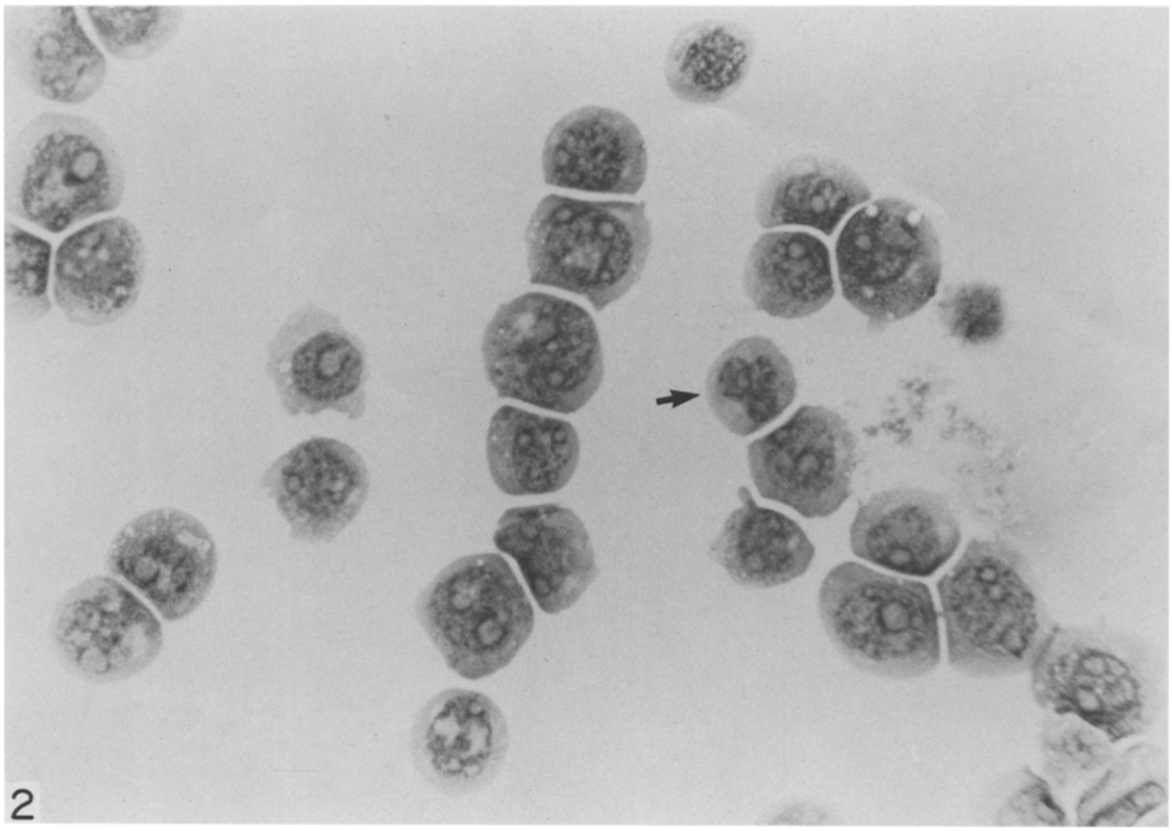

Fig. 2. HL60 cells unstimulated. The arrow shows one example of a differentiated cell.

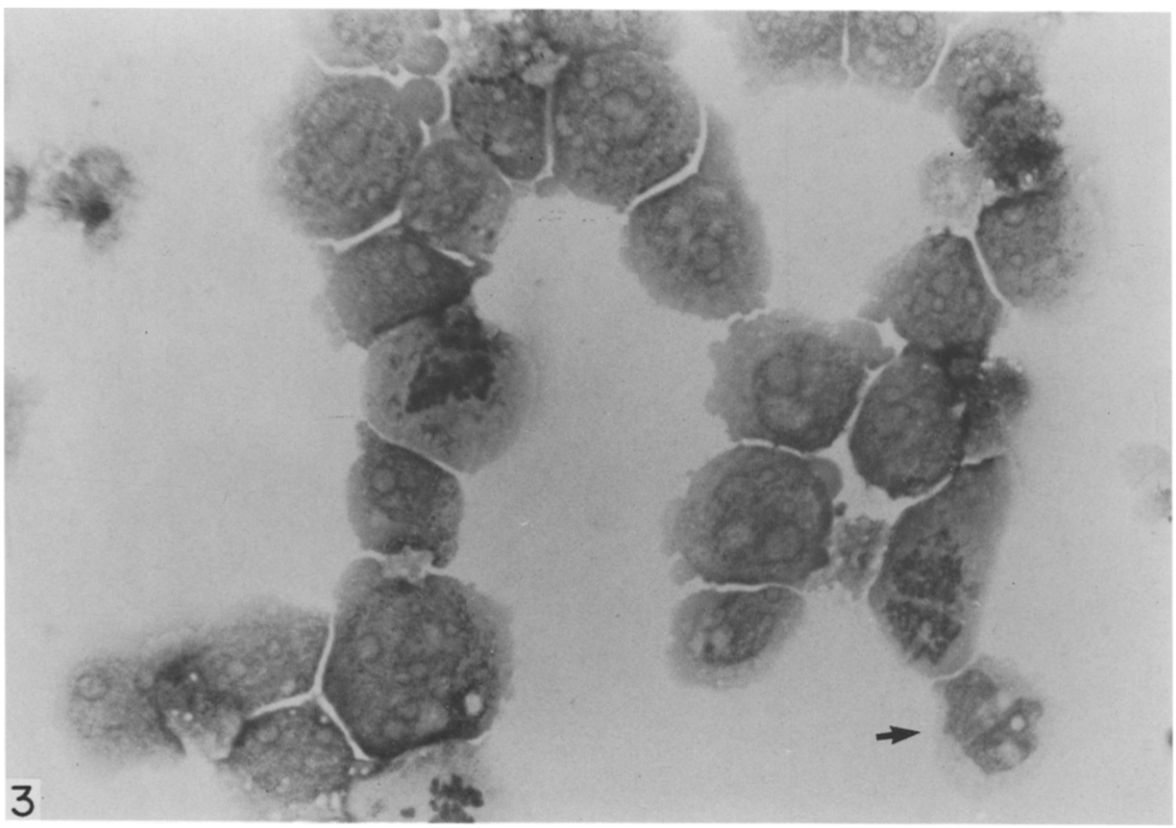

Fig. 3. HL60 cells after 4 days exposure to cytosine-arabinoside. The cells are increased in size. The arrow shows one example of a differentiated cells.

The percentage of markedly differentiated cells after exposure to ARA-C was 8.7 ± 5.2 (mean ± S.D.). The percentage of spontaneous differentiation in control experiments was 4.0 ± 2.5 (non-significant difference). With vitamin D3 used as a differentiation inducer in a simultaneous experiment differentiation in monocytic direction was reached in 80.2 ± 5.8% [16].

The staining for Sudan Black, chloroacetate esterase and non specific esterase did not reveal a significant difference between the HL60 cells exposed to ARA-C and control experiments (Fig. 4).

Cell count

Exposure to ARA-C inhibited cell proliferation; after 4 days the number of cells in control experiments was 3.2 ± 0.3 against 1.9 ± 0.2 in ARA-C treated cells ($P < 0.001$).

Intracellular enzyme activity

Total cell protein increased after exposure to ARA-C by 50% (Table 1). An increase in alpha naphthyl acetate esterase activity expressed as mU per mg protein as well as expressed in mU per 10^6 cells was reached (Table 1). The same is noticed by measuring the amount of alpha naphthyl butyrate esterase activity. This increase is more pronounced when the total esterase activity is expressed per 10^6 cells. When expressed as activity per mg protein the increase is less. Figures 5 and 6 show these values in comparison with the normal values for human moncytes. The amount of esterase activity in ARA-C treated HL60 cells is much less than in normal human monocytes. In simultaneous experiments with vitamin D3 the amount of esterase activity reached the value of normal monocytes [16]. LDH and myeloperoxidase activity expressed as units per mg protein were not significantly different from control experiments.

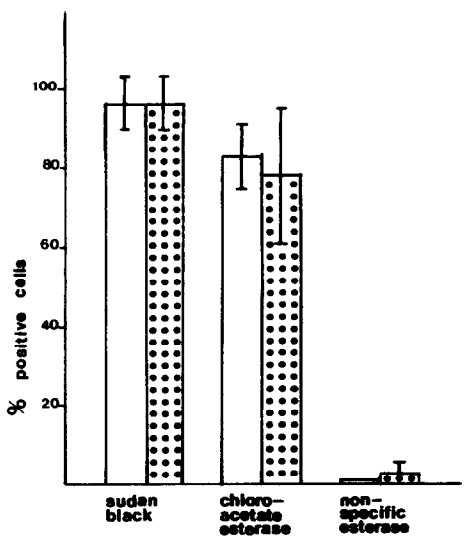

Fig. 4. Specific staining after 4 days exposure to ARA-C (open) and in control HL60 cells (shaded). Means ± S.D. were determined from nine data points.

Latex phagocytosis

Normal mature monocytic or granulocytic cells have the capacity to ingest foreign material. HL60 cells developed in a small percentage the ability to phagocytose. A mean of 27.8 ± 19.9% of the cells ingested five or more latex particles after culture with ARA-C. An average of 19.6 ± 13.5 of the cells phagocytosed particles in the absence of ARA-C. There was no statistically significant difference between cultures with and without ARA-C.

NBT reduction

Reduction of NBT is dependent on production of hydrogen peroxide and superoxide. Monocytes and granulocytes are able to reduce NBT in contrast to their less mature progenitors. A mean of 10.6 ± 3.2% of the HL60 cells cultured in the presence of ARA-C reduced NBT. An average of 8.9 ± 4.8% of the cells in control cultures reduced NBT. This represents non-statistically significant difference between the cultures with and without ARA-C.

Cell diameter

The cell diameter of non-stimulated HL60 cells amounted to 10.66 ± 0.24 μm (mean ± S.D.). After 4 days of exposure to ARA-C there was a statistically significant increase in cell diameter: mean 12.57 ± 0.23 μm ($P < 0.001$). In other experiments showing obvious maturation after exposure to vitamin D3 or DMSO, the cell diameter became significantly smaller.

Table 1. Intracellular enzyme activity after exposure of HL60 cells to ARA-C. The standard deviation was determined from nine data points

	Protein	Alpha naphthyl acetate esterase		Alpha naphthyl butyrate esterase	
	μg/10^6 cells	mU/10^6 cells	mU/mg prot.	mU/10^6 cells	mU/mg prot.
Control	61.8 ± 9.9	0.40 ± 0.22	6.28 ± 3.16	0.53 ± 0.20	8.53 ± 2.99
ARA-C	90.8 ± 10.8	1.28 ± 0.38	14.29 ± 4.5	1.07 ± 0.27	11.97 ± 3.58
P	<0.001	<0.001	<0.001	<0.001	n.s.

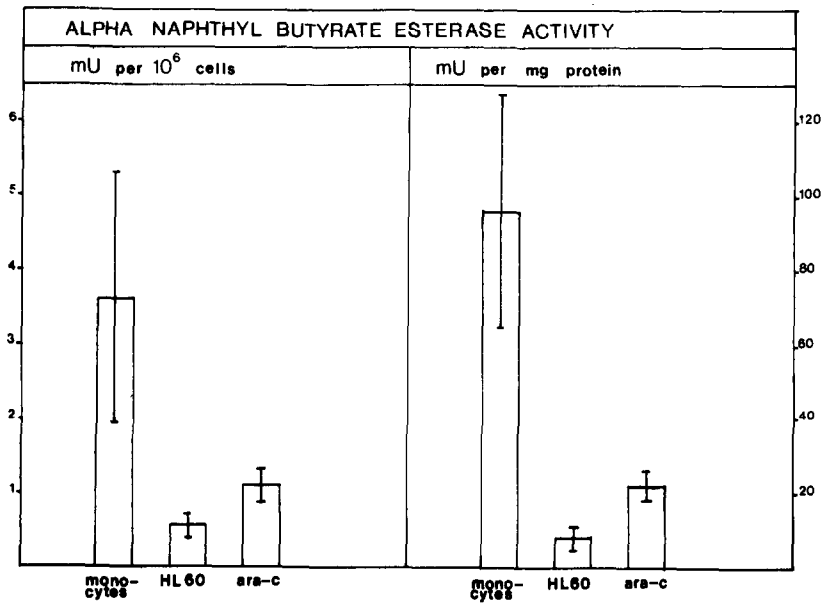

Fig. 5. Alpha naphthyl acetate esterase activity expressed as mU *per* 10^6 *cells and per* mg *protein in normal monocytes, control HL60 cells (HL60) and ARA-C treated HL60 cells (ARA-C). Means ± S.D. were determined from nine data points.*

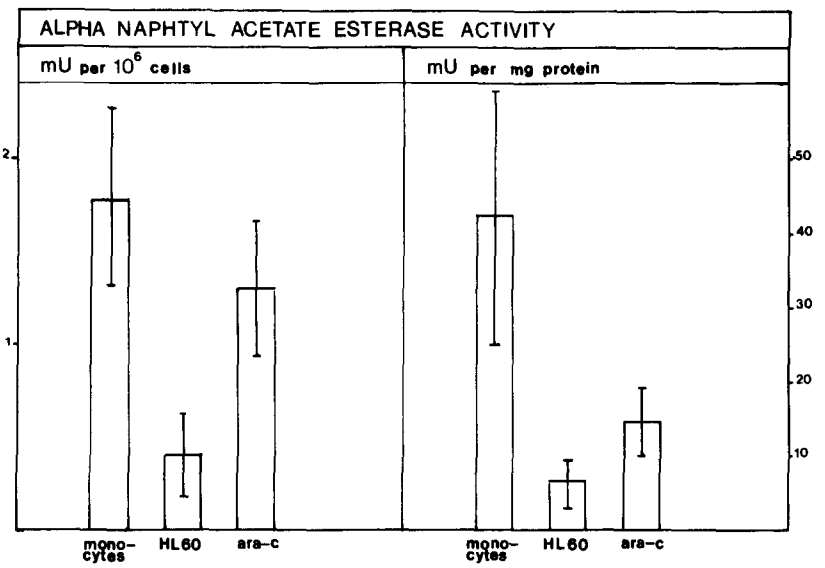

Fig. 6. Alpha naphthyl butyrate esterase activity expressed as mU *per* 10^6 *cells and per* mg *protein in normal monocytes, control HL60 cells (HL60) and ARA-C treated HL60 cells (ARA-C). Means ± S.D. were determined from nine data points.*

Pulse cytophotometry

A mean of 47.6 ± 4.9% of HL60 cells cultured in the presence of ARA-C was in S-phase, as opposed to 33.0 ± 5.7% of HL60 cells in control cultures. The unequal shape of the DNA histograms suggests an arrest of the cells exposed to ARA-C in early S-phase. The differences (see Table 2) are significant ($P < 0.001$).

Clonogenic survival

In two out of 10 experiments there was a nearly 100% decrease in clonogenic survival. In the other experiments the decrease ranged from 0 to 58% (Table 3).

DISCUSSION

Maturation induction of HL60 cells could not be demonstrated on morphological grounds after exposure to ARA-C. Only minor changes were observed in the May Grünwald Giemsa staining. No increase in NBT reduction was noticed. Cell diameter increased considerably whereas decrease of cell diameter could have been expected if differentiation had occurred. The increase of esterase

Table 2. Percentage of HL60 cells in G1 and S phase measured by pulse cytophotometry. The mean ± standard deviation was determined from nine data points

	G1 phase	S phase
Control	49.6 ± 4.1	33 ± 5.7
ARA-C	35.1 ± 4.8	47.6 ± 4.9
P	<0.001	<0.001

Table 3. Colonies (containing more than 40 cells) after exposure to ARA-C and in control cultures

HL60 Control	HL60 ARA-C
7.5×10^3	0.12×10^3
7.6×10^3	0
8.3×10^3	6.6×10^3
6.8×10^3	2.9×10^3
7.8×10^3	8.0×10^3
4.5×10^3	3.1×10^3
6.8×10^3	3.0×10^3
4.0×10^3	4.0×10^3
4.7×10^3	3.3×10^3
11.2×10^3	8.7×10^3

activity was small and even less when related to the increase in protein content. Esterase activity did not reach at all the amount measured in normal monocytes. The activity was not inhibited in control and ARA-C treated cells by natriumfluoride as could have been expected after differentiation in monocytic direction. The cells were arrested in S-phase, a phenomenon well known following the administration of ARA-C. When differentiation occurs most cells are found to be in G_1-phase. The results of the clonogenic assay are not uniform, in two out of 10 experiments a sharp decrease of clonogenic capacity was noticed, while it persisted in the other eight experiments. Persistance of clonogenic capacity is expected when maturation does not occur. Loss of clonogenic capacity, however, does not differentiate between maturation and cytotoxic action.

One has to conclude from this summing up of our results that differentiation does not occur to a significant extent when HL60 cells are exposed to ARA-C for 4 days. Impeded cell division while synthesis of protein continues is probably the underlying mechanism for the observed changes. This unbalanced cell growth was described before [22]. These results are at variance with other published data. Griffin *et al.* [3] showed terminal differentiation of HL60 cells exposed to ARA-C. Differentiation was only demonstrated with increase of surface expression of the myeloid antigen MY-4 and with the induction of non-specific esterase activity by cytochemical staining. An increase in the percentage of non-specific esterase positive cells was also noticed by Ross [22] after exposure of HL60 cells to ARA-C for 5 days. No other differentiation parameters were recorded. On the contrary, Boyd and Sullivan [23] showed minimal morphological changes in HL60 cells exposed to ARA-C. These changes seemed to be granulocytic in type. Leyden *et al.* [4] showed that, while ARA-C produced some monocytic differentiation in HL60 cells, this occurred only in the presence of considerable cell death.

The effect of ARA-C on freshly isolated leukemia cells *in vitro* has been reported by several authors. In leukemia cells from five patients the effect of ARA-C was predominantly cytotoxic with little or no differentiation induction [4]. Michalewicz *et al.* [24] observed differentiation *in vitro* in the granulocytic direction in one patient with acute myeloblastic leukemia and in monocytic direction in another patient with acute monoblastic leukemia. In a short-term culture of freshly isolated leukemia cells Ishikura *et al.* [25] induced differentiation in granulocytic direction after exposure to ARA-C in three out of eight patients.

Some other data pointing to the cytotoxic effect of low dose ARA-C have been published recently. Exposure of HL60 cells to ARA-C did not induce any alteration in *N*-acetyl-glucosaminyltransferases (GlcNAc transferases). These GlcNAc transferases form the enzymatic basis for changes in the asparaginase-*N*-linked oligosacharides reflecting differentiation dependent structural cell surface alterations. Clear changes were found after monocytic maturation via vitamin D3 [26]. The protooncogenes c-myc and c-myb rapidly fall in HL60 cells induced to differentiation. Sato *et al.* found no changes in protooncogen expression in HL60 cells exposed to ARA-C [27]. With functional tests of immunophagocytosis, chemotaxis and antigen presentation as well as with electron microscopy no changes pointing to a maturational effect of ARA-C could be detected by our group [28]. As we published elsewhere, we found no Fc-receptor development, HLA-Dr expression or change in EBM-11 expression (characteristic for monocytic differentiation) [29]. It is obvious from the data mentioned that there is much controversy in the literature on whether ARA-C induces differentiation and if so whether it is a maturation in the granulocytic or monocytic direction. The reason for this debate could well lie in the techniques used. Also a wide diversity of phenotypic characteristics and biological behavior of various HL60 sublines could account for this controversy. It is our experience as well as that of others that interpretation of cell morphology is difficult after exposure to differentiation inducers [30, 31]. In all

of the studies mentioned above very few maturation parameters were taken into account. In our study using a panel of maturation markers we could not demonstrate differentiation to a significant extent in HL60 cells exposed to ARA-C.

For the moment we believe as others do [11–13] that the remission achieved with low dose ARA-C in patients with AML or MDS does not occur by halting proliferation through differentiation induction but just by the cytotoxic effects of the agent.

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