5-Aza-2′-Deoxycytidine Induces Terminal Differentiation of Leukemic Blasts From Patients With Acute Myeloid Leukemias

By Antonio Pinto, Vincenza Attadia, Alfredo Fusco, Felicetto Ferrara, Orlando Antonio Spada, and Pier Paolo Di Fiore

In this study, the effects of 5-aza-2′-deoxycytidine on differentiation of human leukemic cells in primary suspension culture are reported for the first time. Morphological and functional differentiation was induced in cells from two acute monoblastic leukemias and two of three acute myeloid leukemias following repeated exposures to 1 μmol/L 5-aza-2′-deoxycytidine. The observation that nontoxic concentrations of the drug are able to induce the in vitro differentiation of both monoblastic and myeloblastic leukemic cells into mature elements may encourage the exploitation of the differentiating properties of 5-aza-2′-deoxycytidine in chemotherapy protocols for acute non-lymphoblastic leukemias.

A CUTE LEUKEMIAS are regarded as disorders characterized by a differentiative block of the hemopoietic precursors, which results in an unregulated accumulation of immature blood cells. Several in vitro experiments have shown that this block can be overcome by a wide variety of chemical compounds; based on these reports, new strategies of treatment have been proposed for leukemia therapy. Such protocols involve the use of inducers of differentiation, in addition to (or in place of) aggressive chemotherapy. One of the most potent inducers of leukemic cell differentiation, 12-0-tetradecanoyl-phorbol-13-ace-tate, cannot be used in humans because of its potentially harmful effects; other inducers are poorly characterized with regard to their mode of action, pharmacokinetics, and toxicity. It is presently of the utmost importance to obtain clinically useful and fully characterized chemical compounds that are able to exert differentiative effects in vitro with low or no toxicity.

5-Azacytidine (5azaCR) and its 2′-deoxy derivative (5azaCdR) might be regarded as good candidates for this role. These compounds have, in fact, been shown to be able to trigger gene expression in several systems. They can induce the transcription of the metallothioneine I gene in a mouse thymoma cell line and the human hypoxanthine-guanine-phosphoribosyltransferase gene from structurally normal, inactive human X chromosome. They are also capable of triggering endogenous retroviral loci in chicken DNA and of reactivating the viral thymidine kinase gene in mouse cells transformed with herpes simplex virus. Moreover, 5azaCR is capable of driving the in vitro differentiation of murine fibroblasts to functional muscle cells, chondrocytes, and adipocytes, thus activating the complex program needed for cell differentiation. Finally, 5azaCR and 5azaCdR have been shown to promote the in vitro differentiation of leukemic cell lines either of murine or human origin; in particular, these compounds can induce the human promyelo-cytic cell line HL-60 to differentiate along the myeloid pathway and the human erythroleukemic cell line K562 to differentiate terminally into hemoglobin-containing erythroblasts. Recent evidences have shown that 5azaCR is capable of triggering globin gene expression when given in vivo to thalassemia and sickle cell anemia patients. Such effects have been attributed to the ability of these compounds to cause the synthesis of hypomethylated DNA, especially in the light of recent findings correlating the extent of gene methylation to its expression.

Thus far, it seems that 5azaCR and 5azaCdR may act on the differentiative process by interfering with a well-known mechanism, such as DNA methylation, which is in turn supposed to control the normal development of the differentiative program. In addition, 5azaCR and 5azaCdR have both been used in therapeutic protocols for acute leukemias with cytoreductive results, so that their metabolism, pharmacokinetics, and toxicity in humans are presently well known.

In light of these evidences, we investigated whether or not these compounds could exert differentiative effects when tested on fresh leukemic blasts in vitro. Because toxicity is one of the major problems connected with the use of 5azaCR as a differentiation inducer, we used only 5azaCdR in our study. It is well known, in fact, that 5azaCR is actively incorporated into mRNA and tRNA, thus producing its major toxic effect. Conversely, 5azaCdR has been shown to...
be much less toxic than 5azaCR, which strongly inhibits macromolecular synthesis and shows more remarkable cytotoxic and growth-inhibitory effects. In this study, we report that nontoxic concentrations of 5azaCdR are able to induce the in vitro terminal differentiation of blasts from patients with acute monoblastic and myeloblastic leukemias.

MATERIALS AND METHODS

Cells and Culture Conditions

Blood specimens were obtained prior to treatment and after informed consent from two patients with acute monoblastic leukemia (M5a, FAB) (patients 1 and 2) and from three patients with acute myeloblastic leukemia (M2, FAB) (patients 3, 4, and 5). Diagnoses were based on cell morphology and standard cytochemical stainings. All of these patients had a WBC of at least 30 x 10^9/L, with more than 80% blasts. Mononuclear cells were purified on a Ficoll-Hypaque gradient, and nonadherent cells, obtained from two cycles of incubation in tissue culture dishes for one hour at 37 °C, were plated at a concentration of 5 x 10^5/mL and 1.2 x 10^6/mL for monoblastic and myeloblastic leukemias, respectively. Enrichment in nonadherent blast cells was actually carried out to remove normal monocytes and leukemic adherent blasts, whose behavior might have jeopardized the interpretation of results. This procedure removed 15% to 30% of the Ficoll-purified cell population. Because patients were chosen who had a peripheral differential WBC with more than 80% blasts (monocytes averaged between 1% and 8%), these in vitro experiments were carried out on a truly leukemic cell population that was not significant depleted following such a treatment.

Viability and purity of the cell preparations were checked by trypan blue dye exclusion test and May-Grünwald-Giemsa stain on cytospin slide preparations, respectively. Purified populations at zero time of incubation showed 84% to 93% of blasts. Culture medium was Coon's modified Ham's F-12 supplemented with 10% fetal calf serum (GIBCO, Grand Island, NY). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO2 in air. 5azaCdR (a generous gift of Dr R. Di Lauro, Naples), dissolved in normal saline, was added to the culture medium at a final concentration of 1.0 μmol/L and every 12 hours, six times, unless otherwise indicated, the culture fluid was replaced with fresh medium containing the same amount of the drug. Cells were then washed free of the drug.

Morphology and Cytochemistry

Cytocentrifuged smears from treated and untreated cellular populations were stained with May-Grünwald-Giemsa for general morphology. Cytochemistry for α-naphthyl acetate esterase (α-NAE), naphthol-ASD-chloroacetate esterase (N-ASD-CAE), myeloperoxidase (MPO), and α-NAE sodium fluoride inhibition, was performed as described. The adherent populations were removed from the plate by means of a rubber policeman, as necessary.

Functional Tests

Nitroblue tetrazolium (NBT) reduction and latex bead phagocytosis were performed according to published procedures. When necessary, the adherent populations were removed from the plate by means of a rubber policeman.

Immunofluorescence Studies

Indirect immunofluorescence was performed following published methods. Monoclonal antibodies, recognizing antigenic specificities of different blood cell lineages, were a generous gift of Dr G. Rovera (Wistar Institute, Philadelphia). As a second-step reagent, a fluorescein-conjugated goat F(ab')2, anti-mouse Ig (heavy and light chains) (Cappel, Cochranville, Pa) was used. When adherent cells were assayed, immunofluorescence was performed on cells grown on glass coverslips.

RESULTS

Monoblastic Leukemia Cells

Treatment with 5azaCdR did not result in any toxic effect on the cultured cells (Fig 1A); during the first week of treatment, in fact, no differences in viability (dye exclusion test < 5%) or in cell concentration were detectable between the treated and untreated population.

The first noticeable effect of 5azaCdR on monoblastic cell cultures was represented by a dramatic change in cellular adherence to the plastic dish. In comparison to the untreated cells, which remained floating in the medium, by the sixth day of culture, more than 85% of the treated cells from patient 1 (Fig 1B) and more than 60% for patient 2 were firmly adherent to the plate. Under phase-contrast microscopy, 5azaCdR-treated cells appeared to have lost their round shape, becoming flattened with pseudopoda extending from the cellular margins (Fig 2B). This aspect closely resembles that of normal human macrophages in culture.

Morphological examination of cytospin slide preparations revealed that the untreated populations mainly consisted of immature forms that were nearly indistinguishable from blasts seen in the peripheral blood from donor patients (Table 1 and Fig 2, A and D). In contrast, the presence of more than 80% and more than...
60% monocytes/macrophages was evidenced in 5azaCdR-treated cells from patients 1 and 2, respectively (Table I and Fig 2, C and E).

Sodium fluoride-inhibitable α-NAE activity (a specific monocyte marker) was present in both treated and untreated populations, showing scanty, diffuse, fine brown granules in control cells, whereas the induced cells displayed a more marked positivity with larger and darker dots (data not shown).

Functional characteristics of monocytes and macrophages, such as the ability to phagocytize latex particles and to reduce NBT, were present in at least 72% of 5azaCdR-treated monoblastic cells, in contrast to a low positivity for both tests in the untreated populations (Table I and Fig 3, A and B).

The absence of toxicity of 1.0 μmol/L 5azaCdR ruled out the possibility that differentiation was the result of a selective cell killing. It is noteworthy that 5azaCdR, in the case of cells from patient 1, appreciably prolonged cell survival in culture, so that treated cells acquired the typical lifespan of normal macrophages in vitro, whereas their untreated counterparts showed the limited survival expected for leukemic blasts in suspension culture not supplemented by any specific blood cell growth factor (Fig 1A). 

An overall analysis of reactivity with specific monoclonal antibodies gave results consistent with the occurrence of terminal differentiation (Table 2). The monoclonal antibody (MoAb) R1.B19, which has no unequivocal behavior if tested on M5 leukemic blasts, but is negative on peripheral blood monocytes, did not react with 5azaCdR-treated monoblastic cells. The lack of reactivity with the MoAb L13.1 showed that both treated and untreated M5 cells did not express antigens that are present on late myeloid elements, and a maturation process for treated cells was indicated by the acquired reactivity with the MoAb S5.25, which recognizes determinants expressed on mature myeloid cells and monocytes.

Myeloblastic Leukemia Cells

Morphological and functional differentiation has also been observed in 5azaCdR-treated cells from two
Table 1. Morphology, Cytochemistry, and Functionality of Leukemic Cells Before and After Induction With 5azaCdR

<table>
<thead>
<tr>
<th>Patient</th>
<th>Morphology</th>
<th>Functionality</th>
<th>Cytochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>α-NAE t</td>
</tr>
<tr>
<td></td>
<td>FAB</td>
<td>Blasts (%)</td>
<td>Mono (%)</td>
</tr>
<tr>
<td>Patient 1</td>
<td>M5a</td>
<td>Untreated cells</td>
<td>92.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5azaCdR-treated cells</td>
<td>18.3</td>
</tr>
<tr>
<td>Patient 2</td>
<td>M5a</td>
<td>Untreated cells</td>
<td>96.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5azaCdR-treated cells</td>
<td>37.6</td>
</tr>
<tr>
<td>Patient 3</td>
<td>M2</td>
<td>Untreated cells</td>
<td>87.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5azaCdR-treated cells</td>
<td>82.0</td>
</tr>
<tr>
<td>Patient 4</td>
<td>M2</td>
<td>Untreated cells</td>
<td>78.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5azaCdR-treated cells</td>
<td>23.7</td>
</tr>
<tr>
<td>Patient 5</td>
<td>M2</td>
<td>Untreated cells</td>
<td>68.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5azaCdR-treated cells</td>
<td>15.0</td>
</tr>
</tbody>
</table>

α-NAE, α-naphthyl acetate esterase; N-ASD-CAE, naphthol-ASD-chloroacetate esterase; MPO, myeloperoxidase; NBT, nitroblue tetrazolium; 5azaCdR, 5-aza-2’-deoxycytidine 1.0 μmol/L six pulses (cells from patient 5 were exposed to only four pulses); Mono, monocytes; Macro, macrophages; Prom, promyelocytes; Myel, myelocytes; Meta, metamyelocytes; Segm, segmented (including band forms and polymorphonucleates); FAB M5a, acute monoblastic leukemia; FAB M2, acute myeloblastic leukemia with maturation; NT, not tested.

*Viable cells as determined by trypan blue exclusion.
†Cells phagocytizing more than five latex particles were scored as positive.
§α-NAE was inhibitable by sodium fluoride in cells from patients 1 and 2.
§$18.6\%$ of segmented forms were eosinophils.

of three patients with acute myeloblastic leukemias, both of whose cells, when incubated in the absence of 5azaCdR, did not morphologically differentiate (Table 1 and Fig 4, A and C). By the seventh day of culture, morphological examination of cytocentrifuged smears of treated cells revealed the presence of 54.3% mature forms (myelocytes, metamyelocytes, band forms, and polymorphs; Table 1 and Fig 4B) in the culture from patient 4 and 83.3% mature forms in the culture from patient 5 (Table 1 and Fig 4D). It is noteworthy that, in the case of patient 5, about 32% of the segmented forms were eosinophils, even though no
Table 2. Surface Antigenic Pattern of Leukemic Cells, Before and After Induction With 5azaCdR, as Detected by Monoclonal Antibodies

<table>
<thead>
<tr>
<th>Lineage and Stage Specificity of the Monoclonal Antibodies Tested*</th>
<th>Patient 1 (M5a FAB)</th>
<th>Patient 5 (M2 FAB)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated Cells</td>
<td>5azaCdR-Treated Cells</td>
</tr>
<tr>
<td><strong>MoAb</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1.B19</td>
<td>78.5</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>L13.1</td>
<td>10.9</td>
<td>5.7</td>
</tr>
<tr>
<td>S5.25</td>
<td>&lt; 5</td>
<td>91.3</td>
</tr>
<tr>
<td>Ia</td>
<td>77.5</td>
<td>96.5</td>
</tr>
</tbody>
</table>

*MoAb, monoclonal antibody; M5a FAB, acute monoblastic leukemia; M2 FAB, acute myeloblastic leukemia with maturation; Myelobl, myeloblasts; Prom, promyelocytes; Myel, myelocytes; Meta, metamyelocytes; Segm, segmented; Mono, monocytes; 5azaCdR, 5-aza-2'-deoxycytidine 1.0 µmol/L six pulses (cells from patient 5 were exposed to four pulses only).

**Sign of Eosinophil Differentiation**

Sign of eosinophil differentiation was present in the peripheral blood or in the untreated cells from the same patient. This could be attributed to the 5azaCdR-driven differentiation of very immature eosinophil precursors, which were already committed yet not detectable in the peripheral blood with routine staining techniques.

**Treated Cells from Both Patients**

Treated cells from both patients showed a dramatic increase in the ability to reduce NBT, a property of mature granulocytes that is absent in less mature myeloid precursors (Table 1). The cells from patient 3, observed at the seventh day of treatment, showed no significant differentiation. Also in the case of myeloblastic leukemia cells, differentiation occurred without cell death (Table 1), thus excluding the possibility that it might be the result of a selective enrichment for differentiated cells.

**Phenotypic Characterization**

The phenotypic characterization of one of the M2 leukemias in culture (Table 2) showed, for treated cells, the appearance of late myeloid antigens recognized by the MoAb L13.1; a maturative shift was indicated by the reactivity of the induced cells with the MoAb S5.25, which does not react with early myeloid cells. The MoAb R1.B19, which may or may not react with leukemic blasts from the bone marrow of patients with M2 acute myeloblastic leukemia and recognizes antigens present on myeloid cells, was remarkably reactive with 5azaCdR-treated cells.

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**Fig 4. Morphological appearance of uninduced and 5azaCdR-induced leukemic blasts from patients with acute myeloblastic leukemias.** After seven days of culture, cytospin preparations were stained with May-Grünwald-Giemsa. (A) Untreated cells from patient 4; (B) 5azaCdR-treated cells from patient 4; (C) untreated cells from patient 5; (D) 5azaCdR-treated cells from patient 5. Leukemic blasts from patient 5 were exposed only four times to 1 µmol/L 5azaCdR (×250).
DISCUSSION

Many agents have been identified that are able to revert the differentiative block of leukemic cells either in animal or human systems in vitro.\(^{1,3-6}\) Even though leukemia therapy has been aimed at achieving a radical tumor reduction by the use of cytotoxic drugs, alternative strategies have been suggested that tend to exploit the differentiation-inducing potential of such agents.\(^{1,6}\) In this regard, it is noteworthy that low doses of cytosine arabinoside and aclacinomycin A, which are known as potent cytotoxic agents, are able to exert a remarkable differentiative effect on leukemic cells either in vitro or in vivo.\(^{14,37-40}\)

We report that 5azaCdR, formerly used as a merely cytotoxic agent, is able to induce terminal in vitro differentiation of leukemic blasts from patients with acute nonlymphoblastic leukemias. In the present study, we demonstrated that, upon treatment with 5azaCdR, almost all of the leukemic blasts from two M5 (FAB) leukemias acquired the morphological appearance and cytologic characteristics of fully differentiated monocytes/macrophages, as also evidenced by means of enzymic marker analyses. Differentiation was also observed from the functional point of view, as treated cells acquired the ability to phagocytize latex beads with a high efficiency, like tissue macrophages, and to extensively reduce NBT, thus indicating the presence of an active respiratory burst capable of production of hydrogen peroxide and superoxide.

Myeloblastic leukemia cells showed a remarkable differentiative response to 5azaCdR treatment in two of three cases. Myeloid maturation was evidenced in treated cultures by the presence of mature segmented cells, which were able to reduce NBT, but not to adhere to the culture dish (data not shown). This result seems to be of particular interest in that, even though granulocytic differentiation of leukemic cells in culture has been reported, it is a rare event, if compared to the induction of differentiation along the monocytic pathway (for review, see Koeffler\(^{9}\)).

The occurrence of differentiation in 5azaCdR-treated cells was confirmed also by the analysis of the surface antigenic pattern with lineage-specific and stage-specific monoclonal antibodies. This clearly indicated a shift toward a more mature phenotype for both monoblastic and myeloblastic cells, following induction.

Even though spontaneous differentiation of leukemic monoblasts in short-term culture has been described, in terms of the appearance of cells morphologically resembling monocytes/macrophages that were NBT positive,\(^{1,3}\) in our control cultures, morphological differentiation was observed in less than 10% cells, and cells adhering to the plastic dish and reducing NBT were less than 25%. This might be explained by the depletion of adherent cells obtained by means of two cycles of incubation in tissue culture dishes, prior to cell seeding (see Materials and Methods).

The effectiveness of the compound in inducing cell differentiation was increased by repeated pulses of the drug, thus confirming data previously obtained in our laboratory on the K562 cell system.\(^{16,17}\) Treatment of this erythroleukemic cell line with 5azaCdR resulted in erythroid differentiation and increased globins synthesis. Dose–response experiments showed that the optimal differentiation-inducing concentration was 1.0 \(\mu\)mol/L and that repeated pulses (up to six) of 1.0 \(\mu\)mol/L 5azaCdR every 12 hours produced a maximal differentiative effect. The explanation of the requirement for a multiple exposure of the cells to the drug probably lies in the ability of 5azaCdR to produce maximal induction effects on gene expression when cells are treated in late S phase, with concomitant hypomethylation of newly synthesized DNA detectable 48 hours after treatment.\(^{21}\) Moreover, leukemic cells in primary culture show a slow replicating activity,\(^{41}\) as confirmed by a low \(^{3}\)H-thymidine incorporation (in our case, three- to sevenfold higher than in control nonreplicating cells, such as unstimulated peripheral blood lymphocytes) (data not shown). They can also be considered as a steady-state population in which dividing cells are not synchronized. It is thus feasible that repeated pulses of the drug are needed to achieve significant 5azaCdR incorporation into DNA.

Moreover, it should be taken into account that 5azaCR and 5azaCdR are unstable in aqueous solution\(^{22}\) and that the drugs are rapidly inactivated by cytidine deaminase, a catabolic enzyme present at high concentrations in mammalian sera and cells.\(^{43,45}\) All these conditions reduce the actual availability of the drug for the incorporation into DNA. It is thus conceivable that repeated pulses of 5azaCdR are needed to recruit as many cell fractions as possible to obtain the optimal differentiative effect.

The mechanism by which 5azaCdR induces cell differentiation most likely involves its DNA hypomethylating ability. Synthesis of hypomethylated DNA takes place only after the incorporation of the drug into the nucleic acid, and it is due in part to the chemical structure of the compound\(^{21}\) and, mainly, to its ability to trap DNA methyltransferases, thus blocking their action.\(^{27,46}\) Even though the leukemic cell cultures used in this study show a low rate of DNA synthesis, as measured by \(^{3}\)H-thymidine incorporation, it is feasible that such a rate suffices to produce at least a 0.3% substitution of 5azaCdR for deoxycytidine in DNA, which has been demonstrated to reduce the
DNA methyltransferase level by 95%. However, mechanisms of action of the drug, other than DNA hypomethylation, cannot be ruled out.

It is known from the literature that 5azaCR is able to induce differentiation only in a small percentage of the cell population and with a noticeable toxicity.10 As far as our study is concerned, 5azaCdR, at the concentration used, did not exert any toxic effect, as monitored by daily cell counts and trypan blue dye exclusion test. Moreover, in our study, differentiation has been achieved in a major fraction of the cell population. Further investigations are required to study comparatively the effect of 5azaCdR and 5azaCR on fresh leukemic cells in culture; it is interesting to note, however, that experiments carried out in our laboratory with the K562 cell line showed that 5azaCR is much less effective than 5aza CdR in inducing cell differentiation.11 The known differences regarding the molecular targets of the two drugs could account for their different effect on cell differentiation. In fact, it is well known that 5azaCR is actively incorporated into mRNA and tRNA,22-26 thus producing its major toxic effects. It is therefore likely that the concentrations of 5azaCR required to produce an extensive differentiative effect may prove to be toxic to the cells.

The absence of any detectable toxicity of 5azaCdR at the concentrations tested in this study may prompt its use in clinical trials (either alone or in combination with other drugs) in those patients who are not eligible for aggressive chemotherapy, such as elderly individuals and frequently relapsing leukemia patients.

It would also be interesting to investigate whether or not this drug can be used to delay the occurrence of malignant evolution in patients with preleukemic disorders and myelodysplastic syndromes.

The role that 5azaCdR could play in leukemia therapy seems to be of utmost importance in light of the modern trend to use differentiation-inducing agents when cytotoxic compounds cannot be employed. In this regard, it is interesting to note that the concentration of 5azaCdR and/or the time of exposure to the drug employed in the present work are significantly lower than those needed in cytotoxic chemotherapy to achieve an appreciable clinical effect.23

So far, in analogy with the results obtained with cytosine arabinoside,47 one could exploit the differentiation-inducing properties of 5azaCdR by using a low dosage schedule, thus avoiding the problems of toxicity connected with the use of aggressive chemotherapy.

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