In Vitro and In Vivo Effects of Deferoxamine in Neonatal Acute Leukemia

By Zeev Estrov, Akio Tawa, Xin-Hua Wang, Ian D. Dubé, Hassan Sulh, Amos Cohen, Erwin W. Gelfand, and Melvin H. Freedman

A six week old infant with acute leukemia failed to attain remission with chemotherapy. Because we previously demonstrated that the iron chelator deferoxamine (DFO) has antiproliferative properties and modulatory effects on cell differentiation, a protocol was designed for in vitro study and for clinical use in the patient. At diagnosis, blast cells were morphologically undifferentiated, had nondiagnostic cytochemistry, showed an abnormal karyotype ([t(4;11)], expressed markers of B cell lineage, and demonstrated Cμ gene rearrangement. Tissue culture of marrow or blood cells yielded colonies of leukemic blasts. Increasing concentrations of DFO produced a dose-dependent suppression of patient’s blast colony growth in vitro, and blasts within colonies showed a marked change in surface antigen expression from lymphoid to myelomonocytic markers, became monocytic in appearance, and developed intense staining for nonspecific esterase. When DFO was given intravenously to the patient as a single agent for 48 hours, blasts no longer expressed lymphoid antigens and became strongly positive for myelomonocytic markers, identical to the in vitro findings. Intravenous DFO halted rising peripheral blood blast cell numbers and allowed a several-fold increase in normal hematopoietic progenitor colony growth. When combined with low-dose cytosine arabinoside in the treatment protocol, DFO caused striking leukemic cytoreduction. Our findings indicate that DFO has antileukemic properties by virtue of its effects on proliferation and differentiation, and they prompt further experimental and clinical studies with this agent.

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Feeder cells for the culture were prepared from PB of normal cellular composition and immunologic markers.

Preparation of cells. Heparinized BM or PB cells were layered over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ) and centrifuged (200 × g, 4 °C) for 20 minutes to eliminate neutrophils and red cells.

Brain culture procedure. 2 × 10^5 T-depleted PB cells were suspended in RPMI, 10% FCS, 10% PHA-TCM, irradiated feeder cells (10^7/mL), and methylcellulose at a final concentration of 0.9% in 35-mm Petri dishes. The culture dishes were placed into a specialized modular incubator chamber (Billups-Rosenberg, Del Mar, CA) in a humidified atmosphere of 5% O_2, 5% CO_2, and balanced nitrogen. After six days of incubation, colonies were counted. A cluster of more than 20 cells was defined as a colony. Individual colonies were plucked from the culture plates and smeared on glass slides for further evaluation, using Wright’s stain and nonspecific esterase staining. For control, 2 × 10^5 irradiated feeder cells were cultured in the same assay system in each experiment to exclude their potential for colony formation.

Surface Markers

Immunocytochemical method. The cell surface antigens of air-dried smears were detected using monoclonal antibodies (MoAbs) and intestinal alkaline phosphatase, as previously described. This assay was performed to detect surface antigens on patients’ PB and BM smears and colonies plucked from cultures. For colony characterization, each MoAb was tested on five to ten individual colonies that had been plucked at random from a culture plate and smeared on a glass slide. The slides were counterstained in hematoxylin for light microscopy, and the presence of surface antigen was indicated as bright red granular cellular inclusions. Morphological details were well preserved, and a high degree of correlation between morphology and surface antigen expression was observed. The intensity of staining was defined as strong (+ + +), moderate (+ + ), and weak (+).

Immunofluorescence method. The cell surface phenotype was determined by indirect cell-surface fluorescence; a fluorescein-conjugated goat antimouse IgG(Fab), was used as second antibody. For each MoAb tested, 1 × 10^5 cells were stained and 200 cells examined. The labeling was performed on ice in the presence of 0.05% sodium azide. The percentage of cells with surface fluorescence was determined by counting cells using an inverted phase-fluorescent microscope.

Monoclonal antibodies. The following MoAbs were used in these studies: anti-O1a (Ortho Diagnostic Systems, Raritan, NJ) to detect HLA-D framework antigen; anti-MY-7, anti-Mo1 and anti-MY-4 (Coulter Immunology, Hialeah, FL) to identify myelomonocytic markers; anti-BA1, anti-BA2, anti-B1 and anti-B4 (Becton Dickinson, Oxnard, CA) to recognize cells of the B cell lineage; anti-J5 (Coulter Immunology) to recognize cells bearing common ALL (CALLA) antigen; and anti-Leu-1 and anti-Leu-4 (Coulter Immunology) to recognize cells of the T cell lineage.

RESULTS

In vitro

Blast colony assay. Prior to therapy with DFO, patient’s PB blast cells were cultured using the ALL blast colony assay; they yielded a mean of 700 colonies/2 × 10^5 cells plated. Individual colonies were plucked from the culture plates and were examined for cellular composition. Morphologically they were undifferentiated blast cells identical to the appearance of marrow cells at diagnosis. The cells were negative for nonspecific esterase by cytochemistry. Surface antigen studies of PB cells confirmed lymphoblastic markers and absent myelomonocytic markers (Table 2).

Antiproliferative effect of DFO. As shown in Fig 1, when increasing concentrations of DFO were added to
Immunologic surface markers were determined before and after 48 hours of deferoxamine treatment on PB smears using the immunocytochemical method.

**Table 2. Surface Marker Analysis of Leukemia Cell Colonies (% Positive Cells)**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Control</th>
<th>2.5 μmol Dferoxamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia</td>
<td>80% (+++)</td>
<td>80% (+++)</td>
</tr>
<tr>
<td>CALLA</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>B4</td>
<td>80% (+++)</td>
<td>30% (+++)</td>
</tr>
<tr>
<td>Mol</td>
<td>0%</td>
<td>&gt;90% (+++)</td>
</tr>
<tr>
<td>MY-4</td>
<td>0%</td>
<td>60% (+)</td>
</tr>
<tr>
<td>Leu-1</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Leu-4</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

PB leukemia cell colonies were plucked at random from cultures containing 2.5 μmol deferoxamine and were compared to cultures without deferoxamine using the immunocytochemical assay.

Cultures of patient's PB blast cells, there was a dose-dependent suppression of blast colony growth. Significant decline in blast colonies was observed with DFO concentrations greater than 1.25 μmol, and with 2.15 μmol DFO, a 70% reduction in blast colonies was observed. In a control study of normal marrow obtained from a healthy volunteer, DFO also produced a dose-dependent suppression of normal hematopoietic colonies, CFU-C, CFU-E, and BFU-E. However, 10 μmol DFO was necessary to achieve a 70% reduction in colony counts.

**DFO and blast cell differentiation.** Individual blast colonies from the culture plates containing DFO 2.5 μmol were characterized (Table 2). Compared to colonies grown without exposure to DFO, a marked change in cell surface antigen expression was observed. Blast colonies from the DFO plates strongly expressed the myelomonocytic markers Mol and MY-4 as determined by immunocytochemistry, and numbers of B-4 positive cells were markedly reduced. Moreover, individual cells from colonies exposed to DFO were changed in morphology, appearing monocytic, and stained intensely for nonspecific esterase that could be inhibited by fluoride.

**In Vivo**

DFO was administered therapeutically according to the protocol outlined in Table 1.

**DFO and blast cell differentiation.** After 48 hours of continuous infusion of DFO as a single agent, striking changes in blast cell surface markers were detected immunocytochemically (Table 3). Prior to treatment no blasts expressed Mol antigen, and only 8% weakly expressed MY-4. After 48 hours of treatment, these two antigens, Mol and MY-4, were detected in peripheral blood blast cells. Concomitantly with DFO therapy there was a sharp decline from 37% to 0% of cells bearing B4 antigen. These data were confirmed by immunofluorescence studies.

At the conclusion of the 20-day treatment protocol, a marrow aspirate showed 60% blasts that were negative for PAS, Sudan-black, and acid phosphatase. In contrast to the marrow aspirate prior to DFO therapy, 60% of the blasts were positive for nonspecific esterase, and staining could be inhibited by fluoride. Surface marker studies performed concurrently demonstrated expression of myelomonocytic antigens that were not detected in marrow cells prior to treatment (Table 4).

**Antiproliferative effect of DFO.** The WBC and blast number had increased several-fold in the three days prior to DFO treatment (Table 1). After the first 48 hours of DFO therapy, the counts reached a plateau. Significant decline of WBC and blast numbers was observed on day 8, that is, six days following combination DFO-ARA-C therapy. By day 15, PB blasts almost completely disappeared.

Several CFU-GEMM assays were performed on PB during the first 48 hours of DFO treatment (Table 5). Impressive increases in normal hematopoietic progenitor colony growth were documented. Erythroid and granulocytic progenitor colony formation increased several-fold over pre-DFO treatment colony numbers.

**Fig 1. The effect of increasing concentrations of deferoxamine on patient's PB ALL blast colony numbers.**

**Table 3. Surface Marker Analysis Pre- and Post-Therapy (% Positive Cells)**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Before Dferoxamine Treatment</th>
<th>After Dferoxamine Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia</td>
<td>80% (+++)</td>
<td>80% (+++)</td>
</tr>
<tr>
<td>CALLA</td>
<td>2% (+)</td>
<td>0% (+)</td>
</tr>
<tr>
<td>B4</td>
<td>37% (+)</td>
<td>0%</td>
</tr>
<tr>
<td>Mol</td>
<td>0%</td>
<td>40% (+++)</td>
</tr>
<tr>
<td>MY-4</td>
<td>8% (+)</td>
<td>60% (+++)</td>
</tr>
<tr>
<td>Leu-1</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Leu-4</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Immunologic surface markers were determined before and after 48 hours of deferoxamine treatment on PB smears using the immunocytochemical method.
Table 5. Numbers of CFU-GEMM Colonies Pre- and Post-Therapy

<table>
<thead>
<tr>
<th>Colony Type</th>
<th>Before Deferoxamine</th>
<th>After Deferoxamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treatment</td>
<td>Treatment</td>
</tr>
<tr>
<td>BFU-E</td>
<td>9 ± 1</td>
<td>113 ± 14</td>
</tr>
<tr>
<td>CFU-C</td>
<td>45 ± 6</td>
<td>126 ± 17</td>
</tr>
<tr>
<td>Mixed</td>
<td>1 ± 1</td>
<td>5 ± 2</td>
</tr>
</tbody>
</table>

CFU-GEMM colony culture of patient's PB was performed before and after 48 hours deferoxamine treatment.

DISCUSSION

The infant who was studied had leukemia that was unresponsive to conventional chemotherapy. At the conclusion of induction therapy, blasts were still present in PB and in marrow, and when chemotherapy was stopped, PB blast counts increased several-fold in three days.

The blasts were undifferentiated according to morphological and cytochemical criteria and expressed antigens of B lineage at diagnosis. Further confirmation of their origin from B cells was by analysis of DNA from the leukemic cells that revealed rearrangement of one of the immunoglobulin heavy-chain genes. The presence of an abnormal karyotype, t(4;11), as found on cytogenetic analysis of BM cells, has been previously reported in neonatal ALL.16 This form of leukemia is characterized by PB leukocytosis, blast cells with an undifferentiated appearance, and poor prognosis.17 Although the cytogenetic abnormality has been found in ALL,18 it has also been seen in cases of acute leukemia of myelomonocytic lineage.17

We were prompted to use DFO in an experimental treatment protocol because of our previous finding that the agent was a reversible early S-phase inhibitor of human lymphocyte proliferation.4 Although widely used as a chelator of ferric iron in disorders of iron overload, we demonstrated that the drug is a potent inhibitor of DNA synthesis by B and T lymphocytes in vitro, likely by impairing the activity of the iron-containing enzyme ribonucleotide reductase through a chelating mechanism.2 More recently we have shown that DFO can induce monocyte–macrophage cell differentiation in the human promyelocytic leukemia cell line, HL-60, as judged by altered expression of cell surface antigens, nonspecific esterase activity, and morphological changes combined with irreversible inhibition of colony formation.5 The mechanism appeared to be related to inhibition of replicative DNA synthesis or due to an increase in number of single-strand DNA breaks. Because DFO has antiproliferative properties as well as modulatory effects on cell differentiation, it may be potentially useful for experimental and therapeutic applications.

In vitro we were able to demonstrate the antiproliferative effects of DFO on leukemic cell growth from our patient using a blast colony assay. Because the plating efficiency of PB blast cells was very high, clear-cut suppression of colony growth by DFO could be readily demonstrated. The DFO-induced change in blast cell surface antigen expression from lymphoid to myelomonocytic was striking and confirmed the modulatory effect of DFO on cell differentiation.

The in vitro changes correlated closely with the findings during the first 48 hours of continuous infusion of DFO when used as a single agent. The PB WBC and blast counts, which had risen dramatically for three days prior to DFO therapy, leveled off 48 hours after initiation of DFO infusion, suggesting an in vivo antiproliferative effect. Moreover, surface marker antigen changes were observed on the blast cells that were identical to those seen after DFO treatment in vitro. There was convincing evidence for expression of myelomonocytic antigens and concurrent disappearance of lymphoid markers. The possibility that during the 48 hours of DFO treatment a change in PB cell distribution was responsible for the change in surface markers was unlikely, since the blast count remained stable during this period. With the HL-60 cell line, Boyd and Metcalf showed that the differentiation process is associated with irreversible loss of proliferative ability, inhibition of DNA synthesis, and G2/M cell cycle arrest.19 Since identical changes in the same cell line were induced by DFO,3 this could be the mechanism by which DFO affected our patient’s leukemic cells. Although the loss of proliferative ability was suggested by our data, it was not definitively proven using the colony assay technique.

The cellular lineage of the blasts is an important aspect of the case because there was a change in differentiation markers with therapy. It is unlikely that two separate leukemic populations were responsible for the dual marker findings because the abnormal karyotype found at diagnosis was still detected at the conclusion of therapy, suggesting persistence of the same leukemic clone. There have been reports of conversion with chemotherapy of cases of non-T ALL to myelomonocytic leukemia20 and from T-lymphoblastic leukemia to promyelocytic leukemia.21 In the latter case it was shown that the leukemia was actually derived from a stem cell.11 Although we have observed the pattern of immunoglobulin heavy-chain gene rearrangement only in cases of ALL,4 the abnormal karyotype found in our patient’s blast cells has been seen in both ALL and in ANLL.14-18,20 Moreover, the 4;11 translocation is remarkable for its association with biphenotypic expression of differentiation markers.22-24 In our case it is possible that the blasts showed “lineage infidelity”25 and expressed markers from two lineages at different times. With regard to the mechanism, one may speculate that the 4;11 syndrome is a stem cell disorder that can differentiate along one lineage given a set of growth factors and in another lineage given others.

A low plating efficiency of normal pluripotent hematopoietic precursors in acute leukemia, as demonstrated in our patient, has been previously described.26,27 The significantly higher plating efficiency observed using the CFU-GEMM assay of erythroid as well as granulocyte-macrophage elements from our patient’s PB following DFO treatment occurred in spite of persistently high numbers of PB blast cells. It is unlikely that a high percentage of the blast-like cells were actually normal hematopoietic progenitors because of their increased numbers and morphological features. It is possible that DFO in vivo induced a change in the leukemic blasts allowing their differentiation toward the erythroid as well as the granulocyte–macrophage lineage as previously described in Ph1-positive chronic myelogenous leukemia.28,29 Another interpretation is that DFO treatment in some manner permitted the proliferation of normal hema-
topoietic progenitors that were suppressed by the leukemic cells as described by Broxmeyer et al.30,31

The rationale for using ARA-C with DFO in the treatment protocol was based on the hypothesis that if DFO could reversibly block leukemic cells in S-phase, after removal of DFO, ARA-C should destroy the cells progressing through S-phase because its cytotoxic action is S-phase-dependent.

From the available data it is still unclear whether the apparent synergism between DFO and ARA-C is attributed to S-phase synchronization or to the differentiating action of DFO. The marked clinical reduction in PB blast numbers, the changes in leukemic cell marker expression, and the in vitro and in vivo antiproliferative properties prompt further experimental and clinical studies with DFO.

REFERENCES

In vitro and in vivo effects of deferoxamine in neonatal acute leukemia

Z Estrov, A Tawa, XH Wang, ID Dube, H Sulh, A Cohen, EW Gelfand and MH Freedman