Deferoxamine: A Reversible S-Phase Inhibitor of Human Lymphocyte Proliferation


Deferoxamine is widely used therapeutically as a chelator of ferric ion in disorders of iron overload. This study demonstrates that this drug is a potent inhibitor of DNA synthesis by human B and T lymphocytes in vitro, but has relatively little effect on the synthesis of RNA and protein. The inhibitory effects of deferoxamine are completely reversible by washing or by adding stoichiometric amounts of Fe³⁺. Micromolar concentrations of deferoxamine decrease intracellular levels of deoxyribonucleoside triphosphates, which is similar to the effects of hydroxyurea. The binding of iron by deferoxamine likely causes an inhibition of ribonucleotide reductase activity, thereby preventing cells from completing the S phase of the cell proliferation cycle. As a reversible and nontoxic S-phase inhibitor, it may have important experimental and therapeutic applications.

MATERIALS AND METHODS

Cells

Fragments of thymuses were obtained from children aged 6 months to 16 years who were undergoing cardiac surgery. As previously described in detail,1 mononuclear cell suspensions were prepared, and a subpopulation of thymocytes with a high proliferative index was obtained by density gradient centrifugation over a solution of 25% bovine serum albumin.

Peripheral blood was obtained from healthy volunteers; tonsillar tissue was obtained from children undergoing tonsillectomy. Mononuclear cell suspensions were prepared from both sources and further divided into E rosetting (E⁺) and non-E rosetting (E⁻) populations by rosette depletion on Ficoll-Hypaque gradients.14 Tonsil B8 is a cloned B cell line that was derived in our laboratory from a culture of normal human tonsillar lymphocytes. The cells have cytoplasmic and surface IgG and are IgG secretors. MOLT 3 is a T cell line derived from a patient with T cell leukemia.15

Radioisotope Incorporation

Lymphocytes (10⁶) were cultured in RPMI 1640 (Ontario Cancer Institute, Toronto) with 10% fetal calf serum (FCS, M.A. Bioproducts, Walkersville, Md). T cells were incubated with phytohemagglutinin (PHA-P; Difco, Detroit) and B cells were incubated with formalized S aureus Cowan 1 strain (STA, Pansorbin, grade B; Calbiochem-Behring, La Jolla, Calif) as described.6 On day 3, thymidine uptake was measured after a four-hour pulse with 1 μCi [³H]-thymidine (6.7 Ci/mmol; New England Nuclear, Lachine, Quebec). For measurement of protein synthesis, cells were washed and resuspended in valine-threonine-leucine-free RPMI 1640 (Ontario Cancer Institute), pulsed with 1 μCi [¹⁴C]-valine, [¹³C]-threonine, [¹⁴C]-leucine, and harvested after overnight incubation.

Cell Cycle Analysis

Lymphocytes were fixed with ice-cold 70% ethanol. Following degradation of RNA by RNase (50 μg/mL; Worthington Biochemical Corp, Freehold, NJ) for ten minutes at room temperature, DNA was stained for five minutes with propidium iodide (50 μg/mL; Sigma Chemical Co, St Louis). Cellular DNA content was assessed by flow cytometry using the EPICS V fluorescence activated cell sorter (Coulter Electronics, Hialeah, Fla). Data analysis to determine the percentage of cells in each phase of the cell cycle was performed with the aid of the EASY computer system (Coulter).
Deferoxamine Inhibits Proliferation of Human Lymphocytes

Human thymocytes, a human B lymphocyte cell line (tonsil B8), and a human T lymphocyte cell line (MOLT 3) were incubated for 16 hours with graded concentrations of deferoxamine. DNA synthesis by these spontaneously dividing lymphocytes was measured by subsequent incorporation of \(^3\)H-thymidine (Fig 1). Deferoxamine caused a dose-dependent inhibition of \(^3\)H-thymidine incorporation in each of the lymphocyte populations tested. Fifty percent inhibition was achieved at a drug concentration of 20 \(\mu\)mol/L for Tonsil B8, and 40 to 50 \(\mu\)mol/L for thymocytes and MOLT 3. Cell viability, assessed by trypan blue dye exclusion, was unaffected.

Deferoxamine (Desferal, CIBA-Geigy Canada Ltd. Mississauga, Ontario) was reconstituted with distilled H\(_2\)O to a concentration of 150 mmol/L, and dilutions were prepared in RPMI. Reconstituted drug was stored in the dark at 4 °C and used within 48 hours of preparation. Hydroxyurea and ferric chloride were purchased from Sigma Chemical Co.

Deoxyribonucleotide (dNTP) Assays

Nucleotides were extracted in 0.4 mol/L perchloric acid for 30 minutes on ice and neutralized with 1.0 N KOH. dNTP was assayed by a modification of the DNA polymerase assay,\(^\text{7}\) measuring the incorporation of labeled nucleotides into the alternating copolymer of deoxyadenylate and deoxycytidylate (poly[d(A,T)]) and into the alternating copolymer of deoxyguanylate and deoxycytidylate (poly[d(G,C)]). Details of the dNTP assays have been described previously.\(^\text{14}\) The dNTP assays were linear between 0.5 pmol and 20 pmol and were reproducible within 10% SD.

Reagents

Deferoxamine (Desferal, CIBA-Geigy Canada Ltd. Mississauga, Ontario) was reconstituted with distilled H\(_2\)O to a concentration of 150 mmol/L, and dilutions were prepared in RPMI. Reconstituted drug was stored in the dark at 4 °C and used within 48 hours of preparation. Hydroxyurea and ferric chloride were purchased from Sigma Chemical Co.

Table 1. Deferoxamine Inhibits Mitogen-Induced Proliferation

<table>
<thead>
<tr>
<th>Deferoxamine ((\mu)mol/L)</th>
<th>Cells*</th>
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<tbody>
<tr>
<td></td>
<td>PBL</td>
</tr>
<tr>
<td>125</td>
<td>310 ± 100</td>
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<tr>
<td>62</td>
<td>170 ± 30</td>
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<tr>
<td>31</td>
<td>420 ± 150</td>
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<td>16</td>
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<td>4</td>
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</tr>
<tr>
<td>2</td>
<td>10,020 ± 540</td>
</tr>
<tr>
<td>0</td>
<td>36,320 ± 2,500</td>
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</tbody>
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* cpm \(^3\)H-thymidine incorporation.

PBL (5 \(\times\) 10\(^4\)/well) or tonsil E* (2 \(\times\) 10\(^5\)/well) were cultured for 72 hours with PHA and graded concentrations of deferoxamine; tonsil E* (2 \(\times\) 10\(^5\)/well) were cultured with STA plus deferoxamine. Cultures were pulsed with 1 \(\mu\)Ci \(^3\)H-thymidine for the final four hours of culture. Results represent mean ± SD for three replicate wells in one of three representative experiments.
The Inhibitory Effects of Deferoxamine Are Reversible

Spontaneously proliferating human thymocytes were incubated with deferoxamine for varying time intervals during a 24-hour period (Fig 2). All cultures were pulsed with $^3$H-thymidine during the final four hours of culture. When 40 μmol/L deferoxamine was present throughout the 24-hour culture (Fig 2C), there was greater than 90% inhibition of $^3$H-thymidine uptake. Deferoxamine-induced inhibition was reversible by washing: when the drug was present for the first two hours (Fig 2A) or the first 18 hours (Fig 2B), then removed by washing, $^3$H-thymidine incorporation was equal to that in control cultures. In fact, after an 18-hour incubation with 40 μmol/L deferoxamine, there was a reproducible and statistically significant enhancement of thymidine incorporation ($P < .001$; Student's $t$ test). The drug did not appear to have an inhibitory effect on the cellular transport of thymidine. Addition of drug two hours prior to the four-hour pulse with $^3$H-thymidine (Fig 2D) did not inhibit, but slightly enhanced, the incorporation of isotope ($P < .01$).

Deferoxamine could inhibit thymocyte proliferation by direct drug action or indirectly by chelating ferric ion, an essential intracellular cation. We examined the latter by attempting to reverse the drug effect by the addition of ferric ion (Fig 3). When human thymocytes were incubated with deferoxamine (Fig 3C), $^3$H-thymidine incorporation was inhibited approximately 90%, as compared to control cultures without drug (Fig 3A). The addition of FeCl₃ to deferoxamine-treated cultures caused a dose-dependent increase in $^3$H-thymidine incorporation (Fig 3, D,E,F) as compared to deferoxamine-treated controls. Thymidine incorporation reached control levels when FeCl₃ was at approximately the same molar concentration as deferoxamine. FeCl₃ alone had no inhibitory or stimulatory effects on $^3$H-thymidine incorporation (Fig 3B).

Deferoxamine Exerts Its Major Effect on DNA Synthesis

The rates of DNA synthesis ($^3$H-thymidine incorporation), RNA synthesis ($^3$H-uridine incorporation), and protein synthesis ($^{14}$C-valine, $^{14}$C-threonine, $^{14}$C-leucine incorporation) were measured in parallel cultures containing graded concentrations of deferoxamine. The drug caused a dose-dependent inhibition of DNA synthesis in cultures of spontaneously proliferating thymocytes (Fig 4A) and in cultures of the B lymphocyte cell line (Fig 4B). The effect of RNA synthesis was smaller, and the effect on protein synthesis was negligible. At deferoxamine concentrations that caused 90% inhibition of DNA synthesis, the synthesis of RNA was inhibited by approximately 50% and the synthesis of protein was inhibited by only 10%.

Deferoxamine Decreases Intracellular Levels of Deoxyribonucleoside Triphosphates

Deferoxamine, used at micromolar concentrations, exerts its major effect by inhibition of DNA synthesis. The drug effect is completely reversible by the addition of equimolar concentrations of FeCl₃, suggesting that

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**Fig 2.** Human thymocytes ($10^5$) were incubated with (■) or without (□) deferoxamine for varying periods of time during a 24-hour period. All cultures were pulsed with 1 μCi $^3$H-thymidine for the final four hours of culture. Results represent the mean ± SEM in one of three similar experiments.

**Fig 3.** Human thymocytes ($10^5$) were incubated overnight with deferoxamine (100 μmol/L) and/or varying concentrations of FeCl₃ (50 to 150 μmol/L). Thymidine incorporation was measured after a four-hour pulse with 1 μCi $^3$H-thymidine. Results represent the mean ± SEM of three replicate cultures for each group.

**Fig 4.** Lymphocytes ($10^5$) were cultured overnight with graded concentrations of deferoxamine. Cultures were pulsed with 1 μCi $^3$H-thymidine (O), 1 μCi $^3$H-uridine (●), or 1 μCi $^{14}$C-valine, $^{14}$C-threonine, $^{14}$C-leucine (▲). (A) Thymocytes. (B) Tonsil B8 cell line.
Fig 5. A subpopulation of large human thymocytes (4 × 10⁸/mL) was incubated overnight with varying concentrations of deferoxamine or with 0.1 mmol/L hydroxyurea. At the end of the incubation period, cells were harvested, nucleotides were extracted, and levels of deoxynucleotides were measured. dTTP (○–○), dATP (△–△), dGTP (□–□), dCTP (△–△). Results represent data from a single experiment done in duplicate. Two additional experiments gave similar results.

inhibition of DNA synthesis is the result of iron chelation. Because ribonucleotide reductase is a rate-controlling enzyme in DNA synthesis and requires iron as a cofactor, we examined the effect of deferoxamine on this enzyme by measuring intracellular deoxyribonucleotide pools in cultures of thymocytes (Fig 5). Deferoxamine caused a marked depletion of dATP (92%) and dGTP pools (95%); there were smaller effects on dTTP (35%) and dCTP (25%) pools. Incubation of thymocytes with 0.1 mmol/L hydroxyurea, a known inhibitor of ribonucleotide reductase, resulted in a virtually identical pattern of deoxyribonucleotide pool depletion (Fig 5).

**Deferoxamine Blocks Lymphocytes in Early S Phase**

We confirmed the inhibitory effect of deferoxamine on DNA synthesis by examining the effect of the drug on the cell proliferation cycle of T cell-enriched (E⁺) PBLs cultured with PHA. When cultures were initiated, greater than 99% of PBL-E⁺ were in the Gₓ/G₁, or pre-DNA synthesis, phases of the cell cycle. In the presence of PHA for 48 hours, the lymphocytes were triggered to synthesize DNA. The proliferative index was 29.3%, with 9.6% of cells in the S phase and 19.7% in Gₓ/M (Fig 6A). Cells were equally distributed throughout the early, middle, and late stages of the S phase. When PBL-E⁺ were cultured with PHA plus deferoxamine (Fig 6B), there was an increase in S-phase cells from 9.6% to 12%. Furthermore, in the presence of deferoxamine, almost all of these S-phase cells were in the early stage of S phase. The drug also caused a decrease in the percentage of Gₓ/M-phase cells from 19.7% to 3%. These data are consistent with a deferoxamine-induced block of cell cycle progression at early S phase.

**DISCUSSION**

Our investigation of the biologic effects of deferoxamine has utilized lymphoid cell populations with rapid rates of proliferation. We have used human T and B lymphocytes in which proliferation was induced by mitogens, lymphoblastoid cell lines, and a subpopulation of spontaneously proliferating thymic lymphocytes with a proliferation index of approximately 90%. This approach has allowed us to observe short-term effects of deferoxamine using sensitive indicator cell populations.

We have demonstrated that deferoxamine is a potent inhibitor of cell proliferation in fresh human thymocytes and in T and B cell lymphoblastoid cell lines. Ninety-percent inhibition of ³H-thymidine incorporation occurs with micromolar concentrations of deferoxamine in overnight cultures. There does not appear to be a significant difference in the drug sensitivity of T lymphocytes vs B lymphocytes in this system. We have not yet assessed the minimum time required for drug action, but the inhibitory action of deferoxamine appears to be rapidly reversible. ³H-thymidine uptake of spontaneously proliferating thymocytes is completely recovered within two to four hours after deferoxamine is removed by washing.

The rapid and completely reversible effects of deferoxamine in this assay system have allowed us to define the mechanism of drug action. Inhibition of ³H-thymidine incorporation by deferoxamine is prevented by simultaneous addition of equimolar concentrations of FeCl₃, although FeCl₃ appears inert by itself at these concentrations. Furthermore, we have demon-
Strated that deferoxamine is a potent inhibitor of DNA synthesis, but has relatively little effect on the synthesis of RNA and protein. At concentrations of deferoxamine causing 90% of \(^{3}H\)-thymidine incorporation, there was 50% inhibition of RNA synthesis (\(^{3}H\)-uridine incorporation) and only negligible inhibition of protein synthesis (\(^{14}C\)-valine, \(^{14}C\)-threonine, \(^{14}C\)-leucine incorporation). Taken together, these findings suggest the deferoxamine exerts its primary effect by chelating ferric ion and subsequently inhibiting DNA synthesis. Because ATP, GTP, UTP, and CTP levels were normal in treated cells, the inhibition of RNA synthesis is likely to be secondary to the inhibition of DNA synthesis.

Ribonucleotide reductase, a rate-controlling enzyme in the DNA synthetic pathway, is one possible target for the action of deferoxamine. Mammalian ribonucleotide reductase has recently been purified. It consists of an 84,000 mol wt subunit (M1) and a 58,000 mol wt subunit (M2). Neither subunit has any enzyme activity when assayed alone, but activity is recovered upon recombination. It is the M1 subunit that has the receptors for nucleoside triphosphates. The M2 subunit contains a tyrosine free-radical structure that is essential for enzyme activity, but has a half-life of only ten minutes. Regeneration of the free radical requires the continual presence of oxygen and iron. Indeed, the regeneration of the free radical on M2 has been proposed as one way in which a cell may rapidly regulate ribonucleotide reductase activity and thereby regulate DNA synthesis. We demonstrated that thymocytes treated with deferoxamine had markedly decreased intracellular pools of dATP and dGTP and smaller decreases in dCTP and dTTP pools, which is in keeping with an inhibition of ribonucleotide reductase. Similar findings are shown after incubation with hydroxyurea, a known inhibitor of ribonucleotide reductase.

Inhibition of DNA synthesis by deferoxamine has previously been demonstrated in several mammalian cells. Robbins et al documented that deferoxamine decreased DNA synthesis in HeLa cells without affecting RNA or protein synthesis, and that these effects paralleled the drug-induced inhibition of iron uptake by the cells. Deferoxamine has been reported to decrease \(^{3}H\)-thymidine uptake and inhibition of ribonucleotide reductase in PHA-stimulated PBLs, but the concentrations of drug used were ten- to 20-fold higher than in the present study and may have resulted in cell death. We have confirmed the finding that deferoxamine inhibits the proliferation of T lymphocytes in response to the mitogen phytohemagglutinin, and we have documented similar effects on B lymphocytes stimulated with a B cell mitogen, as well as on B and T lymphoblastoid cell lines. Cell cycle analysis of mitogen-stimulated lymphocytes reveals that the cells are blocked early in the S phase of the cell proliferation cycle, a finding that is consistent with our hypothesis that deferoxamine exerts a primary effect on the activity of ribonucleotide reductase. Our finding of enhanced thymidine incorporation after a 18-hour preincubation with deferoxamine (Fig 2) supports the conclusion that deferoxamine is an S-phase blocking agent.

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REFERENCES


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HM Lederman, A Cohen, JW Lee, MH Freedman and EW Gelfand