The Effect of Desferrithiocin, an Oral Iron Chelator, on T-Cell Function

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Desferrithiocin is a new, potent, orally available iron chelator. To determine whether this drug might be useful not only for iron-overload but also for immunosuppression, we studied the in vitro effects of desferrithiocin on T-lymphocyte function. Like deferoxamine, desferrithiocin inhibited, in a dose-dependent fashion, mitogen- and lectin-induced proliferation of both human and murine T cells. It was active at a concentration of 10 μg/mL. The inhibition of proliferation was reversed by ferrous chloride, but not by other metal salts, racemibinant IL-2, or conditioned medium. Desferrithiocin also inhibited proliferation of constitutively dividing, and factor-independent EBV-transformed B cell and leukemic T cell lines. Although desferrithiocin inhibited the induction of cytotoxic T lymphocyte (CTL) activity, it did not inhibit CTL- or natural killer-induced cytotoxicity. The agent did not inhibit the expression of activation antigens such as the IL-2 receptor on T cells, nor early measures of T-cell activation such as the influx of intracellular calcium. Thus, desferrithiocin, like deferoxamine, is a potent and reversible inhibitor of T-cell proliferation. This anti-proliferative effect inhibits T-cell function. Bioavailability after oral administration is a unique property of desferrithiocin, and would make it an attractive alternative to deferoxamine. Its immunomodulating properties may therefore be exploited in vivo to inhibit graft rejection or autoreactive T cells.

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IRON IS AN ESSENTIAL nutrient for cell growth, cell division, and cellular differentiation. It is necessary not only for heme-containing proteins and oxidative enzymes but also for enzymes that require iron as a coenzyme. In vivo, it is not only necessary to treat joint inflammation in rodent models of rheumatoid arthritis, but also its efficacy in humans has been limited. Desferoxamine has been shown to inhibit the development of experimental allergic encephalitis in the rat, associated with diminished T-cell responsiveness and inhibition of the development of inflammatory lesions. Deferoxamine also appears to control allograft rejection in murine allograft models and to treat graft-versus-host disease in human allogeneic bone marrow transplant recipients. The hydroxamic acid deferoxamine (M, 656.8) is produced by Streptomyces pilosus (Fig 1). The agent binds an equimolar concentration of iron to form a stable complex, ferrioxamine. Deferoxamine is poorly absorbed from the gastrointestinal tract and must therefore be administered parenterally, limiting patient compliance and acceptability. Recently, a new, potent, orally active iron chelator, desferrithiocin, has been developed, and shown to be considerably more effective than deferoxamine in promoting iron excretion in a simian model. The sodium salt of desferrithiocin (M, 260.3), a siderophore, was generated from a product of Streptomyces antibioticus (Fig 1). Unlike deferoxamine, it is readily absorbed from the gastrointestinal tract. In order to determine its immunosuppressive capacity, we studied the effect of desferrithiocin on T cell function in vitro. Like deferoxamine, desferrithiocin is a potent, reversible inhibitor of T-cell proliferation and mixed lymphocyte reactivity in vitro. It does not affect the expression of activation antigens on the surface of stimulated T cells, IL-2 production, or early measures of T-cell activation such as a rise in intracellular calcium ([Ca²⁺]). While it inhibits the generation of cytotoxic T cells (CTL), it does not inhibit CTL- or natural killer (NK)-mediated cytotoxicity once these cells are formed. Its profound immunomodulating effects may have therapeutic applications.

MATERIALS AND METHODS

Reagents. Deferoxamine mesylate (Desferal), M, 656.8, was obtained from Ciba-Geigy (Basel, Switzerland). The sodium salt of desferoxamine, M, 260.3, was also kindly provided by Ciba-Geigy. Both compounds were diluted into culture media immediately before use, and filtered sterilized. Ferrous chloride and other metal salts were obtained from Sigma Chemical Co (St Louis, MO).

Monoclonal antibodies. The anti-CD3 monoclonal antibody (MoAb) OKT3 (American Type Culture Collection, Rockville, MD) was subcloned, and grown as ascitic fluid of pristane-primed BALB/c mice. The concentration of OKT3 MoAb used, 1:40,000 dilution of ascites fluid, induced maximal proliferation of peripheral blood mononuclear cells not depleted of accessory cells (data not shown). The MoAbs TS2/18, directed against CD2, and LB3.1, directed against monomorphic determinants of class II major histocompatibility complex proteins respectively, were used at a final concentration of 1:1000 dilution of ascites fluid. Anti-Tac MoAb recognizing the IL-2 receptor (CD25) was the kind gift of Dr Thomas Waldmann (NIH, Bethesda, MD).

Cell culture and preparation. Human venous blood from normal...
healthy volunteers was collected in preservative-free heparin (Sigma). Peripheral blood mononuclear cells (PBMC) were separated on a Ficoll-Hypaque (density 1.077) gradient (Lymphocyte Separation Medium, Bionectics, Kensington, MD) and thoroughly washed. Cells were grown in RPMI-1640 media (M.A. Bioproducts, Walkersville, MD) containing 10% heat-inactivated-fetal bovine serum (FBS, Sigma), 100 units/mL penicillin (GIBCO, Grand Island, NY), 100 μg/mL streptomycin (GIBCO), 10 mmol/L Hepes (M.A. Bioproducts), 2 mmol/L glutamine (GIBCO) and 50 μmol/L 2-mercaptoethanol (Sigma), termed complete medium. PBMC were cultured in either anti-CD3 MoAb (OKT3, American Type Culture Collection); phytohemagglutinin (PHA, Bacto, PHA-P, Difco Laboratories, Detroit, MI), 3 μg/mL; PHA plus recombinant IL-2 (Amgen Co, Thousand Oaks, CA), 10 U/mL; PHA plus 5% IL-2-containing human conditioned medium; or phorbol 12-myristate 13-acetate (PMA, Sigma), 10 ng/mL, plus ionomycin (Behring Diagnostics, La Jolla, CA), 0.2 μmol/L, as described below. Human conditioned medium was prepared from 72-hour culture supernatants of pooled PHA-stimulated PBMC as described in detail previously. The supernatants were concentrated by ammonium sulfate precipitation (50%, 75%), followed by exhaustive dialysis against phosphate-buffered saline (PBS), filter-sterilized, and stored at −20°C.

Murine splenocytes were obtained from B6AF1 mice. Mice were killed by cervical dislocation, the spleen was dissected, and mononuclear cell suspensions were prepared. Cells were cultured in either anti-CD3 MoAb, 145-2C11, recognizing the ϵ chain of the murine CD3 complex (the kind gift of Dr J. Bluestone, University of Chicago, Chicago, IL), or concanavalin A (Con A, Sigma), in the presence or absence of IL-2-containing rat Con A supernatant, as described below.

T-cell proliferation studies. Human PBMC were isolated, washed, plated at 10^5 cells/well in triplicate in flat-bottomed 96-well microtiter plates (Costar, Cambridge, MA). Anti-CD3 MoAb, PHA, Con A, staphlococcal enterotoxin B (SEB, Sigma), PMA, and ionomycin, in the presence of other reagents where indicated, were added at the start of the assay, unless otherwise stated. Cultures were incubated at 37°C with 5% CO₂ in a humidified atmosphere for 72 hours and labeled for the final 18 hours with 1 μCi/well [³H]-thymidine, and harvested for incorporated radioactivity.

Cell surface immunofluorescence and flow cytometry. Cells were washed twice in PBS containing 2% heat-inactivated FBS and 0.02% sodium azide (PBS-azide). Approximately 5 × 10⁵ cells were incubated on ice in the dark with saturating concentrations of detecting MoAb. After 30 minutes of incubation, the cells were washed twice with PBS-azide and then incubated with 5 μL of fluorescein (FITC)-conjugated goat F(ab′), anti-mouse IgG and IgM antibody (Tago, Burlingame, CA) for another 30 minutes in the dark. The cells were again washed twice and fixed with 1% paraformaldehyde. Cells were stored at 4°C in the dark until analysis on an EPIC V Coulter counter.

Stimulation of IL-2 production. PBMC from normal controls, 3 × 10⁵ cells/well, were cultured in 0.2 mL complete medium alone or in the presence of anti-CD3 MoAb (OKT3) in the absence or presence of an iron chelator in flat-bottom 96-well microtiter wells (Costar). Cells were cultured for 18 hours at 37°C in humidified atmosphere of 5% CO₂ in 95% air. In other experiments, the T-lymphoblastoid cell line Jurkat, 2 × 10⁵ cells/well, were cultured with media, OKT3, or OKT3 plus PMA in the presence or absence of iron chelator in duplicate in 96-well flat bottom plates (Costar) for 24 hours. Culture supernatants were collected, frozen at −80°C to lyse residual cells, and thawed. IL-2 activity was assessed by the ability to support proliferation of an IL-2-dependent murine cell line, CTLL-2. 25 CTLL-2, 5 × 10⁴ cells/well, was cultured with supernatants in duplicate in 96-well flat-bottom plates and proliferation was assessed by the incorporation of [³H]-thymidine during a 4-hour pulse following a 20-hour incubation. Excess concentrations of FeCl₃ were added at the time of CTLL-2 assay to neutralize the effect of iron chelators present in the supernatants. The amount of IL-2 present in the test supernatant was assessed by the [³H]-thymidine incorporation of CTLL-2 cells relative to a standard rat Con A supernatant.

Assay for CTL activity. The ⁵¹Cr release assay was performed in triplicate in V-bottom microtiter wells (Nunc, Roskilde, Denmark). Effector cells were counted and added in threefold dilutions in the presence or absence of iron chelator. Target cells, preincubated with 0.1 mCi of ⁵¹Cr (Na ⁵¹CrO₄, New England Nuclear, Boston, MA) for 2 hours, were washed three times, and added at 10⁶ cells/well. Microtiter plates were centrifuged at 200 × g for 5 minutes and incubated for 4 hours at 37°C in 5% CO₂. After incubation, the plates were again centrifuged. One hundred and twenty-five microliters of supernatant was harvested, and counted in a Packard gamma counter.

Mixed lymphocyte reactivity. PBMC, 1.5 × 10⁵ cells/well, were cocultured in 96-well flat-bottom plates (Costar) with irradiated (4.2 Gy from a cesium source) allogeneic stimulator cells, 1.5 × 10⁵ cells/well, derived from normal Leukopak donors. Cultures were incubated at 37°C for 6 days, labeled for the final 18 hours with 1 μCi/well [³H]-thymidine, and harvested for incorporated radioactivity.

Cell surface immunoassay of Fe. In other experiments, the expression of Fe was assessed by the ability to support proliferation of an IL-2-dependent murine cell line, CTLL-2. 25 CTLL-2, 5 × 10⁴ cells/well, was cultured with supernatants in duplicate in 96-well flat-bottom plates and proliferation was assessed by the incorporation of [³H]-thymidine during a 4-hour pulse following a 20-hour incubation. Excess concentrations of FeCl₃ were added at the time of CTLL-2 assay to neutralize the effect of iron chelators present in the supernatants. The amount of IL-2 present in the test supernatant was assessed by the [³H]-thymidine incorporation of CTLL-2 cells relative to a standard rat Con A supernatant.
counter to determine $^{51}$Cr radioactivity released. The specific lysis is calculated by the formula: % cytotoxicity = 100 \{(cpm experimental release - cpm spontaneous release)/(maximum cpm release - cpm spontaneous release)\}.

Determination of $[Ca^{2+}]$. The T-lymphoblastoid cell line Jurkat or PBMC from normal volunteers were incubated with 5 $\mu$mol/L of the acetoxymethyl ester of the calcium sensitive fluorophore Indo-1 (Molecular Probes, Junction City, OR) for 45 to 60 minutes at 37°C on a shaker platform. The Indo-1 loaded cells were washed twice, resuspended in complete medium and stored in the dark at 37°C until analysis. Cytoplasmic calcium was monitored by flow cytometry using an Epics V Coulter as described. The ratio of fluorescence emissions, 405 nm/480 nm, reflects the relative $[Ca^{2+}]$, and is plotted on the X-axis, cell number on the Y-axis, as a function of time on the Z-axis. The resting relative $[Ca^{2+}]$, was measured and is displayed as the first 30 seconds of each panel, after which anti-CD3 MoAb, 1:1000 dilution of ascites fluid, was added. Responses were monitored for 7 minutes. Temperature was maintained at 37°C.

RESULTS

Like deferoxamine, desferrithiocin inhibits T-cell proliferation. Human PBMC were incubated with the mitogenic anti-CD3 MoAb OKT3 and various concentrations of either deferoxamine or desferrithiocin (Fig 2A). Both iron-chelating agents caused a marked and dose-dependent inhibition of proliferation as measured by the incorporation of $[^{3}H]$-thymidine. Concentrations of 10 $\mu$g/mL of either agent completely suppressed T-cell proliferation.

Like deferoxamine, desferrithiocin inhibited proliferation stimulated not only by anti-CD3 MoAb, but also by the mitogens PHA and Con A (data not shown) and by the toxin staphylococcal enterotoxin B (SEB) (Fig 2B). Similarly, stimulation of PBMC proliferation by the combination of PMA and ionomycin, which together bypass the requirement for receptor expression at the cell surface, was also inhibited by the addition of either deferoxamine or desferrithiocin (Fig 2C). The concentration of each iron chelator required to inhibit cell proliferation was similar regardless of the mitogenic stimulus.

The anti-proliferative effects of desferrithiocin and deferoxamine were not species-specific. Murine spleen cells were isolated by density gradient centrifugation and stimulated with either Con A or the anti-CD3 MoAb 145-2C11 in the presence or absence of rat Con A-stimulated supernatant (RCM) as a source of exogenous growth factors. Both desferrithiocin and deferoxamine inhibited the proliferation of murine spleen cells as effectively as that of human resting peripheral blood leukocytes (data not shown).

Desferrithiocin-induced inhibition of proliferation is reversed by iron. To ensure that the iron-chelating ability of desferrithiocin was responsible for the inhibition of PBMC proliferation, human cells were incubated with anti-CD3 MoAb, desferrithiocin or deferoxamine at varying concentrations, and excess concentrations of ferrous chloride (Fig 3). The addition of ferrous chloride completely reversed the inhibition of proliferation induced by either desferrithiocin (Fig 3A) or deferoxamine (Fig 3B) at all concentrations except 100 $\mu$g/mL, at which concentration modest inhibition of proliferation persisted. It appeared that these compounds were toxic to the cells at the 100 $\mu$g/mL concentration. For future studies, a concentration of 10 $\mu$g/mL deferoxamine or desferrithiocin was chosen.

The addition of ferrous chloride to cultures stimulated not only with anti-CD3 MoAb but also with PHA, PMA plus ionomycin, or SEB effectively reversed the inhibition of proliferation observed with desferrithiocin or deferoxamine (data not shown). In contrast, the addition of other metal salts, including MgCl$_2$, ZnSO$_4$, CuSO$_4$, or CaCl$_2$ failed to restore proliferation in desferrithiocin or deferoxamine-treated cultures, at concentrations that had little or no direct inhibitory effect to the cultures (data not shown).

Mitogen-induced stimulation of proliferation was measured at 72 hours after initiation of culture. Inhibition of
anti-CD3 MoAb-induced proliferation by the iron chelators could be reversed if ferrous chloride was added as late as 24 hours after the addition of anti-CD3 MoAb (Fig 4). Therefore, the iron chelators were not directly toxic to the cells, and proliferation could be restored after long exposure to either agent. In addition, PBMC pretreated with either desferrithiocin or deferoxamine for two hours, washed, and then stimulated with either anti-CD3 MoAb or PHA were able to proliferate comparably to control cultures, suggesting that the effects of the agents were fully reversible (data not shown).

Desferrithiocin and deferoxamine inhibit lymphocyte proliferation after transmembrane signaling and after expression of activation antigens. The interaction of T cells with antigen, mimicked by binding anti-CD3 MoAbs to the TcR-CD3 complex or by lectins such as PHA, triggers T-cell activation. Activation is associated with an early rise in the concentration of intracellular calcium ([Ca^{2+}]_i) and hydrolysis of phosphatidylinositol bisphosphate, generating diacylglycerol and inositol triphosphate (reviewed in 28-30), which, in turn, induces release of calcium from intracellular stores. These early signals are associated with transcriptional activation leading to interleukin-2 (IL-2) receptor expression, lymphokine production, and other T-cell effector functions. The early biochemical events were modeled by the human T-lymphoblastoid cell line Jurkat, and the effect of desferrithiocin and deferoxamine on T-cell activation was analyzed.

Jurkat cells were preincubated for two hours with either desferrithiocin or deferoxamine, each at 10 μg/mL, and then loaded with the calcium sensitive fluorophore Indo-1 AM. The relative [Ca^{2+}]_i was measured by the ratio of fluorescence emission at 405 nm (Ca^{2+}-bound) to 480 nm (Ca^{2+}-free) using flow cytometric analysis and was plotted versus time. Despite 2-hour preincubation, the addition of either agent failed to inhibit an early rise in [Ca^{2+}]_i induced by triggering the TcR-CD3 complex with anti-CD3 MoAb (Fig 5). Similarly, neither desferrithiocin (10 μg/mL) nor deferoxamine (10 μg/mL) inhibited the characteristic rise of [Ca^{2+}]_i stimulated by PHA in freshly isolated PBMC despite complete inhibition of mitogen-induced proliferation at this concentration (data not shown).

It has been previously shown that the T-cell stimulation is associated with the expression of activation antigens, including the IL-2 receptor. The expression of cell surface activation antigens on PBMC cultured in the presence of PHA in the presence or absence of desferrithiocin and deferoxamine was determined. The addition of either agent did not affect the induction of IL-2 receptor (CD25) expression by PBMC cultured for 3 days with PHA (Fig 6). Similarly, neither the mean fluorescence intensity nor the percentage of positively
Fig 5. Lack of inhibition by either desferrithiocin or deferoxamine on the relative intracellular calcium ([Ca^{2+}]_i) response of Jurkat cells to anti-CD3 MoAb. Jurkat cells were preincubated for two hours at 37°C in the absence (panel A) or presence of 10 µg/mL desferrithiocin (panel B) or 10 µg/mL deferoxamine (panel C). Cells were then loaded with the calcium-sensitive fluorophore Indo-1 AM as described in Materials and Methods. Cytoplasmic calcium was monitored by flow cytometry by the ratio of fluorescence at 405 nm to 480 nm, which reflects the relative [Ca^{2+}]_i. An increase in the ratio corresponds to an increase in [Ca^{2+}]. The ratio, plotted on the X-axis, and cell number, plotted on the Y-axis, are shown as a function of time on the Z-axis. The resting relative [Ca^{2+}]_i was measured and is displayed as the first 30 seconds of each panel, after which anti-CD3 MoAb, 1:1000 dilution of ascites fluid was added. Responses were monitored for 7 minutes. Temperature was maintained at 37°C.

staining cells for CD25, HLA-DR, CD3, or CD2 was significantly affected by culture with either desferrithiocin or deferoxamine (data not shown).

The production of IL-2 is not affected by desferrithiocin or deferoxamine. The production of IL-2 by PHA-stimulated PBMC may be assessed by determining the ability of stimulated culture supernatants to support the growth of an IL-2 dependent cell line CTLL-20. To this biologic assay, excess FeCl₃ was added to neutralize the effect of the iron chelators on the proliferation of CTLL-20. There was no significant difference between the IL-2 content of PHA-stimulated cultures in the absence (112 U/mL) or presence of either 10 µg/mL desferrithiocin (125 U/mL) or 10 µg/mL deferoxamine (134 U/mL). The addition of exogenous recombinant IL-2 to mitogen-stimulated PBMC did not restore proliferation inhibited by either desferrithiocin or deferoxamine (Table 1). Furthermore, the addition of human conditioned medium containing cytokines in addition to IL-2 also failed to restore proliferation, suggesting that a deficiency of another required growth factor besides IL-2 was not likely to be responsible for the inhibition of proliferation (data not shown). Taken together, these data suggest that neither desferrithiocin nor deferoxamine inhibit early measures of transmembrane signaling such as a rise in [Ca^{2+}]_i, or later events such as IL-2 receptor expression and IL-2 production.

Given that these early events in T-cell activation did not appear to be influenced by iron chelation, we sought to determine how long after initiation of culture inhibition of proliferation by desferrithiocin or deferoxamine could be demonstrated. PBMC were stimulated with anti-CD3 MoAb, and desferrithiocin or deferoxamine was subsequently added at later, varying time intervals. All cultures were pulsed with [³H]-thymidine at 72 hours and harvested 8 hours later. Greater than 50% suppression of proliferation was observed if either agent was added up to 50 hours after initiation of the

Fig 6. Single parameter immunofluorescence of responding PBMC upon stimulation with PHA. After 3 days, PBMC cultured with PHA (2 µg/mL) in medium alone (A), desferrithiocin (10 µg/mL) (B), or deferoxamine (10 µg/mL) (C), were stained with anti-CD25 (anti-Tac) MoAb followed by FITC-conjugated goat anti-mouse F(ab')₂. Stained cells are shown in comparison with cells stained with FITC-goat anti-mouse F(ab')₂ alone.
DESFERRITHIOCIN AND T CELL FUNCTION

Table 1. Inhibition of Stimulation Is Not Reversed by Recombinant IL-2

<table>
<thead>
<tr>
<th>[3H]-Thymidine Incorporation (cpm)</th>
<th>Medium</th>
<th>OKT3</th>
<th>PHA</th>
<th>PMA + Ionomycin</th>
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<tbody>
<tr>
<td>Medium</td>
<td>450</td>
<td>62,030</td>
<td>87,650</td>
<td>41,200</td>
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<tr>
<td>- rIL-2</td>
<td>5,000</td>
<td>78,520</td>
<td>103,780</td>
<td>53,400</td>
</tr>
<tr>
<td>- FeCl₂</td>
<td>450</td>
<td>37,670</td>
<td>112,860</td>
<td>80,450</td>
</tr>
<tr>
<td>Desferrithiocin</td>
<td>240</td>
<td>370</td>
<td>1,010</td>
<td>610</td>
</tr>
<tr>
<td>- rIL-2</td>
<td>180</td>
<td>830</td>
<td>1,070</td>
<td>200</td>
</tr>
<tr>
<td>- FeCl₂</td>
<td>370</td>
<td>17,630</td>
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<td>Deferoxamine</td>
<td>340</td>
<td>730</td>
<td>720</td>
<td>390</td>
</tr>
<tr>
<td>- rIL-2</td>
<td>260</td>
<td>440</td>
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</tr>
<tr>
<td>- FeCl₂</td>
<td>540</td>
<td>37,070</td>
<td>120,610</td>
<td>84,740</td>
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</table>

PBMC were cultured in medium or anti-CD3 MoAb, OKT3 1:40,000 dilution of ascites fluid, PHA 2 μg/mL, or PMA 10 ng/mL plus ionomycin 0.5 μM/L in the absence or presence of desferrithiocin, 10 μg/mL, or deferoxamine, 10 μg/mL. Cultures were harvested at 72 hours after a 9-hour pulse with [3H]-thymidine as described in Materials and Methods.

Because mixed lymphocyte reactivity (Fig 7). No significant effect was observed if either agent was added at 60 hours or later. The data demonstrate that iron chelation can inhibit an ongoing proliferative response.

Like deferoxamine, desferrithiocin inhibited mixed lymphocyte reactivity. Because mixed lymphocyte reactivity depends on proliferation, we wished to determine whether this reactivity was also affected by the presence of iron chelation. Freshly isolated PBMC were stimulated with irradiated allogeneic stimulator cells, and harvested for proliferation on day 6. Both desferrithiocin and deferoxamine markedly suppressed DNA synthesis, an inhibition that was reversed by the addition of ferrous chloride (data not shown).

The effect of desferrithiocin on cell-mediated cytotoxicity was tested. Cells were stimulated by the stimulator cell line, JY, an Epstein-Barr virus-transformed human B-cell line. At six days, there was no generation of cytolytic T-cell ability in the presence of either desferrithiocin, 33 μg/mL, or deferoxamine, 33 μg/mL. (Fig 8). Thus, iron chelation inhibited the development of a mixed lymphocyte response and of CTL effector cells.

Iron chelation did not inhibit CTL or NK-mediated cytotoxicity. Because iron chelation inhibited the generation of CTL activity from naive T cells, we tested whether desferrithiocin inhibited cytotoxicity of preformed CTL. The ability of a long-term CTL line to kill its target cell was not inhibited by the addition of the iron chelator (Table 2). Similarly, freshly isolated PBMC were able to kill the natural killer (NK)-sensitive erythroblastoid cell line K562 in the presence of the iron chelator (Table 2). Therefore, neither desferrithiocin (Table 2) nor deferoxamine (not shown) inhibited CTL- or NK-mediated cytotoxicity.

Iron chelation inhibits proliferation of spontaneously dividing, and factor-independent cell lines. To confirm that the inhibition of proliferation was not secondary to any exogenous factor required for DNA synthesis besides iron, we tested the ability of desferrithiocin and of deferoxamine to inhibit proliferation of spontaneously dividing cells. The T-lymphoblastoid cell line Jurkat, the EBV-transformed cell

Table 2. Effect of Desferrithiocin on Cytolytic Ability

<table>
<thead>
<tr>
<th>Desferrithiocin (μg/mL)</th>
<th>NK Effector Cells</th>
<th>CTL Effector Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>46</td>
<td>76</td>
</tr>
<tr>
<td>0.1</td>
<td>52</td>
<td>80</td>
</tr>
<tr>
<td>1</td>
<td>53</td>
<td>73</td>
</tr>
<tr>
<td>10</td>
<td>49</td>
<td>75</td>
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Cytolytic activity is expressed as percent specific 61Cr release in four hours as described in Materials and Methods. NK effector cells, freshly isolated PBMC, were used at a ratio of 33:1 against 51Cr-labeled K562 cells. The CTL effector cell line was used at a ratio of 6:1 against 51Cr-labeled JY cells.
line JY, and the erythroleukemia cell line K562 were each cultured in the absence or presence of the iron-chelating agents. Like deferoxamine, desferriothiocin inhibited, in a dose-dependent fashion, the proliferation of constitutively dividing, and factor-independent cell lines (data not shown).

**DISCUSSION**

In this report, we have shown that the oral agent desferriothiocin, like deferoxamine, inhibited, in a dose-dependent fashion, mitogen- and lectin-induced proliferation of both human and murine T cells. The inhibition was reversed by ferrous chloride, but not by other metal salts, recombinant IL-2, or human conditioned medium. Desferriothiocin also inhibited proliferation of spontaneously dividing, and factor-independent, EBV-transformed B-cell and leukemic T-cell lines. Similar concentrations of iron chelator was required to inhibit proliferation of PBMC and transformed B- and T-cell lines. While the iron chelators inhibited both the generation of a mixed lymphocyte response and the induction of CTL activity, they did not inhibit CTL- or NK-induced cytotoxicity. The agents did not inhibit the expression of activation antigens, such as the IL-2 receptor, transferrin receptor, or HLA-DR on T cells, or early measures of T-cell activation such as influx of [Ca+]2. The effect of iron chelation was nontoxic and reversible; saturating concentrations of iron could reverse the inhibition of proliferation if added as late as 24 hours after initiation of culture. Thus, desferriothiocin, like deferoxamine, is a potent and reversible inhibitor of T-cell proliferation. This anti-proliferative effect inhibits T-cell differentiation and CTL formation, but T-cell functions such as IL-2 production and the function of preformed cytotoxic cells are preserved.

The studies reported here extend previous reports of the effect of iron chelation on T-cell function in vitro. Iron chelators inhibit proliferation and differentiation of naive T cells, but do not inhibit either lymphokine release or CTL- or NK-mediated killing of preformed or mature T cells. Thus, iron chelation does not eliminate the T-cell response, but rather limits the development of and recruitment of new alloreactive or autoreactive T cells. Previous studies have shown that deferoxamine inhibits DNA synthesis, PBMC proliferation, and the MLR. Our data are consistent with those previously reported, which indicate that the agent acts, at least in part, as an inhibitor of ribonucleotide reductase; the latter uses iron as a coenzyme and is essential for DNA synthesis. Our data do not distinguish between inhibition of cell cycle progression from G0 to G1 and a block in early S phase.

The addition of deferoxamine, 10 µg/mL or 15 µmol/L, (and of desferriothiocin) to PHA-stimulated PBMC did not inhibit IL-2 production or IL-2 receptor expression. This latter observation is in disagreement with that reported for the effect of deferoxamine on PHA-stimulated PBMC by Lederman et al. These authors used deferoxamine at a concentration of 250 µg/mL or 360 µmol/L, a concentration that, in our hands, appears to be toxic to the cells.

Somewhat higher concentrations (33 µg/mL) of either desferriothiocin or deferoxamine were required to completely inhibit the generation of CTL-mediated cytotoxicity than to inhibit T-cell proliferation (10 µg/mL). Whether this relates to residual natural killer (NK) activity, which is not inhabitable by desferriothiocin or deferoxamine (Table 2), in the culture remains to be determined.

Desferriothiocin appears to be effective at a concentration of 10 µg/mL, or 38 µmol/L, while deferoxamine is effective at the same concentration, 10 µg/mL, or 15 µmol/L. Deferoxamine is able to bind an equimolar concentration of iron to yield the stable complex ferrioxamine. Two molecules of deferrithiocin are, however, required to bind one molecule of iron. Therefore, the molar concentration of desferriothiocin required to bind one mole of iron is twice that of deferoxamine. This explains the apparent discrepancy between the molar efficacy of the two compounds.

The immunomodulating properties of deferoxamine have been exploited in vivo to inhibit graft rejection and autoreactive T cells. Using a model of murine pancreatic allografts, deferoxamine administration appeared to reduce chronic islet cell rejection. The administration of continuous deferoxamine reduced the severity and duration of experimental allergic encephalomyelitis in rats, a cell-mediated autoimmune demyelinating disease. Delayed type hypersensitivity was diminished and T-cell number was reduced in the tissue lesions, suggesting that the immunosuppressive properties of the agent might be responsible for the attenuation of the disease. Administration of deferoxamine has been used to treat graft-versus-host disease in human bone marrow transplant allograft recipients. Furthermore, deferoxamine has been used to treat a patient with refractory acute leukemia, demonstrating its antiproliferative effect on dividing cells. In this patient, the suppression of blast colony growth in vitro and treatment in vivo were accompanied by differentiation from lymphoid to myelomonocytic surface antigen expression. Bioavailability after oral administration is a unique property of desferriothiocin. Oral delivery would make desferriothiocin an attractive alternative to deferoxamine as an immunosuppressant and, possibly, as a cytoreductive agent.

In an iron-loaded Cebus monkey model, desferriothiocin was found to have significant activity as an oral chelator. But chronic administration of this powerful chelator desferriothiocin to non-iron-overloaded mice and rats is known to be toxic perhaps because essential iron stores are compromised. Careful study of dose and of schedule will be required if this orally active chelator desferriothiocin is to emerge as potential therapy not only for iron-overload but also for a variety of inflammatory conditions.

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