Specific Toxicity of 2-Chlorodeoxyadenosine Toward Resting and Proliferating Human Lymphocytes

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2-Chlorodeoxyadenosine (CdA), an adenosine-deaminase-resistant purine deoxynucleoside, is markedly toxic toward human T-lymphoblastoid cell lines in vitro and is an effective agent against L1210 leukemia in vivo. The present studies have examined the toxicity, and in some cases, metabolism, of CdA in (1) multiple established human cell lines of varying phenotype, (2) leukemia and lymphoma cells taken directly from patients, (3) normal bone marrow cells, and (4) normal peripheral blood lymphocytes. Nanomolar concentrations of CdA blocked the proliferation of lymphoblastoid cell lines with a high ratio of deoxycytidine kinase to deoxynucleotidase. The drug had virtually no effect on the growth of cell lines derived from solid tissues. The CdA inhibited the spontaneous uptake of tritiated thymidine by many T and non-T, non-B acute lymphoblastic leukemia cell specimens at concentrations \( \leq 5 \ nM \). The same concentrations did not impair either thymidine uptake or granulocyte-monocyte colony formation by normal bone marrow cells. In common with deoxyadenosine, but unlike several other agents affecting purine and pyrimidine metabolism, CdA was lethal to resting normal T lymphocytes and to slowly dividing malignant T cells. In both resting and proliferating lymphocytes, the CdA was phosphorylated by deoxycytidine kinase and entered a rapidly turning over nucleotide pool. Dividing lymphocytes also incorporated abundant CdA into DNA. The selective toxicity of CdA toward both dividing and resting lymphocytes may render the drug useful as an immunosuppressive or antileukemic agent.

An Inherited Deficiency of Adenosine Deaminase (ADA, adenosine aminohydrolase, E.C.3.5.4.4), selectively impairs lymphocyte growth and function in human beings, while sparing other tissues. Several studies have implicated deoxyadenosine nucleotides in the pathogenesis of the immunodeficient state. It has been proposed that lymphospecific toxicity in ADA-deficient patients might result from the preferential uptake of deoxyadenosine by T lymphocytes with high deoxyadenosine phosphorylating activity, and low deoxyadenosine monophosphate (deoxyAMP) dephosphorylating activity. The resulting accumulation of deoxyadenosine 5'-triphosphate (dATP) is lethal to both dividing and resting lymphocytes, probably via more than one mechanism.

It is conceivable that analogs of deoxyadenosine resistant to cellular deamination might mimic the ADA-deficient state and selectively harm human lymphocytes at concentrations that do not affect other cell types. In an earlier study, one such agent, 2-chlorodeoxyadenosine (CdA), inhibited the growth of malignant human T-lymphoblastoid cell lines at concentrations in the nanomolar range. The toxicity of the drug required phosphorylation by deoxycytidine kinase. Optimal dosages of CdA also yielded 50% long-term survivors in the murine L1210 lymphocyte leukemia system. These results have prompted the suggestion that CdA and related 2'-deoxyribonucleosides may have the necessary selectivity for neoplastic cells to be clinically useful as anticancer or immunosuppressive agents.

To determine the potential lymphocyte-specific action of CdA, we have studied the effects of the deoxyribonucleoside on (1) multiple established human cell lines of varying phenotype, (2) leukemia and lymphoma cells taken directly from patients, (3) normal bone marrow cells, and (4) normal peripheral blood lymphocytes. The results document that CdA can inhibit the proliferation and survival of malignant T and non-T, non-B lymphocytes at concentrations that spare normal bone marrow cells and other cell types. Moreover, the CdA was lethal to resting lymphocytes. The latter effect distinguished the deoxyribonucleoside from several other anticancer agents that modify purine or pyrimidine metabolism.

Materials and Methods

Synthesis of CdA

CdA was prepared by transfer of the deoxyribose moiety from thymidine to 2-chloro adenine, catalyzed by a partially purified transdeoxyribosylase from Lactobacillus helveticus, followed by ion exchange chromatography, as previously described. Purity was confirmed by spectral analysis and by high performance liquid chromatography (HPLC). The CdA was stored at \(-20^\circ C\) as a 1 mg/ml solution in isotonic normal saline. Under these conditions, there was no detectable breakdown of the deoxyribonucleoside over 1 month.

Similarly, [8-3H]-chloroadenine was reacted with thymidine in


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the presence of the transdeoxyribosylase to yield \([8-3H]\)-CdA. The \([8-3H]\)-chloroadenine had been prepared from \([8-3H]\)-2-chloroadenosine (11 Ci/m mole, Moravek Biochemicals, City of Industry, CA) by the action of adenosine phosphorylase from *Bacillus subtilis.*17 CdA labeled with tritium in the purine ring, and with carbon-14 in the deoxyribose moiety, was made by reacting \([U-\text{14C}]\)deoxycytidine (470 mCi/m mole, Amersham, Arlington Heights, IL) with \([8-3H]\)-2-chloroadenine. All radiolabeled compounds were purified by reverse-phase HPLC prior to use.

Cell Lines

We measured the growth inhibitory effects of CdA toward 10 established cell lines growing in suspension, and seven cell lines growing as monolayers. These included four Epstein-Barr viral transformed normal lymphocyte cell lines, malignant lymphocytes (CCRF-CEM, HPB-ALL, NALL-1, Louckes), malignant hematopoietic cells (K562, HL60), normal fibroblasts (R7000), malignant neural cells (Te671, A172, H2), malignant melanoma cells (Hs695, A375), and breast cancer cells (Hs578). The source of the cell lines, their phenotypic characterization, and their maintenance in tissue culture have been described.18

The effects of CdA on the growth of suspension cultures were estimated as described previously.13-14 To measure the effects of CdA on the growth of monolayer cultures, 105 cells in regular medium supplemented with 1 nmol-1 µM CdA were dispersed in 0.2-ml aliquots in tissue culture trays. After 6-days incubation at 37°C, the increase in cell number was estimated microscopically. Additionally, 1 µCi of \([\text{methyl-3H}]\)-thymidine(ICN Corp., Irvine, CA, specific activity 77 Ci/m mole) was added to each well. Four hours later, the medium was removed and replaced with 0.1% trypsin (Flow Labs., Rockville, MD) to detach the cells. Thereafter, they were collected on glass fiber filters, washed, and radioactivity was measured in a scintillation counter.

Enzyme Assays

Deoxycytidine kinase and deoxynucleotidase activities in cell extracts from suspension cultures were assayed as previously described.45

Effect of CdA on Leukemia Cells and Normal Bone Marrow Cells From Patients

Normal and leukemic bone marrow cells were obtained by aspiration during routine diagnostic procedures. Marrow progenitor and leukemic cells were obtained by centrifugation on Ficoll-Hypaque density gradients.19,20 After the cells were washed twice in RPMI 1640 medium, they were incubated in microtiter plates at a density of 104/ml in the same medium supplemented with 10% FBS, containing various concentrations of CdA. After overnight incubation, 0.1 µCi \([\text{methyl-3H}]\)-thymidine was added to each well. Two hours later, the effects of CdA on the incorporation of radioactivity into macromolecular material was determined as described above for cell lines.

Granulocyte-macrophage colonies (CFU-GM) from normal adult bone marrow were grown in 0.3% agar in medium supplemented or not with CdA, as described by Taettle et al.26 The number of colonies was enumerated microscopically after 10-day incubation.

Immunologic Classification of Acute Leukemia

The surface antigenic phenotype of the isolated leukemia cells was determined by immunofluorescent staining with a panel of monoclonal antibodies, as described earlier.21

Effects of CdA on Resting Human Lymphocytes

The toxicity toward resting human peripheral blood lymphocytes of CdA and other drugs affecting purine and pyrimidine metabolism was determined as previously described for deoxycytidine.19 Briefly, peripheral blood mononuclear cells from normal subjects, suspended at a density of 108/ml in RPMI 1640 medium supplemented with 2 mM l-glutamine, 20% autologous plasma, and twofold increasing concentrations of the respective agents, were dispersed in microtiter trays in 0.2-ml aliquots. After various time periods, nonadherent cells were removed, and the percentages of cells excluding 0.1% trypan blue were enumerated in a hemocytometer.

Fresh malignant T cells from a surgically removed mediastinal lymph node from a patient with mycosis fungoides were teased apart from stromal tissue, isolated by Ficoll-Hypaque centrifugation, and cultured as described for normal lymphocytes.

Metabolism of CdA

To measure incorporation of CdA into nucleotides and nucleic acid, either 105 proliferating CCRF-CEM T lymphoblasts, CCRF-CEM mutants deficient in deoxycytidine kinase,22 or 8 x 107 peripheral blood mononuclear cells were incubated overnight in 8 ml complete medium supplemented with 200 nM \([8-3H]\)-CdA, or with 15 nM \([\text{methyl-3H}]\)-thymidine. After washing the cells 3 times in balanced salt solution, an aliquot was counted, while another was extracted with 1 ml of 0.4 M perchloric acid. After 4 washes in ice-cold 0.4 M perchloric acid, the supernatants were collected, neutralized, and frozen. Subsequently, the nucleotides were separated by HPLC on a Whatman Partisil-SAX column eluted with a gradient, as described.14 The nucleic acid in similarly incubated CCRF-CEM or peripheral blood lymphocyte cultures was extracted by the method of Favaloro,21 as modified by Maniatis.22 Following digestion with either pancreatic deoxyribonuclease-I or with ribonuclease-A (both from Sigma, St. Louis, MO), the nucleic acid was extracted with phenol/chloroform and was reprecipitated with ethanol.23 The incorporation of \([8-3H]\)-CdA into DNA and RNA was measured in a liquid scintillation spectrometer and yielded comparable results with the two enzymatic digestion methods.

To measure the catabolism of CdA phosphates by resting peripheral blood lymphocytes, cells previously incubated with 20 nM \([8-3H]\)-CdA for 24 hr were washed and resuspended at a density of 5 x 108/ml in fresh medium lacking CdA. At various times thereafter, aliquots of the cells were centrifuged, and the radioactivity in the supernatant and pellet was measured.

RESULTS

Toxicity of CdA Toward Dividing Cells

Figure 1 illustrates the concentrations of CdA inhibiting by 50% the proliferation of 10 different human cell lines growing in suspension culture. The 5 sensitive cell lines all had substantial deoxycytidine kinase levels (0.17-0.28 nmole/min/mg protein), and low deoxynucleotidase levels (0.9-4.0 nmole/min/mg protein), expressed in Fig. 1 as a ratio of the two enzymatic activities. In this group were the malignant lymphoblastoid cell lines CCRF-CEM, HPB-ALL, NALL-1, and Louckes, and the promyelocytic leukemia cell line HL60. A 24-hr exposure to CdA was lethal to these cell lines, which never resumed growth after removal of the nucleoside and resuspension in fresh medium (re-
inhibited spontaneous thymidine uptake by 50% in 40 leukemic and 20 normal bone marrow cell suspensions. Notably, 12 of 20 acute lymphoblastic leukemia (ALL) cell suspensions that bore the common acute lymphocytic leukemia antigen (CALLA +), and 4 of 5 T and pre-T acute lymphoblastic leukemia cell preparations, were more sensitive to the inhibitory effects of CdA than any normal bone marrow (ID50 > 200 nM). On the other hand, the pre-B-cell acute lymphocytic leukemias and the acute myelocytic leukemias (AML) varied in their sensitivity to CdA from 2 nM to more than 50 nM. When analyzed by the nonparametric 2 sample rank test, the sensitivity to CdA of the CALLA-positive ALL specimens and the T and pre-T ALL specimens differed significantly from normal bone marrow (p < 0.01 in each case).

The effects of continuous exposure to CdA on granulocyte-monocyte colony formation (CFU-GM) by normal bone marrow cells were measured by Dr. Raymond Taetle. When compared to control cultures, 1 nM and 10 nM CdA actually enhanced CFU-GM formation by 23%–35%. The basis for this phenomenon was not investigated further. One hundred nanomolar CdA suppressed CFU-GM generation by 60%. In the presence of 1 μM CdA, no colonies were visible.

**Toxicity of CdA Toward Nondividing T Lymphocytes**

CdA was lethal toward resting normal human peripheral blood lymphocytes maintained in autologous plasma (Fig. 3). Cell killing was a function of the CdA concentration and the duration of exposure to the deoxyribonucleoside. Thus, peripheral blood lymphocytes incubated with 100 nM CdA for 1 day and then washed retained normal viability, and indeed, responded normally to the mitogen phytohemagglutinin (not shown). In contrast, 8 nM CdA killed 60% of the cells after 7 days incubation. The malignant lymph node cells from a patient with mycosis fungoides were approximately as sensitive as normal lymphocytes to CdA toxicity (Fig. 4). The mycosis fungoides cells had low levels of spontaneous thymidine uptake (1 pmole/10⁶ cells/24 hr) and were relatively resistant to cytosine arabinoside (ID₅₀ = 256 nM). As shown in Table 1, the toxicity of CdA toward nondividing or slowly dividing lymphocytes distinguished it from several other clinically useful anticancer agents affecting purine and pyrimidine metabolism.

**Metabolism of CdA by Dividing and Nondividing Lymphocytes**

Proliferating wild-type CCRF-CEM cells, but not mutants deficient in deoxycytidine kinase, formed the mono-, di-, and triphosphate derivatives of CdA (Table
The malignant T cells also incorporated CdA into a perchloric-acid-insoluble fraction that was sensitive to deoxyribonuclease digestion and was resistant to ribonuclease digestion. When the dividing T lymphoblasts were incubated with CdA labeled with tritium in the 8 position of the purine ring, and with carbon-14 in the deoxyribose moiety, the ratio of the two labels in the perchloric-acid-precipitated fraction was identical to the starting material (results not shown). Neither intact lymphocytes nor cell extracts detectably catalyzed CdA. These results suggest that CdA was phosphorylated by deoxycytidine kinase, converted to the 5'-triphosphate derivative, and incorporated into DNA by the proliferating T cells.

Resting normal human peripheral blood lymphocytes had nearly unmeasurable tritiated thymidine uptake (<0.3 pmoles/10^8 cells), but readily phosphorylated CdA (82 pmoles/10^8 cells) (Table 2). Only minimal amounts of radioactivity (0.36 pmoles/10^8 cells) appeared in the acid-precipitable fraction. When resting peripheral blood lymphocytes were incubated with tritiated CdA for 24 hr, and then washed and resuspended in medium lacking the nucleoside, intracellular radioactivity decreased by 60% after 1 hr (Fig. 5). Thus, the major fraction of the intracellular CdA phosphates were part of a rapidly turning over nucleotide pool.

DISCUSSION

2-Chlorodeoxyadenosine (CdA) inhibited thymidine uptake by T and null lymphoblasts from many patients with acute lymphoblastic leukemia (ALL) at concentrations (≤5 nM) that did not impair thymidine incorporation or granulocyte-monocyte colony formation by normal bone marrow cells. The CdA also blocked the proliferation of malignant hematopoietic cell lines with a high ratio of deoxycytidine kinase to cytoplasmic deoxynucleotidase. In a previous study, the combination of deoxyadenosine and the adenosine deaminase inhibitor, deoxycoformycin, was similarly toxic to cell lines with low deoxynucleotidase levels. Notably, the CdA was approximately 1,000-fold more potent than deoxyadenosine plus deoxycoformycin in blocking the proliferation of fresh ALL cells and T-lymphoblastoid cell lines.\textsuperscript{5,6,26}

<table>
<thead>
<tr>
<th>Agent</th>
<th>ID_{50} Resting Cells (nM)</th>
<th>ID_{50} Proliferating Cells (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Chlorodeoxyadenosine</td>
<td>1</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Cytosine arabinoside</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
<tr>
<td>6-Thioguanine</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Hydroxyurea</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
</tbody>
</table>

The ratio is derived from the concentration of each drug killing 50% of normal human peripheral blood lymphocytes after 5-day incubation ÷ the concentration inhibiting by 50% the growth of a T-lymphoblastoid cell line (CCRF-CEM) over a 72-hr period.
TOXICITY OF 2-CHLORODEOXYADENOSINE

Cell lines derived from normal or malignant solid tissues were relatively impervious to CdA toxicity, but had deoxycytidine kinase levels roughly within the same range as lymphoid cell lines. Levels of deoxynucleotidase in monolayer cell lines and in fresh extracts of solid tissues have proved difficult to quantitate because of variable interference by nonspecific phosphatases. Hence, one cannot yet state with certainty the biochemical basis for the resistance of monolayer cultures to the noxious effects of the deoxyadenosine analog. The application of recently described electrophoretic methods for the separation and semiquantitative measurement of deoxynucleotidase activities in crude tissue extracts may be required to ascertain the exact role of cytoplasmic deoxynucleotidase in CdA metabolism and toxicity.

The ability of a drug to inhibit in vitro thymidine uptake by ALL cells has not been shown definitely to predict in vivo efficacy. This result is not surprising, considering that only a fraction of the lymphoblasts probably synthesize DNA during the brief period of the assay. It is therefore important to emphasize that CdA (4–20 nM) was directly toxic to resting normal human peripheral blood T lymphocytes and to slowly dividing malignant T cells from a patient with mycosis fungoides. Although a 1-day exposure of dividing CCRF-CEM malignant T lymphoblasts to CdA was lethal, the toxicity of CdA toward resting lymphocytes required at least 2 days incubation with the drug. Prior to cell death, both dividing and resting lymphocytes actively phosphorylated CdA and incorporated it into a rapidly turning over nucleotide pool. In addition, the dividing T lymphoblasts actively incorporated CdA into DNA.

The exact mechanism of action of CdA remains obscure. The abundant accumulation of CdA into DNA could contribute to the drug’s antiproliferative effects, but does not explain its toxicity toward resting lymphocytes. Deoxyadenosine and several other adenine nucleoside analogs that are converted to the 5’-triphosphate derivatives are similarly injurious to nondividing lymphocytes. The various nucleotides may interfere with one or several ATP-dependent enzymatic activities that are essential for cell survival. The ultimate death of dividing and resting lymphocytes incubated with CdA and related agents may be the cumulative result of multiple drug effects.

Purine analogs that impair ATP-dependent metabolic conversions would not be expected to have clinical utility as anticancer or immunosuppressive agents because of unacceptable toxicity to metabolically active normal tissues. However, nonspecific toxicity to normal cells may not be encountered with CdA and other purine deoxyribonucleosides that resemble deoxyadenosine. As shown here, these drugs are selectively toxic toward cells with high deoxycytidine kinase levels and low deoxynucleotidase activity, independent of growth rate. Included in the CdA-sensitive population are a substantial number of malignant T and non-T, non-B leukemias and lymphomas, as well as normal T lymphocytes.

In sum total, the following constellation of metabolic characteristics may render CdA useful as an immunosuppressive or anticancer agent: (1) potent cytotoxic and cytostatic effects at nanomolar concen-

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**Table 2. Metabolism of [8-3H]-CdA by Resting and Proliferating Lymphocytes**

<table>
<thead>
<tr>
<th>Uptake (pmole/10⁶ Cells)</th>
<th>Resting Peripheral Blood Lymphocytes</th>
<th>Dividing CCRF-CEM Lymphoblasts</th>
<th>Deoxycytidine-Kinase-Deficient CCRF-CEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total nucleotides</td>
<td>82</td>
<td>553</td>
<td>ND</td>
</tr>
<tr>
<td>CdAMP</td>
<td>71%</td>
<td>68%</td>
<td></td>
</tr>
<tr>
<td>CdADP</td>
<td>8%</td>
<td>14%</td>
<td>ND</td>
</tr>
<tr>
<td>CdATP</td>
<td>21%</td>
<td>18%</td>
<td></td>
</tr>
<tr>
<td>RNA</td>
<td>0.06</td>
<td>2.0</td>
<td>ND</td>
</tr>
<tr>
<td>DNA</td>
<td>0.3</td>
<td>33.1</td>
<td></td>
</tr>
</tbody>
</table>

Eight x 10⁶ normal resting peripheral blood lymphocytes, or 10⁵ CCRF-CEM malignant T lymphoblasts, each in 8 ml complete medium, were incubated for 24 hr at 37°C with 200 nM [8-3H]-CdA. After washing and extraction, CdA incorporation into nucleoside mono-, di-, and triphosphates, and into RNA and DNA, was determined as described in Materials and Methods. ND, not detected (<0.2 pmole).
trations, (2) resistance to cellular catabolism, (3) selective toxicity toward certain normal and malignant lymphocyte populations, and (4) ability to kill equivalently nondividing and dividing cells. In principle, these properties might be most advantageous in the treatment of immune disorders mediated by long-lived helper T lymphocytes and in the elimination of slowly dividing malignant lymphocyte clones resistant to cycle-specific chemotherapeutic agents.

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