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MODULATION OF DEOXYCYTIDYLATE DEAMINASE IN INTACT HUMAN LEUKEMIA CELLS

ACTION OF 2′,2′-DIFLUORODEOXYCYTIDINE

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Abstract—Cellular metabolism studies had demonstrated previously that low cellular concentrations of 2′,2′-difluorodeoxycytidine (dFdC) nucleotides are eliminated by deoxycytidylate deaminase (dCMPD), whereas dCMPD activity is inhibited at high cellular dFdC nucleotide levels (Heinemann *et al.*, *Cancer Res* **52**: 533–539, 1992). An assay for measuring dCMPD activity in intact human leukemia cells has now been developed to permit investigations of the interactions of dFdC nucleotides with dCMPD in intact cells in which the regulated nature of this enzyme was not disrupted. Using [^{14}C]dCyd as the substrate, radioactivity that accumulated in dTTP was quantitated after high-pressure liquid chromotography by a radioactive flow detector. The assay was first characterized using either the dCMPD inhibitor tetrahydrodeoxyuridine (H_4dUrd) which directly inhibits dCMPD, or thymidine and 5-fluoro-2′-deoxyuridine (FdUrd) which indirectly inhibit and activate dCMPD, respectively, by affecting the cellular dCTP:dTTP value. Measured by this *in situ* assay, there was a strong correlation between dCMPD activity and dCTP:dTTP levels. Consistent with previous studies using partially purified enzyme, incubation of cells with dFdC resulted in a concentration-dependent inhibition of dCMPD *in situ*. The mechanism of modulation of dCMPD by dFdC, however, was clearly different from that of thymidine and FdUrd. In addition to the effect of dFdC on cellular dCTP:dTTP, our findings also suggested an additional inhibitory mechanism, possibly a direct interaction between dCMPD and dFdC 5′-triphosphate. Thus, results obtained using this direct assay of dCMPD in intact cells support the hypothesis that dCMPD is inhibited by nucleotides of dFdC.

Deoxycytidylate deaminase (dCMP aminohydrolase, dCMPD†; EC 3.5.4.12) is a key enzyme in pyrimidine deoxyribonucleotide metabolism. It is believed to play an important role in providing a balanced supply of dCTP and dTTP for DNA synthesis [1]. As illustrated in Fig. 1, cells have two pathways to make dCMP: either by reducing CDP through ribonucleoside diphosphate reductase or by the phosphorylation of exogenous dCyd by deoxycytidine kinase (dCK). Under physiological conditions, the equilibrium among dCMP, dCDP, and dCTP, which is rapidly achieved, is heavily shifted toward dCTP. Therefore, dCTP can be regarded as the substrate pool for dCMPD. After deamination, the product dUMP is converted to dTTP by the successive actions of thymidylate synthase (TS), dTMP kinase, and nucleoside diphosphate kinase. dCMPD is highly regulated in cells by its end products, dCTP and dTTP, which allosterically affect the enzyme activity in extracellular assays. Whereas dCTP serves as an activator, dTTP is an inhibitor [2–5]. Several lines of evidence also indicate that the ratio of dCTP to dTTP is responsible for regulation of dCMPD in living tissues [4, 5].

Since previous studies have indicated that there is an elevated level of dCMPD in transformed cells and tumors [6–9], there has been interest in the possibility that this enzyme may represent another target for cancer chemotherapy [10]. However, only one specific inhibitor, 3,4,5,6-tetrahydro-2′-deoxyuridine 5′-monophosphate (H_4dUMP), has been described up to the present time [11]. The precursor of H_4dUMP, 3,4,5,6-tetrahydro-2′-deoxyuridine (H_4dUrd), though shown to have limited therapeutic value by itself [10], may be used to improve the therapeutic effects of other reagents, such as ara-C [12, 13]. Alternatively, elevated dCMPD in tumors has been used as a tool for the conversion of a nontoxic nucleoside analogue (5-fluoro-2′-deoxycytidine) to a toxic antimetabolite (FdUMP) in a tumor-directed manner [14, 15].

The importance of dCMPD in cancer chemotherapy was also illustrated by studies on 2′,2′-difluorodeoxycytidine (dFdC), another dCyd analogue with antitumor activity [16]. Treatment of cells with dFdC results in accumulation of its nucleotides which are associated with specific inhibition of DNA synthesis [17]. This is attributed to both inhibition of ribonucleoside diphosphate reductase by dFdCDP and the subsequent depletion of dNTP pools [18],

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† Abbreviations: dCMPD, deoxycytidylate deaminase; CD, cytidine (deoxycytidine) deaminase; dCK, deoxycytidine kinase; TS, thymidylate synthase; TK, thymidine kinase; H_4dUrd, 3,4,5,6-tetrahydro-2′-deoxyuridine; H_4dUMP, 5′-monophosphate of H_4dUrd; H_4Urd, 3,4,5,6-tetrahydrouridine; H_4UMP, 3,4,5,6-tetrahydro-2′-deoxyuridine; dFdC, 2′,2′-difluorodeoxycytidine; dFdCDP, 5′-diphosphate of dFdC; dFdCTP, 5′-triphosphate of dFdC; and FdUrd, 5-fluoro-2′-deoxyuridine.

and to inhibition of DNA replication after incorporation of the analogue into DNA [19]. In a related study, we have shown that dFdCTP is eliminated from cells through the action of dCMPD [20]. When the intracellular dFdCTP concentration is high, however, the elimination proceeds at an extremely low level. We have also shown that dFdCTP itself is an inhibitor of dCMPD in assays of the partially purified enzyme. A self-potentiation mechanism for dFdCTP elimination was proposed based on the ability of dFdC nucleotides to affect dCMPD due to changes in dCTP:dTTP and the direct inhibition of the enzyme by dFdCTP[20]. To understand these interactions in greater detail, it is important to study the modulation of dCMPD activity by dFdC under conditions where the regulation of the enzyme is functional.

Although dCMPD has been purified and characterized extensively from several sources [2, 3, 21–24], regulation of the enzyme activity in intact cells has not been well understood. To address this problem, we developed an HPLC method, using [^{14}C]dCyd as the substrate, for measuring dCMPD in intact human leukemia cells. The results are consistent with a dual mechanism of inhibition of dCMPD by nucleotides of dFdC: one involving the dCTP:dTTP value and a second that suggests a direct action of a dFdC metabolite.

MATERIALS AND METHODS

Materials. dFdC was synthesized at Lilly Research Laboratories (Indianapolis, IN) and was supplied by Dr. L. W. Hertel. 3,4,5,6-Tetrahydrouridine (H_4Urd) was provided by Dr. Ven Narayanan, Drug Synthesis and Chemistry Branch, National Cancer Institute, Bethesda, MD. H_4dUrd was synthesized by Behring Diagnostics (La Jolla, CA) on a contract basis. [2-^{14}C]dCyd (25.2 mCi/mmol), [5-^{3}H]dCyd (20–40 Ci/mmol), [5-^{3}H]Cyd (20–40 Ci/mmol), and [8-^{3}H]dATP (10–25 Ci/mmol) were obtained from the New England Nuclear Corp. (Boston, MA). [6-^{3}H]dCyd (5–15 Ci/mmol) and [5-^{3}H]dCMP (20–25 Ci/mmol) were obtained from Moravek Biochemicals Inc. (Brea, CA). Aphidicolin, dCTP, dTTP, and other chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO). DNA polymerase I from *Escherichia coli* (10 U/μL) was obtained from the Boehringer Mannheim Corp. (Indianapolis, IN). Oligonucleotide templates for the assay of dNTPs using DNA polymerase [25] were synthesized by Genosys Biotechnologies, Inc. (The Woodlands, TX).

Cell lines. The human lymphoblastic leukemia cell line CCRF-CEM and the myeloid leukemia cell line KG-1 were obtained from the American Type Culture Collection (Rockville, MD). A thymidine kinase (TK)-deficient CCRF-CEM cell line (CEM-TK$^-$) was obtained from the AIDS Research and Reference Reagent Program (Bethesda, MD). Characterizations of cell lines CCRF-CEM and KG-1 have been described previously [26, 27]. Cells were maintained in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) supplemented with either 10% fetal bovine serum (GIBCO) for CCRF-CEM cells, or 15% fetal bovine serum for KG-1 cells. Cells were routinely checked for contamination of mycoplasma by the American Type Culture Collection and were consistently negative. Cell number and cell volume were determined using a Coulter counter (model ZM) equipped with a model C-1000 particle analyzer (Coulter Electronics, Hialeah, FL).

Assay of dCMPD activity in intact cells. To measure *in situ* dCMPD activity, cells ($1–3 \times 10^7$) in exponential growth were incubated with 0.2 μCi [^{14}C]dCyd or 2.5 μCi [6-^{3}H]dCyd in 5 mL RPMI 1640 medium without fetal bovine serum for 15 min. A non-toxic concentration of aphidicolin (1 μg/mL) was added simultaneously to inhibit incorporation of radioactivity into DNA. The reaction was stopped by adding 10 mL of ice-cold phosphate-buffered saline (containing 140 mM NaCl, 8 mM Na_2HPO_4, 3 mM KCl, and 2 mM KH_2PO_4, pH 7.4). Cells were collected by centrifugation at 5000 rpm for 5 min. Ice-cold 0.4 N $HClO_4$ (0.5 mL) was used to extract acid-soluble nucleotides. The extraction was carried out at 4° for 15 min. After removing the pellet by centrifugation, the supernatant was neutralized with KOH. Portions of the soluble extracts were analyzed using an HPLC Partisil 10-SAX column (250 mm × 4 mm; Whatman Inc., Clifton, NJ) connected with a radioactive flow detector (model A250; Packard Instrument Co., Meriden, CT). Radioactively labeled deoxynucleotides were separated by the following scheme at a flow rate of 1 mL/min: 0–10 min, isocratic 100% buffer A (0.005 M $NH_4H_2PO_4$, pH 2.8); 10–70 min, linear gradient from 100% buffer A to 100% buffer B (0.75 M $NH_4H_2PO_4$, pH 3.7); 70–75 min, isocratic 100% buffer B. The eluant was mixed with scintillation fluid (Flo-Scint IV; Packard Instrument Co.) at a ratio of 1:3. The A-200 series Flo-One/Data II software version 1.6 (Packard Instrument Co.) was used to analyze the results. The dCMPD activity index was defined as the ratio of labeled deamination products (dTTP + dUMP) divided by total labeled deoxynucleotides incorporated in acid-soluble material. Both [^{14}C]dCyd and [6-^{3}H]dCyd were successfully used in the assay. For untreated CCRF-CEM cells, the dCMPD activity index was 0.48 ± 0.06 (N = 5) after labeling with the radioactive dCyd for 15 min.

Assay of dCMPD and cytidine deaminase (CD) activities in cell extracts. CCRF-CEM and KG-1 cells in exponential growth were suspended and disrupted in extraction buffer (50 mM Tris–HCl, pH 7.5, 2 mM $MgCl_2$, 2 mM dithiothreitol, and 10% glycerol), using a Parr cell disruption bomb as described previously [18]. After centrifugation at 50,000 *g* for 60 min, the supernatant was stored at −40° until analysis. Measurement of dCMPD activity was carried out with [^{3}H]dCMP as described previously [20]. CD activity was measured using [^{3}H]dCyd as described by Bhalla *et al.* [20]. [^{3}H]dCyd was separated from [^{3}H]dUrd using a 3 μm Spherisorb ODS2 column (15 cm × 10 mm, Phase Separations, Norwalk, CT), at a flow rate of 0.7 mL/min of isocratic 2.5 mM ammonium acetate containing 1.25% methanol, pH 4.1. All determinations were carried out with triplicates.

Measurement of cellular dCTP and dTTP pools.

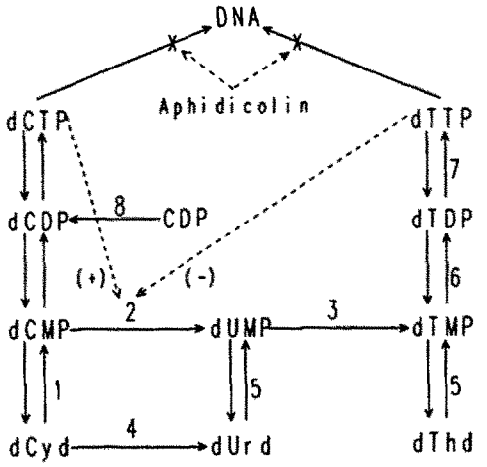

Fig. 1. Metabolism of dCyd in intact cells. The indicated reactions are: (1) dCyd kinase, (2) dCMPD, (3) TS, (4) CD, (5) TK, (6) dTMP kinase, (7) nucleoside diphosphate kinase, and (8) ribonucleoside diphosphate reductase. Regulation of dCMPD by dCTP and dTTP is indicated by arrows. Key: (+) activation, and (−) inhibition. Inhibition of DNA synthesis by aphidicolin is also indicated (×).

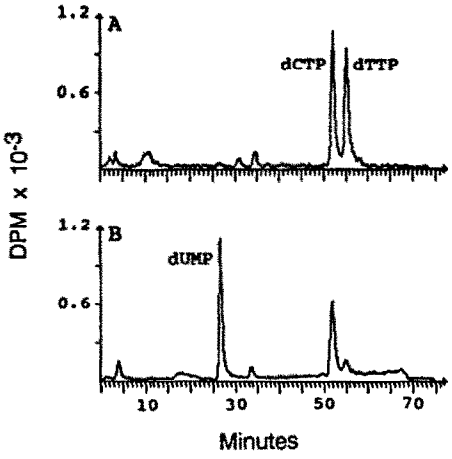

Fig. 2. HPLC profile of $HClO_4$ extracts after labeling with [^{14}C]dCyd. Metabolites were identified by comparing with standard compounds. (A) CEM cells (1×10^7) were pulsed for 10 min with 0.2 μCi [^{14}C]dCyd in 5 mL of culture medium. [^{14}C]dCTP and [^{14}C]dTTP were eluted at 53 and 57 min, respectively. (B) Cells were preincubated with 2 nM FdUrd for 2 hr before labeling with [^{14}C]dCyd. [^{14}C]-dUMP was eluted at 27 min. The distribution of radioactivity among labeled metabolites was as follows: (A) [^{14}C]dCTP (44.5%), [^{14}C]dTTP (and/or [^{14}C]dUTP) (46.8%); (B) [^{14}C]dUMP (49.0%), [^{14}C]dCTP (36.5%), [^{14}C]dTTP (and/or [^{14}C]dUTP) (4.8%).

Deoxyribonucleoside triphosphates were extracted from CCRF-CEM cells with 0.4 N $HClO_4$, and the acid-insoluble material was removed by centrifugation. The supernatant was carefully neutralized to pH 7 with KOH, and the precipitated $KClO_4$ was removed by centrifugation. Samples were stored at −20° until analysis. An assay for dNTPs using DNA polymerase and synthetic oligonucleotide template primers was applied to determine dCTP and dTTP pools as described by Sherman and Fyfe [25]. For untreated CCRF-CEM cells, dCTP and dTTP pools were 23.6 ± 1.6 and 82.8 ± 4.4 pmol/10^6 cells (N = 5), respectively. The experiments were all performed in duplicate, and the experimental errors were less than 15% of the mean values. The experimental data were analyzed and plotted with Graphpad Inplot software version 3.01 (H. Motulsky, University of California, San Diego, CA).

RESULTS

Labeling of cells with radioactive dCyd. Exogenous dCyd can serve as a precursor of dCTP and dTTP through the salvage pathways. After dCyd is transported into CCRF-CEM cells, which have only low levels of CD activity (Fig. 1, reaction 4), it is phosphorylated to dCMP by dCK (Fig. 1, reaction 1). At this branch point, dCMP can be either phosphorylated to dCTP or deaminated to dUMP by dCMPD (reaction 2) dUMP is converted to dTMP by TS (reaction 3), and is subsequently phosphorylated to dTTP. When cells were labeled with [^{14}C]dCyd in the presence of aphidicolin, radioactively labeled triphosphates (i.e. [^{14}C]dCTP and [^{14}C]dTTP) were the dominant products (Fig. 2A). No accumulation of dTMP and dTDP was observed. However, if TS was inhibited by 5-fluorodeoxyuridine (FdUrd), as shown in Fig. 2B, labeled dUMP became a major metabolite. These results indicated that under normal growth conditions, TS activity exceeded that of dCMPD. Therefore, measurement of the end product [^{14}C]dTTP should be proportional to the deamination activity.

Because dTTP and dUTP co-eluted under the separation conditions, it was unclear whether a small amount of dUTP was accumulated. Nevertheless, the radioactivity in dUMP and dTTP (dUTP) can be added as the products of dCMPD. Under our experimental conditions, labeled triphosphates generally represented more than 90% of incorporated radioactivity if TS activity was not inhibited. Labeled monophosphates and diphosphates existed in small amounts and generally were difficult to distinguish from the background. Therefore, for practical purposes only labeled dCTP and dTTP were quantitated for determination of dCMPD activity.

Assay of dCMPD activity in intact cells. The dCMPD activity can be quantitated in intact cells by measuring only the labeled dTTP after pulsing with [^{14}C]dCyd if the incorporation of radioactivity into DNA is blocked. This measurement, however, may be altered by conditions that affect dCyd uptake or phosphorylation by dCK, the critical initiating step. To address this potential problem, we introduced an enzyme activity index, defined by the ratio of labeled dTTP divided by the total radioactivity incorporated (dCTP + dTTP), as an appropriate indicator for measuring dCMPD activity in intact cells.

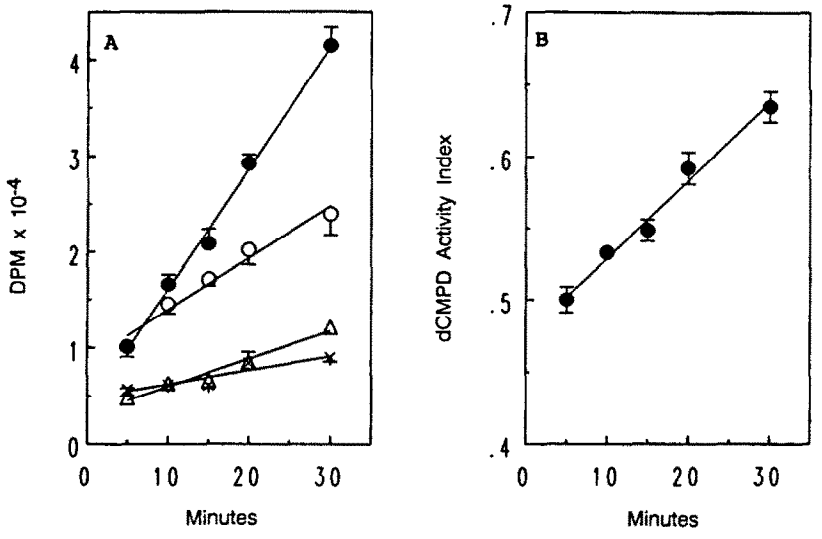

Fig. 3. Assay of dCMPD in intact CEM cells. (A) Cells (1.5×10^7) were pulsed with [^{14}C]dCyd for the indicated times and collected by centrifugation. The nucleotides were extracted with 0.4 N $HClO_4$. The assay was conducted with or without aphidicolin (1 μg/mL). [^{14}C]dCTP and [^{14}C]dTTP were separated by HPLC and quantitated by a radioactive flow detector. Key: [^{14}C]dTTP in the absence (△) or presence (●) of aphidicolin; [^{14}C]dCTP in the absence (×) or presence (○) of aphidicolin. (B) The dCMPD activity index was calculated as described in Materials and Methods. Data are the means ± range of duplicate measurements.

Quantitation of incorporated [^{14}C]dCTP and [^{14}C]dTTP after labeling cells with [^{14}C]dCyd is shown in Fig. 3A. Both [^{14}C]dCTP and [^{14}C]dTTP increased linearly over 30 min. Aphidicolin blocked incorporation into DNA and greatly enhanced the accumulation of labeled deoxyribonucleotide triphosphates. The dCMPD activity index also increased linearly over the 30-min period (Fig. 3B), indicating that deamination continued at a constant rate. Treatment of cells with aphidicolin itself did not result in significant changes in cellular dNTP pools over the duration of the assay (data not shown). Furthermore, the linearity of the assay also suggested that dCMPD activity was not affected by the simultaneous incubation with aphidicolin.

To further characterize this assay, cells were analyzed for dCMPD activity after preincubation for 2 hr with H_4dUrd, the 5′-monophosphate of which is a potent inhibitor of dCMPD [11, 13]. H_4Urd, which inhibits CD but not dCMPD [11, 13, 29], was used as a control. As shown in Fig. 4, dCMPD activity in both CCRF-CEM and KG-1 cells was specifically inhibited by H_4dUrd, whereas little effect was observed with H_4Urd. The previously reported observation by Maley and Maley [11] that H_4dUrd is phosphorylated by the nucleotide phosphotransferase, and not by TK, was supported by the following experiments. When a TK-deficient CCRF-CEM cell line (CEM-TK$^-$) was treated with 10 μM H_4dUrd for 1 hr, we found that dCMPD activity was inhibited by 56%; 100 μM H_4dUrd resulted in 76% inhibition. In wild-type CCRF-CEM cells, a 1-hr incubation with 100 μM H_4dUrd resulted in a similar 72% inhibition of dCMPD.

dCMPD and CD activities in cell extracts. The

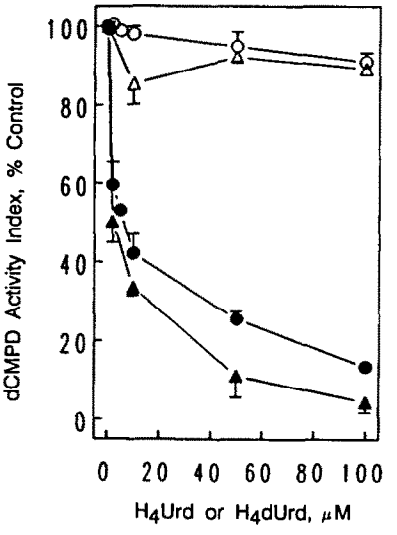

Fig. 4. Inhibition of dCMPD by H_4dUrd. CCRF-CEM (○, ●) and KG-1 (△, ▲) cells were incubated with indicated concentrations of H_4dUrd (●, ▲) or H_4Urd (○, △) for 2 hr. Cells were washed into fresh medium and assayed for dCMPD activity index as described in Materials and Methods. Data are the means ± range of duplicate measurements. The dCMPD activity index was 0.48 ± 0.06 (N = 5) for the untreated CCRF-CEM cells, and an average of 0.35 (0.24 and 0.46) in the KG-1 cells, after labeling with the radioactive dCyd for 15 min.

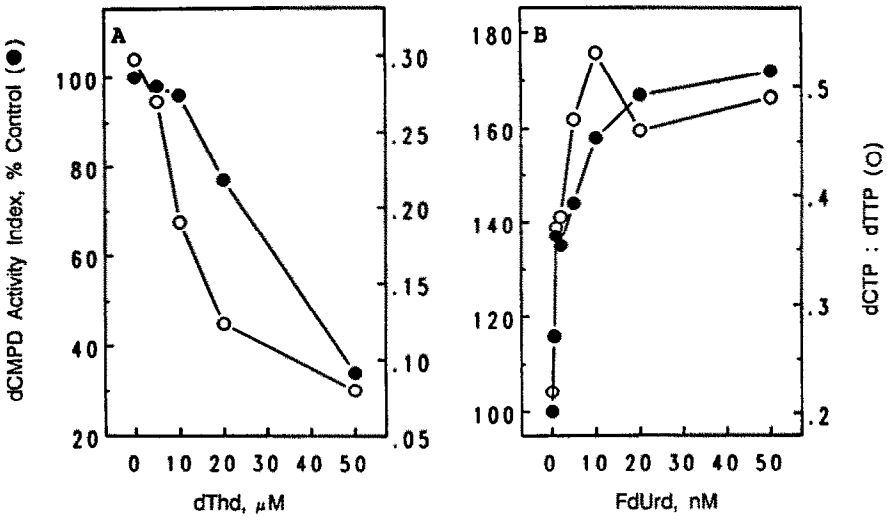

Fig. 5. Effects of dThd and FdUrd on dCMPD. CCRF-CEM cells were incubated with indicated concentrations of dThd (A) or FdUrd (B) for 2 hr. The dCMPD activity index (●) and the ratio of dCTP:dTTP pools (○) were measured as described in Materials and Methods. Because dUMP was a major product of [^{14}C]dCyd metabolism in cells incubated with FdUrd (see Fig. 2), radioactivity associated with dUMP was included in addition to that associated with dTTP in the dCMPD activity index in Fig. 5B. Two separate experiments with duplicates were performed for A. Duplicate measurements were performed for B. SD of the measurements was less than 10%. The control value for the dCMPD activity index was 0.48 ± 0.06 (N = 5) after a labeling with the radioactive dCyd for 15 min.

lack of an effect by the specific CD inhibitor H_4Urd on the *in situ* dCMPD assay may be attributed to either a low level of CD in these cells or the possibility that cellular CD does not interfere with the procedure. Analysis of deaminase activities in CCRF-CEM cell extracts demonstrated dCMPD activity to be 1312 ± 67 nmol dUMP/hr/mg, whereas CD activity was only 0.60 ± 0.11 nmol dUrd/hr/mg (dCMPD:CD = 2187). In extracts of KG-1 cells, dCMPD and CD activities were 1138 ± 75 nmol dUMP/hr/mg and 0.97 ± 0.24 nmol dUrd/hr/mg, respectively (dCMPD:CD = 1173). Our results are consistent with previous reports that human hematopoietic cell lines, such as K-562, HL-60, Molt-4 and U-1568, in addition to CCRF-CEM and KG-1, all contain low CD activities [12, 30, 31]. The potential impact of either high CD activities or a low dCMPD:CD value on this assay will have to be evaluated in other cells.

Correlation of dCMPD activity with cellular dCTP:dTTP. The effect of varying celluar dCTP:dTTP on dCMPD activity was studied using dThd and FdUrd. Previous studies have demonstrated that incubation of cells with dThd resulted in elevation of the dTTP pool, which is associated with a decrease in the dCTP pool, presumably caused by inhibition of the ribonucleoside diphosphate reductase catalyzed reduction of CDP to dCDP [32]. Indeed, when cells were treated with various concentrations of dThd for 2 hr, a concentration-dependent decrease in the cellular dCTP:dTTP value and an associated proportional reduction in the dCMPD activity index were observed (Fig. 5A). Incubation of cells with 50 μM dThd resulted in a 60% inhibition of dCMPD, whereas the dCTP:dTTP value was decreased from 0.30 to 0.08. On the other hand, incubation of cells with FdUrd resulted in a decrease of the dTTP pool and an increase of the dCTP pool presumably because of inhibition of TS [33] (Fig. 5B). The cellular dCTP:dTTP more than doubled after treatment with 10 nM FdUrd for 2 hr; this alternation in the cellular ratio of regulatory nucleotides was associated with a 1.6-fold enhancement of the dCMPD activity index.

Modulation and dCMPD by dFdC. Our recent studies indicated that dFdCTP is an inhibitor of partially purified dCMPD [20]. The inhibition of ribonucleoside diphosphate reductase by dFdCDP also results in a diminished dCTP pool and an altered dCTP:dTTP value [18]. To investigate the relative importance of these separate activities of dFdC, *in situ* dCMPD activity was examined to determine the effect of dFdC treatment on dCMPD activity in intact cells. After cells were incubated with 10 μM dFdC for 150 min, labeled dTTP was decreased by 68% over the time of exposure, whereas labeled dCTP was increased to 128% of control (Fig. 6A), consistent with the interpretation that deamination was inhibited. The dCMPD activity index remained about 0.4 without dFdC treatment. At time zero of treatment, the dCMPD activity index was 0.42; it decreased to 0.08 after cells had been treated with 10 μM dFdC for 150 min (Fig. 6B).

The effect of dFdC concentration on dCMPD activity was also studied. As shown in Fig. 7, dFdC exerted potent inhibition of cellular dCMPD activity. The inhibition was dramatic at low dFdC

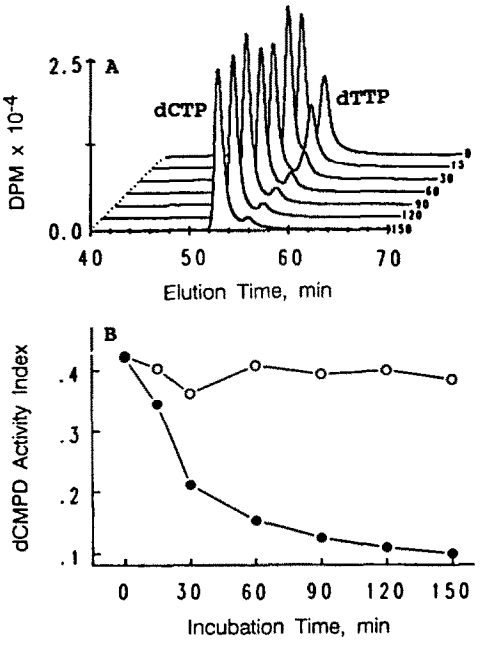

Fig. 6. Effect of dFdC on dCMPD activity. CCRF-CEM cells were incubated with 10 μM dFdC for various times and then pulsed with [6-^{3}H]dCyd for determination of dCMPD activity as described in Materials and Methods. (A) HPLC analyses were performed for $HClO_4$ extracts of dFdC-treated cells at the indicated times (as labeled at the end of each chromatography). Radioactivity labeled dCTP and dTTP were identified by comparison with dCTP and dTTP standards. (B) After dFdC treatment, dCMPD activity index was quantitated as described in Materials and Methods (●). Untreated samples were incubated without dFdC under the same conditions for the indicated times (○). At time zero (0 min), untreated cells had incorporated 7.4×10^5 dpm in [^{3}H]dCTP, and 5.6×10^5 dpm in [^{3}H]dTTP; in the presence of 10 μM dFdC, cells had incorporated 2.2×10^5 dpm into [^{3}H]dCTP, and 1.6×10^5 dpm into [^{3}H]dTTP.

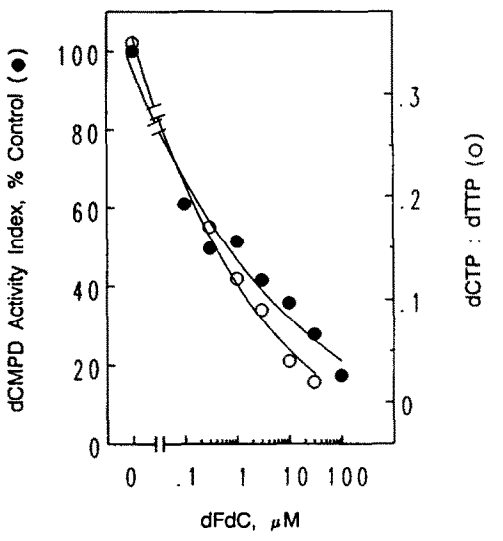

Fig. 7. Concentration-dependent inhibition of dCMPD by dFdC. CCRF-CEM cells were incubated with the indicated concentrations of dFdC for 2 hr. dCMPD activity index (●) and dCTP:dTTP (○) were measured as described in Materials and Methods. Data are the means of duplicate measurements. The control value for the dCMPD activity index was 0.48 ± 0.06 (N = 5) after labeling with the radioactive dCyd for 15 min.

concentrations with an IC_{50} of 0.2 μM. In correlation with the inhibition of dCMPD, cellular dCTP:dTTP was also decreased. Untreated cells had a dCTP:dTTP value of 0.35, which was decreased to 0.03 after incubation with 10 μM dFdC for 2 hr.

To evaluate the action of dFdC on dCMPD, we compared alterations in the dCMPD activity index with changes in the dCTP:dTTP value for dFdC and with those caused by dThd and FdUrd (Fig. 8). The dCMPD activity index was expressed as the function of cellular dCTP:dTTP. A combined linear regression line (slope = 265.6 ± 20.9) was obtained after dThd and FdUrd treatment. On the other hand, a different linear regression line ($P < 0.05$) was obtained for dFdC (slope = 149.5 ± 36.0), which also has a lower position in the graph. The cumulative evidence suggests that the greater inhibition of dCMPD by dFdC is likely due to a direct effect of dFdCTP on dCMPD.

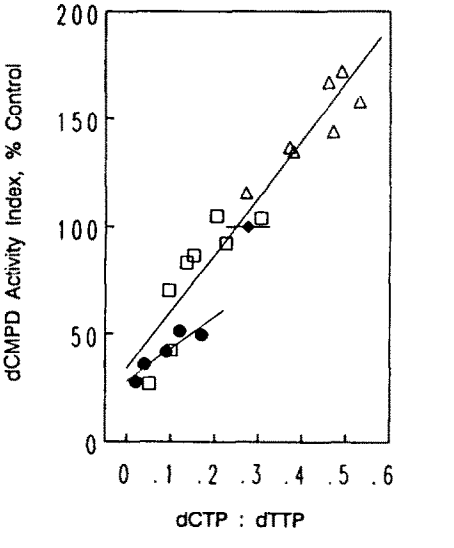

Fig. 8. Relationship between dCMPD activity index and cellular dCTP:dTTP. Data from Figs. 5 and 7 were replotted. Key: (●) dFdC treatment, slope = 149.5 ± 36.0; (□) dThd treatment, (△) FdUrd treatment, slope = 265.6 ± 20.9; and (◆) control. Data are the means of duplicate measurements. The control value for the dCMPD activity index was 0.48 ± 0.06 (N = 5) after labeling with the radioactive dCyd for 15 min.

DISCUSSION

dCMPD and TS are two key enzymes that maintain

a balanced supply of dCTP and dTTP for DNA synthesis. Measurements of TS activity in intact cells using [5-^{3}H]dUrd has been given considerable attention. The assay is based on the fact that the tritium is released into water when dUMP is methylated [34–37]. Previously, use of the method has led to the characterization of various chemotherapeutic agents targeted at TS. On the other hand, measurement of dCMPD in intact cells has not been reported before.

In the present study, we report an HPLC assay of dCMPD using radioactive dCyd as the precursor in intact human leukemia cells. The activity of dCMPD was determined by measuring the end product dTTP (Fig. 1). Although other enzymes, such as TS, dTMP kinase, and nucleoside diphosphate kinase, were also involved in this pathway, dCMPD was clearly catalyzing the rate-limiting step. TS, which lacks allosteric regulation, is generally believed not to be the rate-limiting enzyme in the metabolic pathway [1]. Analysis of dCyd metabolites in intact cells confirmed this view (Fig. 2). If TS was the rate-limiting enzyme, dUMP would have been detected, as in the experiment when TS was inhibited by FdUrd. Because dUMP was undetectable in untreated cells, we concluded that TS activity was in excess of dCMPD activity in CCRF-CEM cells. The low level of labeled monophosphate and diphosphate also excluded the possibility that dTMP kinase or nucleoside diphosphate kinase might play some rate-limiting role. Aphidicolin, a potent inhibitor of DNA polymerases [38], was used to inhibit incorporation of radioactivity into DNA. As shown in Fig. 3, the dCMPD activity index increased linearly over 30 min, indicating that dCMP deamination continued at a constant rate in the presence of aphidicolin.

The assay was further characterized using H_4dUrd, the precursor of the dCMPD inhibitor H_4dUMP [11, 13], and by dThd and FdUrd, two compounds known to alter cellular dCTP and dTTP pools [32, 33] and thus to putatively affect the rate of dCMP deamination. When cells were labeled with [^{14}C]-dCyd, H_4dUrd potently inhibited deamination of labeled dCyd nucleotides (Fig. 4). In controls, H_4Urd, which inhibits CD but not dCMPD, had little effect. This result indicated that [^{14}C]dCyd was metabolized by the dCK and dCMPD pathway, whereas the CD and TK pathway was relatively insignificant in the two leukemia cell lines studied (Fig. 1). This is also consistent with the relatively low activity of CD in these cells. dThd and FdUrd were reported to modulate cellular dCTP and dTTP pools, which led to varied dCMPD activity in Novikoff hepatoma cells [32] and mouse T-lymphoma cells [33]. These effects were confirmed in CCRF-CEM cells using the *in situ* dCMPD assay.

Based on *in vitro* studies, it is known that dCMPD is activated by dCTP and inhibited by dTTP at low dCMP concentrations. At higher substrate concentrations (0.1 to 100 mM), these allosteric effects became diminished [39]. We estimated that the dCMP concentration in CCRF-CEM cells was approximately 0.2 μM (assuming dCTP:dCMP is 100:1 based on studies of the ratios in cells equilibrated with [^{14}C]dCyd; and dCTP concentration is roughly 20 μM). At such a low dCMP concentration, dCMPD is fully expected to be subjected to allosteric regulation by dCTP and dTTP. Indeed, in the present study, we demonstrated that dCMPD activity in the intact CCRF-CEM cells was very sensitive to changes in cellular dCTP and dTTP pools. Our results also indicated a possible correlation between the dCMPD activity level and cellular dCTP:dTTP. As shown in Fig. 8, when dCTP and dTTP pools were modulated by dThd and FdUrd, dCTP:dTTP varied between 0.08 and 0.53, and dCMPD activity was changed linearly from 34 to 172% of control, respectively.

Previous studies have indicated that dCMPD is involved in the elimination of some dCyd analogues, such as dFdC, FdCyd, and ara-C, from tissues or cultured cells [14, 15, 20, 40]. Because the elimination of dFdCTP is concentration dependent, the mechanism involved is of particular interest. As we recently reported, dFdC 5'-monophosphate is a substrate of dCMPD, and dFdCTP acts as an inhibitor of dCMPD in cell-free assays [20]. Evidence also indicated that dFdCDP inhibits ribonucleoside diphosphate reductase [18, 41], an action that contributes to depletion of the dCTP pool, but not the dTTP pool. This effect should further enhance the inhibition of dCMPD activity. These observations supported the proposal of a self-potentiation mechanism for dFdCTP elimination.

Inhibition of dCMPD activity in CCRF-CEM cells after dFdC treatment was confirmed in the present study using the *in situ* assay (Figs. 6 and 7). Cellular dCTP:dTTP was decreased concomitantly with the dCMPD activity index. But is this decreased dCTP:dTTP solely responsible for the inhibition of dCMPD activity observed after dFdC treatment? After results in Figs. 5A and 7 were compared, it was clear that when the dCTP:dTTP value was decreased to an equal extent by dFdC and dThd, dFdC more strongly inhibited dCMPD activity than did dThd. After treatment with 0.2 μM dFdC for 2 hr (150 μM dFdCTP is accumulated under this condition) [20], dCMPD activity was inhibited to 50% of control, whereas the dCTP:dTTP value was 0.16. On the other hand, after treatment with 10 μM dThd for 2 hr, a similar dCTP:dTTP value was observed, but dCMPD activity was inhibited only 5%. Given the proportionality of dCMPD activity over a wide range of dCTP:dTTP values, the difference between the increased sensitivity of dCMPD relative to the dCTP:dTTP induced by dFdC is consistent with a direct inhibition of the enzyme by dFdCTP. Attempts to directly demonstrate this by repleting dCTP in cells containing high dFdCTP levels by incubating with dCyd failed due to simultaneous increases in the cellular concentrations of both dCTP and dTTP [42].

dFdCDP is a potent inhibitor of ribonucleotide reductase [18, 41], an action that probably reduces the cellular concentrations of all dCyd nucleotides. This raises the possibility that the specific activity of these pools is increased upon labeling with [^{14}C]-dCyd during this assay. Expression of the results of this *in situ* assay as an activity index was intended to normalize for such an effect, and therefore avoid a possible underestimation of the degree of dCMPD

inhibition. The strong inhibition of dCMPD in cells treated with dFdC (Figs. 6 and 7) suggests that underestimation of dCMPD activity due to specific activity artifacts was not a limitation of the technique.

Based on the results summarized in Fig. 8, we proposed that dCMPD activity in intact cells has a linear relationship with cellular dCTP:dTTP. Although an aspect of the modulation of dCMPD after dFdC treatment may be attributed to this mechanism, it clearly has an additional component. Consistent with the studies with the partially purified enzyme [20], the data suggest that a direct inhibition resulted from the reaction of dFdCTP with dCMPD. This can account for the inhibition of dCMPD activity over that which would be expected on the basis of a decrease in the dCTP:dTTP alone. The exact mechanism by which dFdCTP inhibits dCMPD, however, remains to be determined.

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