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Original Paper

Treatment of Normal and Malignant Cells with Nucleoside Analogues and Etoposide Enhances Deoxycytidine Kinase Activity

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Deoxycytidine kinase (dCK), one of the rate-limiting enzymes in the intracellular metabolism of many antileukaemic drugs, was shown to be stimulated after treatment of human tonsillar lymphocytes by 2-chloro-2′-deoxyadenosine (cladribine, CdA) (Sasvári-Székely, et al., *Biochem Pharmacol* 1998, 56, 1175–1179). Here we present a comparative study of different normal and malignant cells in respect to the activation of dCK by CdA. G-phase lymphocytes showed a higher sensitivity for dCK stimulation than S-phase cells. Normal and leukaemic peripheral blood mononuclear cells, as well as the pro-myelocytic cell line HL60 responded to CdA treatment by a 2–5-fold increase in activity of dCK. However, no significant stimulation was detected either in CCRF-CEM T-lymphoblastoid cells, or in K562 myeloid cells. Thymidine kinase (TK) activity was not stimulated in any cases. Treatment of these cells with several other analogues beside CdA, such as 2-chloro-2′-arabino-fluoro-2′-deoxy-adenosine (CAFdA), 2-fluoro-1-β-D-arabinosyladenine (Fludarabine, FaraA) and 1-β-D-arabino-sylcytosine (cytarabine, araC) gave similar results to CdA treatment. Enhancement of dCK activity could also be achieved with the topoisomerase II inhibitor, etoposide. In contrast, 2-chloro-riboade-nosine (CrA) had no effect on the dCK at concentrations of 10 μM or less, while dCyd and 5-aza-dCyd caused slight inhibition. These results indicate that treatment of cells with several inhibitors of DNA synthesis potentiates the dCK activity. The drugs widely differ in their stimulatory effect on dCK, and there are also 'responsive' and 'non-responsive' cells with respect to dCK activation. Thus, enhance-ment of the dCK activity by specific drugs in 'responsive' cells might give a rationale for combination chemotherapy.

Key words: nucleoside analogues, deoxycytidine kinase, thymidine kinase, human, lymphocyte, chemotherapy
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INTRODUCTION

Deoxycytidine kinase (dCK, EC 2.7.1.74) is a key enzyme in the intracellular metabolism of numerous drugs used against viral infections, leukaemia and different solid tumours (reviewed in [1]). The biochemical explanation of the outstanding role of dCK in drug activation is its wide substrate specifity [2], which enables the enzyme to phosphorylate a large variety of deoxynucleoside analogues, such as 2′,3′-dideoxycytidine (ddC), 1-β-D-arabinofuranosylcytosine (araC), 2′,2′-difluorodeoxycytidine (dFdC), 2-fluoro-1-β-D-arabino-syladenine (FaraA), 2-chloro-2′-deoxyadenosine (CdA) and 2-chloro-2′-arabino-fluoro-2′-deoxyadenosine (CAFdA) [1–4]. CAFdA is a novel, acid stable 2′-arabino-fluoro derivative of CdA which combines certain attractive properties of FaraA and CdA [3, 4].

The other clinically important property of dCK is its constitutive expression, which is predominant in lymphoid tissues

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(reviewed in [1]). For this reason, nucleoside analogues can be metabolised not only in dividing, but also in non-dividing cells by dCK. In contrast to the thymidine kinase 1 (TK1) [5], dCK is not cell-cycle regulated and this may be explained by the fact that its promoter contains only half of an E2F binding site. The efficacy of metabolism of nucleoside analogues through dCK is highly influenced by the regulation of dCK activity. No increase in dCK mRNA levels is found after mitogen stimulation of peripheral blood T lymphocytes [6], however, a 6-fold increase in dCK activity is seen in undifferentiated lymphnode lymphocytes [7] compared with differentiated ones. Moreover, 2–3-fold higher dCK activity is seen in S-phase enriched cells, which led to the suggestion that dCK might be post-transcriptionally regulated [8, 9].

One of the nucleoside analogues activated by dCK is arabinosylcytosine (araC), the major drug the in treatment of acute leukaemia. Similarly, gemcitabine (dFdC) and cladribine (CdA) are also phosphorylated by dCK in sensitive cells. Furthermore, dCK deficiency has been shown to decrease the sensitivity of rat leukaemic cells to araC [10] and the cytotoxic activity of araC is dependent on the intracellular levels of araCTP. Etoposide (VP16), the semisynthetic derivative of podophyllotoxin, is an anticancer drug frequently used against haematological malignancies and solid tumours. VP16 causes DNA strand breaks (DBS) by interacting with topoisomerase II and inhibiting DNA ligation [11], and this DNA damage leads to apoptosis and cell death. Recently etoposide has been shown to activate dCK in murine lymphocytes [12].

Previously, we have shown that treatment of primary cultures of human tonsillar lymphocytes by CdA (adenosine deaminase resistant deoxyadenosine (dAdo) analogue) led to a substantial increase in the dCK activity that was measured in cell-free extracts, without any influence on the TK1 and TK2 activities [8]. The aim of present study was to investigate the enhancement of dCK activity with several chemotherapeutic drugs in normal and transformed cells of different origin.

MATERIALS AND METHODS

Materials

CdA and CrA were synthesised by Zygmunt Kazimierczuk [13]; CAFdA was a gift from H. Cottam (University of California, San Diego, California, U.S.A.); FaraA, araC, 5-aza-dCyd and dCyd were purchased from the Sigma Chemical Co.; 5-^{3}H-dCyd and (methyl-^{3}H)-dThd were purchased from Amersham Life Science (Buckinghamshire, U.K.), 8-^{3}H-CdA was obtained from Moravek Biochemicals Inc. (Brea, California, U.S.A.). Etoposide (VP16) was kindly provided by Bristol-Myers Squibb Scandinavia (Bromma, Sweden). Other chemicals were of analytical grade and were products of Reanal, Budapest, Hungary.

Cell sources

Tonsillar lymphocytes were prepared from surgically removed tonsils of 3–6-year-old children and were separated on an albumin gradient for blast (S-phase enriched) and small (G-phase enriched) cell fractions according to their density as previously described [14]. Mononuclear cells were isolated by standard Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation from normal (PBMC) or patient (chronic lymphocytic leukaemia, CLL-PBMC, acute myeloid leukaemia, AML-PBMC) heparinised peripheral blood. Human T-lymphoblastic (CCRF-CEM), chronic myelogenous (K562) and promyelocytic (HL60) leukaemic cell lines were maintained in exponential growth in RPMI 1640 medium supplemented with fetal calf serum (10%), penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamate (2 mM). The volume and number of cells were determined by a Coulter Multisizer (Coulter Electronics, Luton, U.K.) or by counting in Turck reagent (tonsillar lymphocytes).

Cell treatment and extraction

Cells were washed twice with serum-free medium and resuspended in the same medium (10×10^6 cell/ml Eagle's minimal essential medium (MEM) for tonsil lymphocytes and $2–10\times10^6$ cell/ml RPMI for others). After addition of drugs, cells were incubated at 37°C for the indicated time periods. After this treatment cells were washed twice in phosphate buffered saline (PBS) and extracted 3 times by fast freezing–thawing in extraction buffer containing 50 mM Tris-HCl pH 7.6, 2 mM dithiothreitol (DTT), 0.5 mM phenyl methyl sulfonyl fluoride (PMSF), 20% glycerol and 0.5% Nonidet P40. The cell-free extracts were then centrifuged for 5 min at 13 000 rpm, 0°C and used immediately for the determination of enzyme activities.

Enzyme assays

Deoxycytidine kinase was assayed with 5-^{3}H-dCyd (10 μM) or 8-^{3}H-CdA (50 μM), TK with (methyl-^{3}H)-dThd (10 μM) as substrate in a reaction mixture containing 50 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$, 5 mM ATP, 2–4 mM DTT, 10 mM sodium fluoride and cell extract. Specific activities of the labelled compounds were in all cases between 500 and 1000 cpm/pmole. The enzyme assays were carried out at 37°C, the appropriate enzyme concentrations and incubation times, to measure at a linear reaction rate, in each case were determined in preliminary experiments. To stop the reaction, aliquots were spotted on DEAE cellulose filters, which were then washed, eluted and counted as previously described [15].

RESULTS

Time course of CdA treatment on dCK and TK levels in G- and S-phase enriched human tonsillar lymphocytes

Human tonsils are natural sources of B lymphocytes, the majority of these cells are in a resting state known as quiescence. However, 10–20% of lymphocytes which have originated from the germinal centres are activated *in vivo* and display relatively high rates of DNA synthesis and activities of related enzymes. Low density (S-phase) and high density (G1-phase) lymphocytes were separated by centrifugation through 25% bovine serum albumin [14]. dCK activity was approximately 3-fold, and TK was nearly 10-fold higher in S-phase (lymphoblasts) than in G-phase (small lymphocytes) cell extracts (Figure 1). Treatment of cells with 1 μM CdA for 120 min increased the dCK activity 2.5-fold in G1-phase lymphocytes, measured with ^{3}H-dCyd as a substrate (Figure 1a). The maximal activation of dCK in the S-phase cell fractions was already achieved after 60 min, but it did not exceed an increase of 1.6-fold. The dCK activity measured initially with ^{3}H-CdA as substrate (Figure 1b) was 4–5-fold higher than the ^{3}H-dCyd phosphorylation, corresponding to the higher V_{max} that has been observed with the purine substrate [15]. However, the stimulation of ^{3}H-CdA phosphorylation was lower (1.6-fold compared with 2.5-fold) in G1-phase cells, and no activation was detected in S-phase cells.

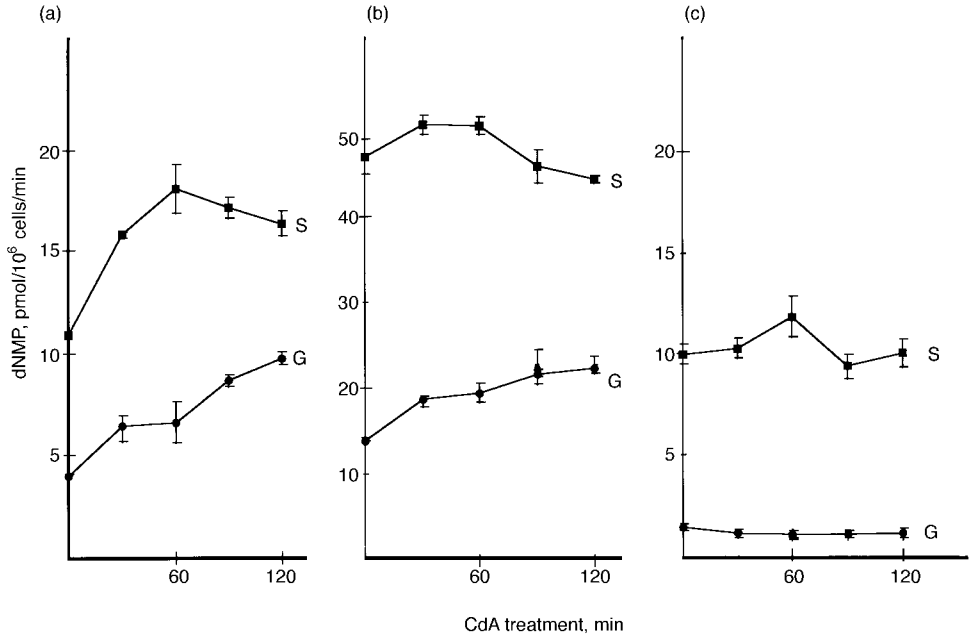

Figure 1. The effect of 2-chloro-2′-deoxyadenosine (CdA) treatment on deoxycytidine kinase (dCK) and thymidine kinase (TK) levels in tonsillar lymphocytes. Lymphocytes were separated into S-phase (–■–) and G-phase (–●–) enriched fractions, incubated in the presence of 1 μM CdA for the indicated time, washed and extracted as described in Materials and Methods. dCK activity was measured with ^{3}H-dCyd (a) or ^{3}H-CdA (b) as substrates, TK activity was determined with ^{3}H-dThd (c).

The phosphorylation of dCyd by TK2 should be blocked by 1 mM dThd in the dCK reaction mixture [15], but no changes were observed either in TK1 (Figure 1c) or in TK2 activities (data not shown) during the CdA treatment. We have measured also the cytidine deaminase, dCMP deaminase activities and the catabolism of ^{14}C-dCMP in control and CdA-treated cell extracts, and no differences were detected (data not presented). One can conclude, that the phosphorylation of deoxycytidine by dCK was effectively increased by CdA in G1-phase lymphocytes, while the higher activity of kinase in S-phase cells showed a smaller increase.

*The concentration dependence of CdA treatment on the dCK activity in PBMC, CCRF-CEM, HL*60 *and K*562 *cells*

Normal (PBMC) and leukaemic (CLL-PBMC) human peripheral blood mononuclear cells were used as sources for resting Go/G1 lymphocytes (70% T cells), and the exponentially growing T lymphoblastoid CCRF-CEM cells can be regarded mainly as a source for S-phase cells. The dCK activity calculated per cell was much lower in blood lymphocytes, as compared with CEM cells. Treatment of either normal, or leukaemic PBMC with 1–3 μM CdA for 2 h resulted in a dose-dependent, 3–4-fold increase of the dCK activity. In contrast, the dCK activity in CCRF-CEM cells decreased upon incubation with CdA (Figure 2a).

Similarly, differences were observed in the promyelocytic cell line HL60 compared with the K562 cell line, derived from a patient with chronic myelogenous leukaemia (Figure 2b). More than 3-fold activation of dCK was measured in the HL60 cells treated with 0.1–10.0 μM CdA, while only a 30% difference was obtained in K562 cells during a similar treatment. Apparently, the mechanisms leading to dCK activation are different in leukaemic cell lines compared with normal lymphocytes.

Similarly with tonsillar lymphocytes, dCK stimulation by CdA in HL60 cells was less pronounced if measured with ^{3}H-CdA than with ^{3}H-dCyd as substrate (data not shown). As the mitochondrial deoxyguanosine kinase (dGK) can also phosphorylate CdA [16], we had to prove that for the increased 3H-CdA phosphorylation only dCK was responsible. We therefore also measured the phosphorylation of CdA also in the presence of excess deoxycytidine (1 mM). The ^{3}H-CdA phosphorylation that remained (in this case, less than 10% of total) was due to dGK and it did not change upon treatment of the cells with CdA (data not shown). Thus, only the activity of dCK was enhanced by the dAdo analogue.

*Time course of dCK activation with different concentrations of CdA in HL*60 *and CLL-PBMC cells*

Maximal activation of dCK was achieved with 1–3 μM CdA in the PBMCs (Figure 2a), after 90–120 min incubation at 37°C (Figure 3). In permanently dividing HL60 cells 0.03 μM CdA already caused a significant increase in the dCK activity (Figure 3), maximal activation was achieved with 0.3–3 μM CdA after 30–60 minutes of treatment, while longer incubation with 3 μM of the analogue slightly decreased the dCK stimulation. These results suggest that CdA has to be metabolised to induce the activation of dCK. Our previous finding that the presence of deoxycytidine during the cell treatment by CdA completely prevented the activation of dCK [8], is in agreement with this hypothesis.

*The effect of CAFdA, FaraA and dCyd treatment on dCK activity in HL*60 *cells*

FaraA, similarly to CdA is used against low grade lymphocytic malignancies (reviewed in [17]). CAFdA is a new deoxyadenosine analogue comprising properties of CdA and FaraA. Treatment of HL60 cells with increasing concentrations of the purine analogues resulted in a significant dose-dependent activation of dCK (Figure 4). Differences in the concentrations of analogues necessary for maximal stimulation, are apparently related to the affinity of these nucleosides

for the kinase [17]. No stimulation was seen with deoxycytidine, moreover, higher dCyd concentrations had a slight inhibitory effect on the level of dCK (Figure 4).

A comparison of the effects of some pyrimidine nucleosides and the ribo-adenosine analogue on dCK activation in primary human tonsillar lymphocytes

The effect of some pyrimidine analogues (araC, 5-aza-araC (AaraC) and 5-aza-dCyd (AdC) on the dCK activity was compared with that of an effective purine derivative (CAFdA) (Figure 5a). Treatment of tonsillar lymphocytes by araC caused a clear-cut increase in dCK activity, although the extent of stimulation was much lower compared with the purine dAdo-analogues. A small stimulation of activity compared with the control could be seen with 5-aza-araC, while 5-aza-dCyd had a slight inhibitory effect. All purine and pyrimidine analogues mentioned above are phosphorylated by dCK. In contrast, 2-chloro-ribo-adenosine (CrA) is activated mainly by adenosine kinase and incorporated into the ribonucleotides, although a minor part can be converted to CdATP by the action of ribonucleotide reductase and nucleoside diphosphate kinase. With 3 μM CrA, there was no effect on the level of dCK (Figure 5b), while a minor stimulation was achieved with 100 μM CrA, possibly due to the accumulation of CdATP.

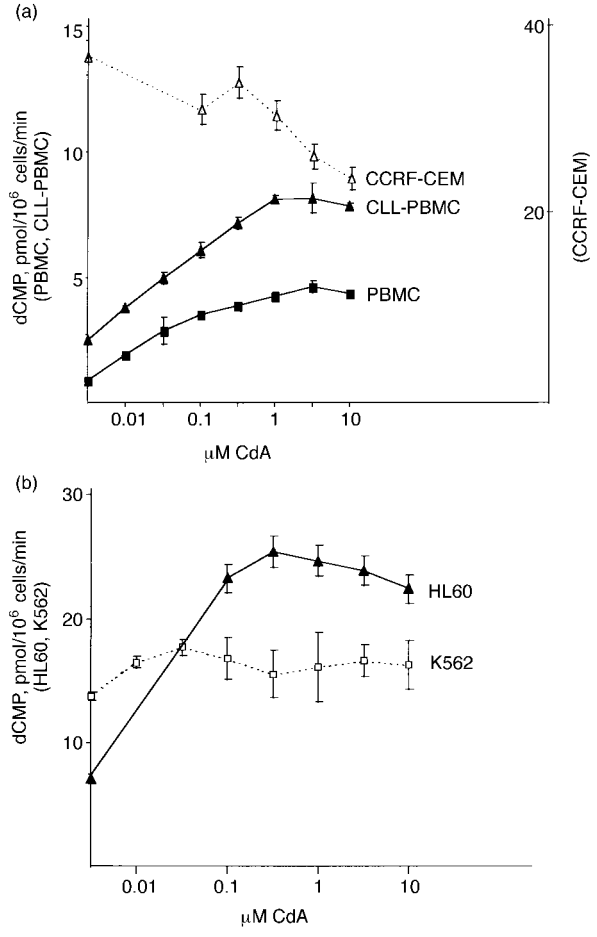

Figure 2. Changes in deoxycytidine kinase (dCK) levels in blood lymphocytes and different leukaemic cell lines after treatment with 2-chloro-2′-deoxyadenosine (CdA). Cells were extracted immediately, or after incubation in serum-free RPMI for 2 h with the indicated concentrations of CdA. Crude extracts were used for activity measurements with ^{3}H-dCyd as described in the Materials and Methods. dCK activity in extracts from: (a) normal (PBMC, –■–) or leukaemic (CLL-PBMC, –▲–) blood lymphocytes (left scale) compared to extracts from CCRF-CEM (···△···, right scale). (b) Myeloid cell lines HL60 (–▲–) and K562 (–□–).

Effect of CdA and etoposide treatment in normal and leukaemic PBMC

The effect of the topoisomerase II inhibitor etoposide on dCK was compared with that of CdA in PBMC from a healthy donor and an AML patient (Figure 6). Similar to effects seen with CdA, VP16 caused a more than 2.5-fold stimulation of dCK in both cell types. However, the concentration of

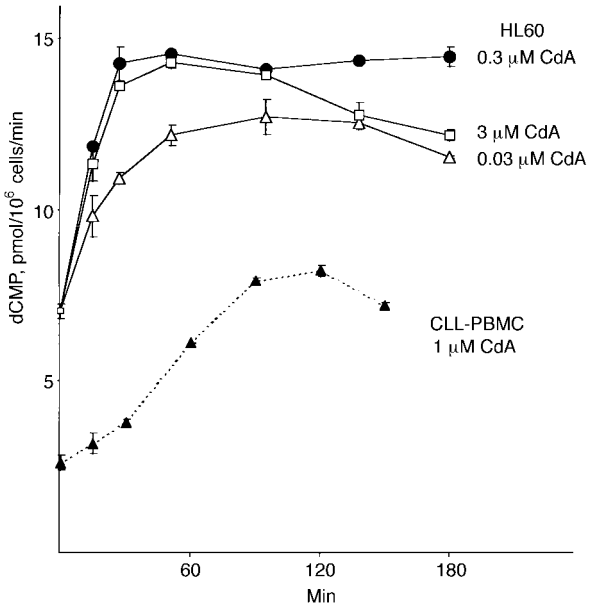

Figure 3. Time-dependent changes in the deoxycytidine kinase (dCK) levels in HL60 and leukaemic blood lymphocytes during treatment with various concentration of 2-chloro-2′-deoxyadenosine (CdA). CLL-PBMC (···▲···, 1 μM CdA) and HL60 cells (–△–, –●– and –□– correspond to 0.03, 0.3 and 3 μM CdA, respectively) were treated with CdA as indicated, washed, extracted and the enzyme activities determined.

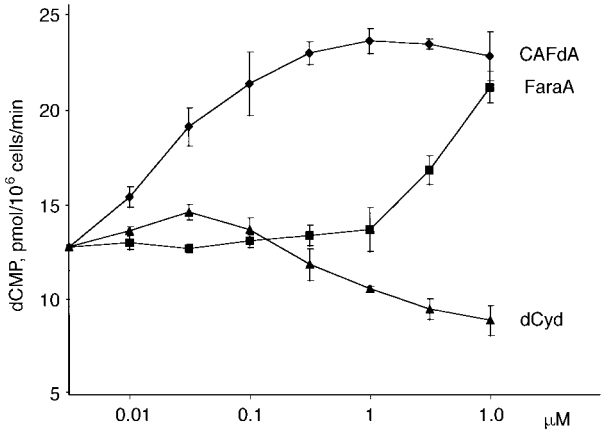

Figure 4. Deoxycytidine kinase (dCK) activities in extracts from HL60 cells treated with dCyd or arabinosyl nucleoside analogues. HL60 cells were extracted immediately or after 2 h incubation with various concentrations of CAFdA (◆), FaraA (■) and dCyd (▲). dCK activity was measured in cell extracts with ^{3}H-dCyd. For abbreviations see Introduction.

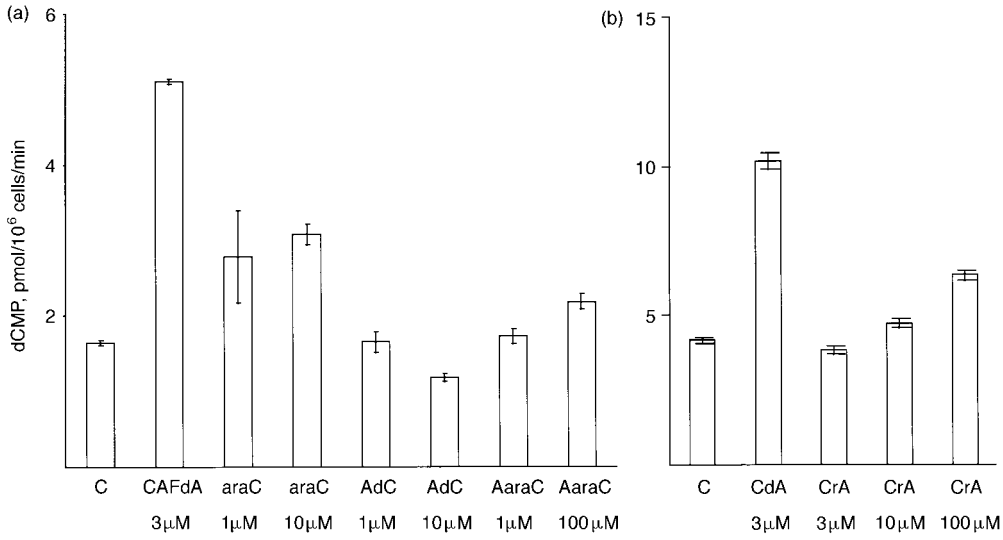

Figure 5. The effect of different nucleosides on the deoxycytidine kinase (dCK) levels in tonsillar lymphocytes. Freshly prepared tonsillar lymphocytes were incubated for 2 h without any addition (C) or with the indicated nucleoside analogues in the indicated concentrations: (a) CAFdA, araC, 5-aza-dCyd (AdC), 5-aza-araC (AaraC); (b) 2-Cl-dAdo (CdA), 2-Cl-Ado (CrA). Then cells were washed, extracted and proceeded for dCK activity measurement with ^{3}H-dCyd. For abbreviations see Introduction.

VP16, needed for maximal dCK stimulation in PBMC (50–100 μM, data not shown), substantially exceeded that of CdA (1–3 μM, Figure 2a).

In summary, our results show that dCK could be effectively stimulated by treating the cells with different nucleoside analogues and inhibitors of DNA synthesis like etoposide, especially in normal and leukaemic peripheral blood lymphocytes. However, stimulation of dCK activity by analogues is not a universal property of cells, leading to a categorisation of 'responder' and 'non-responder' cell types. Moreover, several drugs, like cladribine or CAFdA, are better stimulators of dCK than for example the pyrimidine arabinosides.

DISCUSSION

Deoxynucleosides and their analogues, used in anticancer and in antiviral therapy, are mainly metabolised by dCK. This enzyme is involved in DNA synthesis and repair pathways, as it catalyses the first and rate-limiting step in deoxynucleoside activation tissues. Thus, the sensitivity of malignant cells to these drugs is highly influenced by dCK activity [10, 18, 19], and any stimulation of enzyme activity might be important to increase the sensitivity and efficacy of the chemotherapy regimen. Previously we have shown that the deoxyadenosine analogue CdA enhanced the activity of dCK in human lymphocytes [8]. Here we present new evidence that dCK enhancement can be achieved not only in normal, but also in transformed cells (Figures 2 and 3) and also with several other nucleoside analogues which inhibit DNA synthesis (Figures 4 and 5).

Etoposide (VP16), which is widely used in the treatment of leukaemias, ovarian and testicular cancer, small cell and non-small cell lung cancer does not need dCK for its therapeutic effect. Our experiments showed that VP16 also activates dCK in normal and leukaemic PBMC (Figure 6), as well as in tonsillar lymphocytes and in HL60 (data not shown), in agreement with previously published findings [12]. It has also been shown that araC accumulation was increased by 40% after a CdA infusion, in patients with AML [20], reflecting an increased dCK activity in these cells. Recently, van Moorsel and colleagues [21] demonstrated elevated accumulation of dFdCTP in Lewis Lung cells during cotreatment or short pretreatment with etoposide. Several *in vitro* studies have demonstrated that dCK is feedback inhibited by dCTP [22, 23]. The triphosphates of CdA, FaraA and CAFdA are known to inhibit the ribonucleotide reductase activity *in vitro* [3, 4, 24], and the decrease of the dNTP pools in the cells might be the cause of dCK stimulation, as was suggested in Refs [24, 25]. However, tonsillar lymphocytes display a very low dCTP pool (1–3 pmoles of dCTP/10^6 cells [26]), whilst *in vitro* inhibition of dCK requires micromolar concentrations of dCTP [23]. Moreover, the dCTP pool size was not affected by treatment with either araC or etoposide [21, 26]. Here we have presented data, that both purine and cytosine arabinoside analogues and etoposide caused the activation of dCK in HL60 (Figure 4), in tonsillar lymphocytes (Figure 5) and in PBMC (Figure 6). In contrast, neither the accumulation of CdATP, nor the dNTP pool changes [27] were able to increase the deoxycytidine kinase activity in CEM cells (Figure 2).

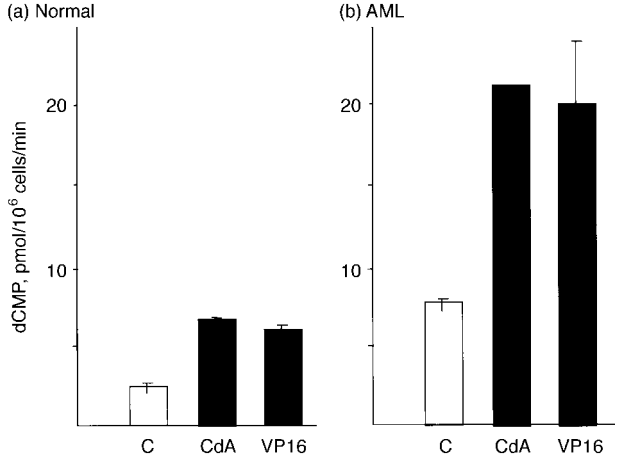

Figure 6. The effect of etoposide (VP16) and CdA on the dCK levels in normal and leukaemic PBMC . Cells were incubated for 2 h in the absence (C) or presence of 1 μM CdA or 100 μM VP16. (a) normal PBMC; (b) PBMC of an acute myelogenous leukaemia (AML) patient.

These results confirm that the changes in feedback regulation alone cannot explain the activation of dCK, in accordance with our previous results suggesting a post-translational modification of the deoxycytidine kinase [8]. Recently, a significant increase in dCK activity was measured after bryostatin 1 treatment of the WSU-CLL cell line, developed from a patient with CLL resistant to fludarabine [28]. *In vitro* phosphorylation and activation of dCK by protein kinase C was shown previously [29], however, the mechanism of dCK activation *in vivo* is still unclear.

In summary, active metabolites, probably triphosphates, of the deoxyribonucleoside analogues investigated (CdA, CAFdA, FaraA, araC and 5-aza-araC) activated dCK in natural lymphocytes and in 'responsive' cell lines, such as HL60. The topoisomerase II inhibitor, etoposide also possessed a comparable dCK stimulating effect (Figure 6).

Conditions leading to an increased dCK activity might be successfully used for the development of new combinations in antitumour chemotherapy. Short pretreatment with CdA might increase the sensitivity and efficacy to all the drugs activated by deoxycytidine kinase (araC, dFdC, etc.) already widely used in human cancer therapy. The finding that VP16 elevates dCK activity in human PBMC (Figure 6), suggests VP16 might be especially effective when used in combined chemotherapy [21] since it does not compete with cytostatic nucleoside analogues for dCK. Finally, several cell lines were 'non-responsive', i.e. their dCK could not be activated by any of these drugs. Further comparison of these 'responsive' and 'non-responsive' cell lines should help to reveal the mechanism behind the activation of dCK.

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