Rapid Communication

Transfection of Wild-Type Deoxycytidine Kinase (dck) cDNA Into an AraC- and DAC-Resistant Rat Leukemic Cell Line of Clonal Origin Fully Restores Drug Sensitivity

By A.P.A. Stegmann, W.H. Honders, R. Willemze, V.W.T. Ruiz van Haperen, and J.E. Landegent

The AraC-resistant rat leukemic cell line RO/1-A has been shown to have a typical deoxycytidine kinase (DCK)-deficient phenotype and cannot metabolize the antileukemic drugs cytarabine (AraC) and decitabine (DAC). To investigate the relative contribution of mutations in the dck gene to the development of in vitro-induced AraC-resistance, a neomycin selectable plasmid construct harboring the wild-type dck coding region was transfected into RO/1-A. Polymerase chain reaction analysis confirmed the presence of vector DNA in the target cells (RO/1-A\textsuperscript{DCK}) that were stably transfected and monitored over a period of 14 weeks. Northern and Western blot analysis showed restoration of dck mRNA and protein expression. Initial rate measurements of DCK activity showed that \( K_m \) values for dck were only slightly altered as a result of transfection, whereas strongly increased \( V_{max} \) values were observed, resulting in a 12-fold increased phosphorylation efficiency for both dC and AraC, compared with the AraC-sensitive parental cell line RO/1 from which the RO/1-A was originally derived. In vitro sensitivity to AraC- and DAC-mediated cytotoxicity was fully restored in RO/1-A\textsuperscript{DCK}. The data pinpoint acquired DCK deficiency caused by mutations of the dck gene as the major cause of AraC resistance in this model.

\( \text{©} \ 1995 \text{ by The American Society of Hematology.} \)

Materials and Methods

Chemicals. 1-\( \beta \)-D-arabinofuranosylcytosine, 2'-deoxycytidine, bovine serum albumin, and adenosine 5'-triphosphate magnesium salt were purchased from Sigma (Sigma Chemical Co, St Louis, MO) and 5-aza-2'-deoxycytidine was a gift from Dr P. Engel (Mack Nachforschung, Karlsruhe, Germany). Creatine kinase and creatine phosphate were obtained from Boehringer (Mannheim, Germany) and Na\textsuperscript{+} was bought from Merck (Darmstadt, Germany). \( [6-\text{H}] \) 2'-deoxy-5'-azacytidine (8 Ci/mmol) was obtained from Moravek Biochemicals Inc (CA), \( [5-\text{H}] \) cytosine \( \beta \)-d-arabinoside (28 Ci/mmol) and \( [125\text{I}] \)-protein A (30 mCi/mg) from Amersham Inc (Amersham, UK), and \( [5-\text{H}] \) deoxycytidine (25.6 Ci/mmol) from NEN Dupont (Boston, MA). G418 sulfate (Geneticin) was from Gibco (Grand Island, NY).

Cell culture conditions. The AraC- and DAC-sensitive rat leukemic cell line RCL/0 was originally purchased from TNO (Rijswijk, The Netherlands). As we described previously, subclone RO/1 was
obtained by means of a limiting dilution assay, and thus originates from a randomly selected single RCL/O cell. To induce resistance, clone RO/1 was exposed to AraC concentrations increasing from 0.1 μmol/L to 10 μmol/L over a 180-day period as detailed earlier. All cell lines were cultured in HEPES-buffered RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (GIBCO), 4 mmol/L L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, and amphotericin-B. RO/1-A was cultured in the continuous presence of 10 μmol/L AraC, representing the IC_{50} value of AraC for RCL/O and RO/1 cells. Cells obtained during exponential growth phase were used for all experiments.

**DCK activity assay.** DCK phosphorylating activity was determined in all cell lines using an assay adapted from Cheng et al. As described earlier, DCK activity for the conversion of the substrates dC, AraC, was determined in crude cell extracts obtained by three cycles of freeze-thawing. The reaction mixture contained MgATP as the phosphate donor and 7 mmol/L NaF, 0.2% bovine serum albumin, and 0.2 mmol/L tetrahydouridine (THU) to block dC-deaminating activity. Reactions were started by the addition of 20 μL of cell lysate supernatant to 150 μL of reaction mixture at 37°C. After 20, 40, and 60 minutes of reaction time 50-μL samples were spotted onto DEAE-coated paper discs (Whatman DE-81; Whatman, Maidstone, UK). Upon scintillation counting in an LKB Rackbeta liquid scintillation counter (LKB, Cambridge, UK), DCK activity was calculated by linear regression analysis of the time-response curves for each of the substrate concentrations and expressed in nanomoles of reaction product formed·min^{-1}·mg protein^{-1}. K_{m} constants and V_{max} values were calculated by nonlinear regression analysis, using the Biosoft (Cambridge, UK) scientific parameter fit software program P Fit, version 5.1 (Fig P Software Corp, Durham, NC).

**Construction of pRC/CVM-DCK.** To clone the entire coding region of dck by PCR, two 25-mer oligonucleotides were synthesized. One (A65) contains six non-dck–specific nucleotides (underlined), an HindIII restriction site (italic), and the 5' end of the dck coding region with the ATG start codon (bold): 5'-TGAGAAGCT-TAGCATGGCCAACCCCA-3'. The other primer (B65) contains 10 non-dck–specific nucleotides (underlined), including an XbaI site (italic) and the 3' end of dck with the termination codon (bold) end: 5'-TCATGAAACACTAACAGATCTGACC-3'. Both primers were used to amplify cDNA from a healthy rat thymus sample, obtained as previously described. The amplification protocol consisted of denaturation for 5 minutes at 95°C, followed by 33 cycles of 95°C: 0.7 minutes, 55°C: 0.7 minutes, 72°C: 0.8 minutes. The amplification product of 809 bp was purified using a Spin-X DNA purification column (Costar, Cambridge, UK). The product was digested overnight at 37°C with 5 U/100 ng HindIII and XbaI I (BRL, Gaithersburg, MD). After purification, the 801-bp fragment was ligated into the HindIII/Xba I linearized pRC/CVM vector (Invitrogen, San Diego, CA). pRC/CVM is a 5.5-kb neomycin resistance selectable expression vector for eukaryotic cells. Ligation was performed at 16°C for 16 hours, using 10 μL of T4 DNA ligase (Amersham, UK) and a 1:1 molar vector/insert ratio. After transformation into the Escherichia coli strain Top10 (Invitrogen), white colonies were checked by polymerase chain reaction (PCR) for the presence of the appropriate insert, using primers for the SP6 and T7 promoters, which flank the multiple cloning site. The inserts were further analyzed for dck specificity and proper orientation within the vector using primer sets A6-B6, A2-B1, A1-SP6, and T7-B2 that were used before. One clone (pRC/CVM-DCK) was selected for large-scale plasmid isolation and purification using CsCl density gradients following standard protocols. The insert was sequenced to confirm proper integration of the full-length DCK coding region.

**Transfection of RO/1-A with pRC/CVM-DCK.** By means of a commercial lipofectin reagent (GIBCO BRL, Life Technologies, Gaithersburg, MD) the pRC/CVM-DCK construct was transfected into the drug-sensitive clone RO/1 and into its AraC-resistant derivative RO/1-A, yielding cell lines RO/1-DCK and RO/1-A-DCK, respectively. As a control, the pRC/CVM vector was transfected into the same cell lines, yielding RO/1 and RO/1-A. For each transfection 45 μg lipofectin reagent and 3 μg plasmid DNA were mixed in a final volume of 0.5 mL serum-free RPMI 1640 medium. Liposome-DNA complexes were allowed to form for 15 minutes at room temperature. Target cells (7.5 × 10^7) were washed with serum-free RPMI 1640 and resuspended in a 2.5 mL volume. The liposome-DNA mixture was added to the cell suspension and incubated in 50-mL tissue culture flasks at 37°C/5% CO₂. After 5 hours, 5 mL of regular culture medium was added, and 6 mL more after another 16 hours. G418 selection was started at 75 μg/mL, 48 hours after transfection, and gradually increased to a final concentration of 800 μg/mL within 5 weeks. Every 2 days, cell growth was monitored and fresh medium added. The presence of constructs was checked by PCR on 100 ng of DNA samples from the cell lines transfected with pRC/CVM-DCK or pRC/CVM. Vector-specific primers SP6 and T7 and the dck–specific primers A2 and B1 were used for amplification, in the protocol described above.

**Detection of dck mRNA by RT-PCR and Northern blot analysis.** cDNA was reverse transcribed from 2 μg RNA, isolated by the guanidinium-isothiocyanate protocol as described by Chomczynski et al with some minor modifications. RT-PCR on the resulting cDNA was performed using primers A6-B6 (794 bp) and A2-B1 (701 bp). Amplification of rat β-actin was used as a control. For Northern blot analysis 20 μg of total RNA was fractionated on a 2.2 mol/L formaldehyde 1% agarose gel, transferred to Hybond-N nylon membranes, and subsequently hybridized at 42°C in a 50% formamide solution following routine procedures. RT-PCR product A2-B1 (701 bp) was purified from a 0.8% low-melting-point agarose gel and used as a hybridization probe. As a positive control, rat thymus RNA was also analyzed, because of its relatively high level of dck expression.

**Immunodetection of DCK protein by Western blot analysis.** Proteins were precipitated from crude cell extracts by a Noristet P-40 scission protocol and 30 μg of total protein was separated for 1.5 hours at 100 V on 12% polyacrylamide gels, as detailed before. Western blotting was performed as described. Briefly, the fractionated proteins were blotted to Immobilon PSQ membranes (Millipore BV, Etten-Leur, The Netherlands). The filters were blocked for 2 hours and then stained overnight by incubation with an affinity-purified rabbit-antihuman DCK polyclonal antibody (a generous gift from Dr S. Eriksson, Swedish University of Agricultural Sciences, The Biomedical Centre, Uppsala, Sweden), followed by a 2-hour incubation with 125I-protein A (0.3 μCi/mL). As a positive control sample, a batch of purified DCK obtained from human spleen (also provided by Dr S. Eriksson) was used. Films were exposed for 48 hours before developing.

**RESULTS**

**DCK activity in RO/1 and RO/1-A.** DCK activity for the phosphorylation of dC and AraC was measured in RO/1 and RO/1-A. In Table 1 K_{m} and V_{max} values, calculated from initial rate measurements, show that the kinetic constants for DCK from RCL/O and its subclone RO/1 are comparable. The RO/1-A has acquired resistance to AraC, marked by a classical DCK-deficient phenotype. AraC phosphorylation could no longer be detected, whereas a residual DCK activity with respect to dC conversion was found, as can be seen.
Table 1. DCK Kinetic Constants

<table>
<thead>
<tr>
<th></th>
<th>dc</th>
<th>AraC</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCL/O*</td>
<td>9.4</td>
<td>5.0</td>
</tr>
<tr>
<td>RO/1</td>
<td>15.0</td>
<td>2.7</td>
</tr>
<tr>
<td>RO/1-A</td>
<td>921.0</td>
<td>10.4</td>
</tr>
<tr>
<td>RO/1-DCK</td>
<td>17.1</td>
<td>4.9</td>
</tr>
<tr>
<td>RO/1-ADCK</td>
<td>21.6</td>
<td>48.6</td>
</tr>
</tbody>
</table>

Kinetic constants for DCK activity with substrates dc and AraC. K_m and V_max values were calculated from initial rate measurements, using the P.Fit software for nonlinear regression analysis, fitted to the Michaelis-Menten model. All values are averages from two independent experiments. K_m values in pmol/L, V_max values in nmol·min^(-1)·mg protein^(-1). <, values below the detection level of the assay.

Values taken from reference 10.

from the extremely increased K_m value for dc in RO/1-A (921 µmol/L v 15 µmol/L in RO/1).

Transfection and detection of the pRc/CMV-DCK+ construct. Previously we have presented a single-strand conformation polymorphism (SSCP) analysis of Roll-A that showed the presence of at least two pointmutations within the dck coding region. RO/1-A was transfected with a construct harboring the wild-type dck coding region, to reverse the resistant phenotype. Transfection of RO/1 and RO/1-A with either pRc/CMV or pRc/CMV-DCK+ did not have a significant growth inhibitory effect. Five hours after transfection, when the serum-free RPM1 was replaced by regular culture medium, approximately 15% of cells were dead, compared with a normal viability count of 90% to 95%. Neomycin selection, started at 75 µg/mL 48 hours after transfection and gradually increased to 800 µg/mL (ie, 20-fold the IC50 value for RO/1 and RO/1-A) within 5 weeks, did not cause major growth inhibitory effects. All transfected cell lines had unaltered doubling times and normal viability counts.

As shown in Fig 1, PCR performed on 100 ng of DNA with the T7-SP6 primer combination resulted in the amplification of an 853-bp band from RO/1-DCK and RO/1-A-DCK, but not from the cell lines transfected with pRc/CMV. The size of this amplicon matches that expected for the insert plus adjacent vector specific nucleotide stretches. The amplified signal was markedly stronger in RO/1-A-DCK, suggesting a higher copy number in this cell line. Using dck-specific primers A2-B1 on the same DNA samples, an amplicon was obtained from only those that were also positive for T7-SP6 amplification and the same difference in the intensity of the observed bands was found.

Detection of dck mRNA expression by reverse transcriptase (RT)-PCR and Northern blot analysis. RT-PCR with primer sets A6-B6 (794 bp, not shown) and A2-B1 (701 bp, Fig 1) yielded products from all cDNAs tested. Under these conditions amplification from RO/1-A cDNA gave rise to bands that are of similar intensity as those in RO/1, suggesting a normal level of dck mRNA expression in the resistant cell line, as was found before. Transfection with pRc/CMV-DCK+ resulted in a strong increase of the expressed message (Fig 1), both in RO/1-DCK and RO/1-A-DCK.

As shown in Fig 2, Northern blot analysis revealed a high expression of dck mRNA from the construct in RO/1-A-DCK (lane 3) and a markedly lower expression in RO/1-DCK (lane 1). mRNA expressed from the plasmid was expected to have a length of approximately 1.15 kb, but was always observed as a smear between 0.4 and 1.4 kb. A titration of RO/1-ADCK RNA resulted in the detection of a more defined band of ±1.2 kb, readily detectable in 2.5 µg of total RNA (not shown). At short exposure times of 2 days (Fig 2A) expression of the normal-length dck mRNA (4.1 kb) could only be detected in thymus cells, which were used as a positive control, but not in any of the other cell lines. At long exposure of 18 days (Fig 2B), the 4.1-kb transcript was detected in all cell lines except RO/1-A. Interestingly, this lack of expression of normal-length dck mRNA appears to be restored in RO/1-A-DCK cells (lane 3). Transfection with pRc/CMV alone did not affect normal expression levels (lanes 2

![Fig 1. Detection of dck sequences by PCR. (A) RT-PCR, using primers A2 and B1 (701 bp). (B) PCR on 100 ng of genomic DNA, using primers T7 and SP6 (853 bp). Loading: RO/1-DCK (1), RO/1 (2), RO/1-A-DCK (3), RO/1-A (4), RO/1 (5), and RO/1-A (6).](image-url)
Fig 2. Detection of dck mRNA by Northern blot analysis. (A) Exposure for 2 days, showing expression of a smear of construct transcribed mRNA (lane 3) and the normal 4.1-kb mRNA (lane 7). (B) Exposure for 18 days. (C) Hybridization of the same filter with β-actin. Loading: 20 µg of RO/1- (2), RO/1DCK (1), RO/1-A (4), RO/1-A_DCK (3), RO/1 (5), RO/1-A (6), and rat thymus RNA (7).

and 4). Hybridization of the same filter with a β-actin probe demonstrated equivalent loading of the slots (Fig 2C).

Detection of DCK protein expression by Western blot analysis. To establish whether the increased dck mRNA expression observed by Northern blot analysis leads to a higher production of DCK protein, Western blot analysis was performed, using a rabbit-antihuman DCK polyclonal antibody. A human protein pool, enriched for DCK, was used as a control (Fig 3, lane C). The antibody detected an approximately 30-kD band, representing the subunit molecular mass of the DCK heterodimeric protein. As can be seen from Fig 3, the antibody also recognized rat DCK, identical in size to human DCK. RO/1 (lane 2) expressed an amount of DCK comparable to the control sample (lane C) that was not changed upon transfection with pRc/CMV-DCK* (lane 1). In RO/1-A cells no DCK could be detected (lane 4). However, after transfection with pRc/CMV-DCK+ a high amount of DCK is present in RO/1-A_DCK (lane 3).

Restoration of DCK activity. To investigate whether the observed expression of DCK protein in the transfected cells led to a restoration of DCK enzymatic activity, the activity of the enzyme toward dC and AraC was measured (Table 1). The assay used to measure initial rate DCK conversion was linear for time and protein. Transfection of RO/1 resulted in a 1.8-fold increase of the V_max value for dC in RO/1_DCK but did not affect the K_m value significantly (1.14-fold). Identical results were obtained in RO/1_DCK with respect to AraC conversion, with a K_m value similar to RO/1 (235.2
μmol/L and 228.5 μmol/L, respectively) and a 2.6-fold increased $V_{\text{max}}$ value.

In contrast, RO/l-A$^{\text{DCK}}$ has a strongly increased affinity for dC (43-fold) compared to RO/1-A, with $K_m$ values of the same magnitude as those observed for its parental sensitive clone RO/1 (21.6 μmol/L and 15 μmol/L, respectively), as well as a 4.7-fold increased $V_{\text{max}}$ value. The conversion rate in RO/l-A$^{\text{DCK}}$, is 18-fold that of RO/1. For AraC, the $K_{\text{m}}$ is increased 2.1-fold and the $V_{\text{max}}$ 26-fold compared with RO/1. Transfection with the pRc/CMV vector alone had no significant effect on DCK kinetic constants in the resulting RO/1$^{-}$ and RO/1-A$^{-}$ cell lines (not shown).

The antiproliferative effect of AraC on RO/1, RO/l-A, and RO/l-A$^{\text{DCK}}$ was studied by the addition of varying concentrations of the drug to the cell culture medium. IC$_{50}$ values were calculated from the resulting growth inhibition curves shown in Fig 4, after 48 hours of continuous exposure. In comparison with RO/1, RO/l-A has become more than 9,000-fold resistant to AraC, with an IC$_{50}$ of >10 mmol/L. As a result of the transfection of pRc/CMV vector alone had no significant effect on DCK kinetic constants in the resulting RO/1$^{-}$ and RO/1-A$^{-}$ cell lines (not shown).

DISCUSSION

Resistance to the antimetabolite AraC is known to be mediated by mutations in the deoxycytidine kinase gene, as we and others have shown. However, it is not clear whether mutations in the dck locus are the sole cause of DCK protein deficiency and whether factors other than functional DCK inactivity may still be involved. In order to investigate the relative role of dck mutations in the development of a resistant phenotype, we have transfected a plasmid construct containing the wild-type dck coding region into an AraC-resistant cell line of clonal origin (RO/1-A). This approach has resulted in the complete restoration sensitivity to AraC in the formerly resistant target cells.

RO/1-A was generated in vitro by exposure of RO/1 to gradually increasing concentrations of AraC. RO/l-A has a DCK-deficient phenotype as can be seen from the kinetic constants presented in Table 1 and has similar characteristics as an ex vivo generated AraC-resistant cell line we described earlier. As was argued before, the residual metabolization of dC that is found may be the result of the presence of another nucleoside phosphokinase in our crude cellular extracts, most likely the mitochondrial thymidine kinase 2, that is known to phosphorylate dC, but not AraC. Earlier we have analyzed the dck gene in RO/1-A and found evidence for the presence of two pointmutations by SSCP analysis.

After transfection, evidence for the presence of the pRc/CMV-DCK$^{+}$ construct in RO/1$^{\text{DCK}}$ and RO/l-A$^{\text{DCK}}$ was obtained by PCR on genomic DNA samples, showing the expected 853-bp product for primer combination T7-SP6 in these cell lines, but not in RO/1$^{-}$ and RO/l-A$^{-}$. Although this assay does not discriminate between amplification from construct-DNA integrated into the host genome or amplification from episomally present pRc/CMV-DCK$^{+}$, both cell lines were stably transfected over a period of 14 weeks to date.

By RT-PCR, high expression of dck mRNA was seen in both transfected lines, with a somewhat stronger signal in the RO/1-A$^{\text{DCK}}$. A much higher difference in expression and activity was observed between RO/1-A$^{\text{DCK}}$ and RO/1$^{\text{DCK}}$ by Northern, Western, and DCK-activity analysis. However, the PCR method used was not expected to provide reliable quantitative data.

Northern blot analysis shows a very high expression of dck-mRNA of vector origin in RO/1-A$^{\text{DCK}}$ and only a marginal expression in RO/1$^{\text{DCK}}$. Bands representing normal-length dck-mRNA of 4.1 kb could only be detected after extremely long exposure times of up to 18 days, except in rat thymus that has a high constitutive expression of dck mRNA. Interestingly, in the RO/1-A$^{\text{DCK}}$ a weak expression of normal-length dck mRNA (4.1 kb) was observed that was not expressed in RO/1-A before transfection. This could suggest the involvement of transcriptional regulation mecha-
nisms in RO/1-A as a second factor contributing to the AraC-resistant phenotype, in addition to the mutational events affecting the dck gene.

Western blot analysis showed that transfection resulted in a very high expression of DCK protein in the RO/1-A DCK that previously did not express any detectable amounts. In the RO/1 DCK no elevation of DCK protein levels when compared with RO/1 was observed, confirming the results of Northern blot analysis. The rabbit-antihuman DCK polyclonal antibody used for immunoblotting recognized rat DCK just as well as it did human DCK. The 30.5-kD subunit of the DCK protein was readily detected in RO/1. This is in agreement with the high homology of the rat amino acid sequence (91.9%) that we reported previously.13 The same antibody has also been used for the detection of mouse DCK, which is 94.6% homologous to the human protein.24

Initial rate measurements of DCK activity show a mild increase in RO/1 DCK (1.6-fold) of the $V_{max}/K_m$ ratio (for dC), demonstrating a marginal effect of transfecting wild-type dck into this wild-type clone. It is not clear why the transfection of dck into RO/1 has apparently been less successful. Despite the presence of substantial amounts of pRC-CMV-DCK*, as assessed by PCR, and the expression of elevated levels of dck transcripts, no substantial elevation of mRNA, protein, or DCK-activity levels were found in RO/1 DCK. Possibly, the normal regulation of DCK expression in these cells also controls the expression of DCK from the vector and strives to maintain normal levels of functional DCK.

The increase in DCK phosphorylating activity was substantial in RO/1-A DCK and could not be adequately measured without diluting the enzyme samples. The $V_{max}/K_m$ ratios for dC conversion in RO/1-A DCK increased 12.5-fold and 204-fold compared with the parental RO/1 and the resistant RO/1-A, respectively, indicating a restoration of DCK activity to levels higher than in wild-type cells. AraC phosphorylation in RO/1-A DCK was restored at a conversion rate level 26.4-fold higher than in the parent RO/1. $K_m$ values for dC and AraC were not significantly altered in RO/1 DCK and RO/1-A DCK, compared with RO/1, indicating that the enzyme translated from the construct and the natural DCK protein have a similar affinity for both substrates. The $K_m$ value for dC decreased from 921 μmol/L in RO/1-A to 17.1 μmol/L in RO/1-A DCK, confirming that the construct-derived protein has a normal affinity for dC. In RO/1-A DCK an unexpected but significant concomitant 2.1-fold increase of the affinity constant for AraC was observed, whereas it was of normal magnitude in RO/1 DCK.

In conclusion, we have shown that transfection of wild-type dck cDNA into an AraC-resistant cell line results in the complete restoration of sensitivity to AraC cytotoxicity. The results indicate that DCK deficiency, mediated by mutations in the dck gene, is the crucial factor in the development of AraC resistance in this cell line.

REFERENCES

3. Estey EH, Kantarjian H, Keating M: Idarubicin plus continuous infusion high-dose cytarabine as treatment for patients with acute myelogenous leukemia or myelodysplastic syndrome. Semin Oncol 20:1, 1993 (suppl 8)
15. Stegmann APA, Honders MW, Willemze R, Landegent J: In vitro induced de novo mutations in the gene encoding deoxycytidine kinase (dck) confer resistance to cytarabine (AraC) and 5-aza-2'-deoxycytidine (DAC) in rat leukemic clonal cell lines. Leukemia (in press)


Transfection of wild-type deoxycytidine kinase (dck) cDNA into an AraC- and DAC-resistant rat leukemic cell line of clonal origin fully restores drug sensitivity

AP Stegmann, WH Honders, R Willemze, VW Ruiz van Haperen and JE Landegent